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Evaluation of net antioxidant activity of mono- and bis-Mannich base hydrochlorides and 3-keto-1,5-bisphosphonates from their ProAntidex parameter

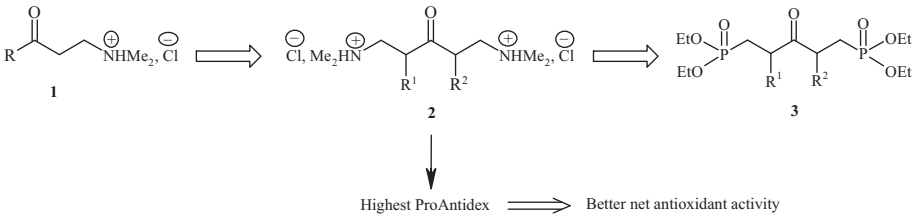
Karima Lahbib *, Mohamed Tarhouni, Soufiane Touil

Laboratory of Heteroatom Organic Chemistry, Faculty of Sciences of Bizerta, University of Carthage, 7021 Zarzouna, Tunisia

HIGHLIGHTS

- We synthesized a series of mono- and bis-Mannich base hydrochlorides and of 3-keto-1,5-bisphosphonates.
- We characterized the synthesis compounds on the basis of their infrared (IR), ^{1}H, ^{13}C and ^{31}P NMR spectral data.
- All the title compounds were tested for their *in vitro* antioxidant activities as well as their chelating abilities.
- We tried to analyze the pro-oxidant capacity of compounds.
- All the tested compounds showed significant antioxidant activity and high ProAntidex.

GRAPHICAL ABSTRACT



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ABSTRACT

A series of mono- and bis-Mannich base hydrochlorides and of 3-keto-1,5-bisphosphonates were prepared and characterized on the basis of their infrared (IR), ^{1}H, ^{13}C and ^{31}P NMR spectral data. All the title compounds were tested for their *in vitro* antioxidant activities by 1,1-diphenyl-2-picrylhydrazyl (DPPH), H_2O_2, hydroxyl radical and Ferric Reducing Power (FRP) methods. The antioxidant activity of these compounds was analyzed simultaneously with their pro-oxidant capacity. The ratio of pro-oxidant to the antioxidant activity (ProAntidex) represents a useful index of the net free radical scavenging potential of the synthesized compounds. Ferrous, calcium and magnesium ion chelating abilities were also evaluated. All the tested compounds showed significant antioxidant activity and high ProAntidex.

Introduction

Pharmaceutical chemistry is a discipline which is interested in the design, the preparation and the interpretation of structure–activity relationship of the bioactive molecules (drugs) resulting from organic synthesis. It is a science which tries to establish relations between the chemical structure of drugs and their biological activity.

Mannich bases are an important class of compounds in pharmaceutical chemistry with a wide range of biological properties including antimicrobial [1], anticancer [2], anti-inflammatory [3], analgesic [4], anticonvulsant [5] and anti-HIV [6] activities.

On the other hand, bisphosphonates have interesting industrial applications as water-softeners, anti-scaling, anti-corrosive and complexing agents, mainly in the textile, fertilizer and oil

* Corresponding author.
E-mail address: lakarima29@gmail.com (K. Lahbib).

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industries. Their use as water softeners was based on their ability to act as sequestering agents for calcium, and in particular their ability to inhibit calcium carbonate precipitation, as do polyphosphates [7,8].

It is only during these forty last years that bisphosphonates were used as drugs and they have been found to possess a wide variety of medicinal applications [9]. Besides their well-known antibone resorption properties [10], antimicrobial [11] and antitumor [12] activities, some of their derivatives inhibit cancer manifestations through antiangiogenic, antiinvasive and immunomodulatory actions [13].

Despite the wide spectrum of biological activities associated to Mannich bases and bisphosphonates, knowledge on their antioxidant properties is limited. Therefore, we report in the present study, the synthesis of a series of mono- and bis-Mannich base hydrochlorides and of 3-keto-1,5-bisphosphonates and the evaluation of their *in vitro* antioxidant activities by 1,1-diphenyl-2-picrylhydrazyl (DPPH), H_2O_2, hydroxyl radical and Ferric Reducing Power (FRP) methods. The antioxidant activity of these compounds was analyzed simultaneously with their pro-oxidant capacity. The antioxidant and pro-oxidant effects are due to the balance of two activities, respectively, free radical-scavenging activity and ferric reducing power (FRP). The pro-oxidant capacities of the compounds, deduced from the FRP measurements, were compared to the IC_{50} (mg/mL) of the antioxidant scavenging activity of DPPH, $OH^{\cdot}$ and H_2O_2. This ratio of pro-oxidant/antioxidant activity (ProAntidex) represents a useful index enabling us to evaluate the net antioxidant potential of the synthesized compounds as this index includes not only the effective free radical scavenging ability, but also the pro-oxidant capacity of the compounds. Ferrous, calcium and magnesium ion chelating abilities were also evaluated. All the tested compounds showed significant antioxidant activity and high ProAntidex.

We shall note here that the search for novel antioxidant agents that prevent or reduce the impact of oxidative stress on cell is a contemporary field. Indeed, antioxidants are considered as first-line therapy that protect organisms from highly reactive free radicals, especially oxygen derived ones (reactive oxygen species: ROS), which are capable of oxidizing biomolecules causing human diseases such as cancer, emphysema, cirrhosis, atherosclerosis and arthritis [14].

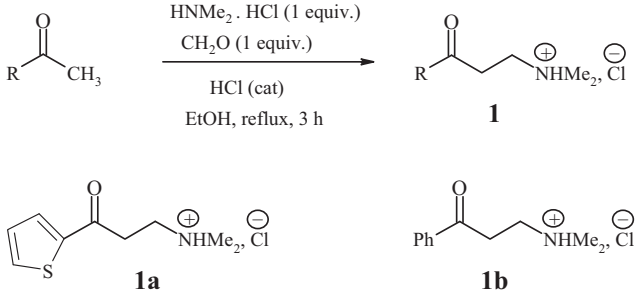

Fig. 1. Synthesis of mono-Mannich base hydrochlorides **1**.

Materials and methods

Chemistry

Synthesis of mono-Mannich base hydrochlorides 1

Mannich base hydrochlorides **1** were prepared according to the reported Mannich synthetic procedure which involves a three component condensation of an enolizable ketone with formaldehyde and a secondary amine [15]. Experimentally, the reaction of the enolizable ketone with equimolar amounts of paraformaldehyde and dimethylamine hydrochloride, performed in refluxing 95% ethanol, for 3 h, in the presence of a catalytic amount of concentrated HCl, led to mono-Mannich base hydrochlorides **1** (Fig. 1).

*General procedure for the synthesis of compounds **1**.* A mixture of the methylketone (0.20 mol), dimethylamine hydrochloride (0.20 mol), paraformaldehyde (0.25 mol), and concentrated HCl (0.50 mL), in 30 mL of 95% ethanol, was refluxed for 3 h. After cooling, acetone (150 mL) was added and the mixture was left overnight in the refrigerator. The crystals formed were filtered and recrystallized from a mixture of acetone and 95% ethanol.

Synthesis of bis-Mannich base hydrochlorides 2

Bis-Mannich base hydrochlorides **2** were synthesized according to the method described in the literature [16]. The reaction also involves a three component condensation of an enolizable ketone on both sides of the C=O group, with two equivalents of paraformaldehyde and dimethylamine hydrochloride. Experimentally the reaction was performed in refluxing 95% ethanol, for 24 h, in the presence of a catalytic amount of concentrated HCl (Fig. 2).

*General procedure for the synthesis of compounds **2**.* A mixture of the ketone (0.2 mol), dimethylamine hydrochloride (0.4 mol), paraformaldehyde (0.5 mol), and concentrated HCl (1 mL), in 60 mL of 95% ethanol, was refluxed for 24 h. After cooling, the solvent was removed under reduced pressure and acetone (300 mL) was added. The mixture was left overnight in the refrigerator. The crystals formed were filtered and dried in a vacuum desiccator.

Synthesis of 3-keto-1,5-bisphosphonates 3

3-Keto-1,5-bisphosphonates **3** were prepared according to the reported procedure developed in our laboratory [17,18] (Fig. 3).

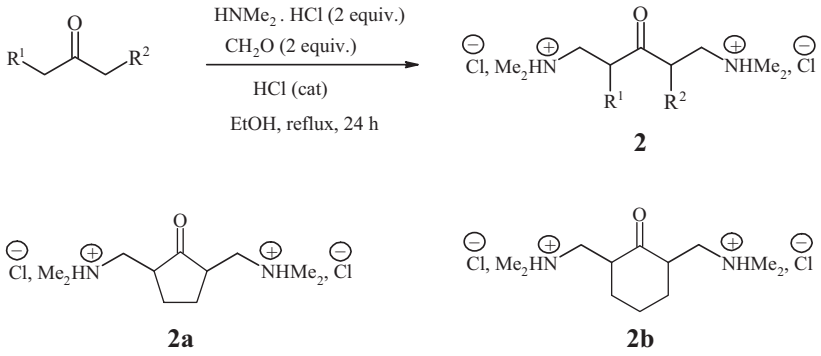

Fig. 2. Synthesis of bis-Mannich base hydrochlorides **2**.

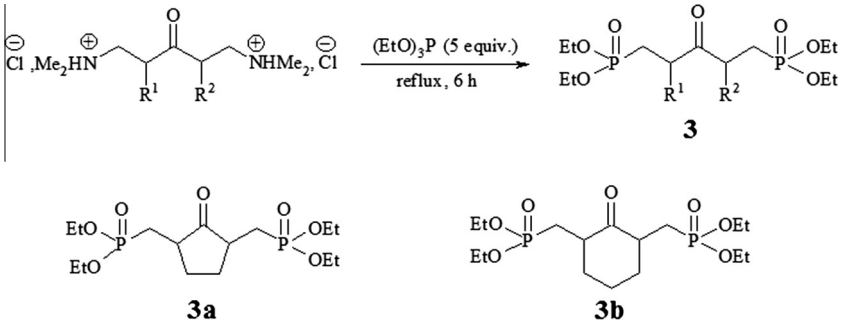

Fig. 3. Synthesis of 3-keto-1,5-bisphosphonates **3**.

Thus, the reaction of bis-Mannich base hydrochlorides **2** with an excess of triethyl phosphite (5 equivalents), performed under reflux, for 6 h, in solvent-free conditions, led to 3-keto-1,5-bisphosphonates **3**.

General procedure for the synthesis of compounds ***3***. A mixture of bis-Mannich base hydrochloride **2** (0.02 mol) and triethyl phosphite (0.1 mol) was heated under reflux for 6 h. After cooling, the excess of triethyl phosphite was removed under reduced pressure, then $CHCl_3$ (100 mL) was added. The organic phase was washed with water (2 × 50 mL), dried over Na_2SO_4 and concentrated under vacuum. The obtained residue was chromatographed on a silica gel column using a mixture of ether and hexane (3:1) as eluent.

Spectral data for the synthesized compounds

The structures of compounds **1**, **2** and **3** were confirmed by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopies. ^1H, ^{31}P, and ^{13}C NMR spectra were recorded with $CDCl_3$ as the solvent for compounds **3** or in a mixture of $CDCl_3$ and DMSO-d_6 for Mannich bases **1** and **2**, on a Bruker AC-300 spectrometer operating at 300.1 MHz for ^1H, 121.5 MHz for ^{31}P and 75.5 MHz for ^{13}C. The chemical shifts are reported in ppm relative to TMS (internal reference) for ^1H and ^{13}C NMR and relative to 85% H_3PO_4 (external reference) for ^{31}P NMR. The coupling constants are reported in Hz. For the ^1H NMR, the multiplicities of signals are indicated by the following abbreviation: s: singulet; d: doublet; t: triplet; q: quartet; quint: quintet; m: multiplet. IR spectra were recorded on a Nicolet IR200 spectrometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA).

Spectral data for compounds **1 (a,b)** and **3 (a,b)** were mentioned in our previous articles [19,20].

Spectral data for compounds **2 (a,b)** are as follows:

2a: White solid; M = 271.23 g mol^{-1}; mp = 180–182 °C; yield = 79%; IR (neat): $\nu_{c=o}$ = 1740 cm^{-1}; ν_{NH} = 3448 cm^{-1}; ^1H NMR: δ = 1.75–2.48 (m, 4H, CH_2—CH_2); 2.91 (s, 3H, $C\underline{H}_3$—N—CH_3); 2.93 (s, 3H, CH_3—N—$C\underline{H}_3$); 3.07–3.72 (m, 3H, CH and CH_2—N); 9.57 (br s, 1H, N—H); ^{13}C NMR: δ = 10.1 (s, $\underline{C}H_2$—CH_2); 20.2 (s, CH_2—$\underline{C}H_2$); 34.6 (s, $\underline{C}H_3$—N—CH_3); 36.8 (s, CH_3—N—$\underline{C}H_3$); 44.6 (s, $\underline{C}H$—CH_2); 58.0 (s, CH_2—N); 216.2 (s, C=O).

2b: White solid; M = 285.14 g mol^{-1}; mp = 175–176 °C; yield = 77%; IR (neat): $\nu_{c=o}$ = 1728 cm^{-1}; ν_{NH} = 3450 cm^{-1}; ^1HNMR: δ = 1.18–2.86 (m, 6H, $(CH_2)_3$); 2.76 (s, 3H, $C\underline{H}_3$—N—CH_3); 2.78 (s, 3H, CH_3—N—$C\underline{H}_3$); 2.94–3.66 (m, 3H, CH and CH_2—N); 10.70 (br s, 1H, N—H); ^{13}CNMR: δ = 22.3 (s, CH_2—$\underline{C}H_2$—CH_2); 27.2 (s, $\underline{C}H_2$—CH_2—CH_2); 32.6 (s, CH_2—CH_2—$\underline{C}H_2$); 40.4 (s, $\underline{C}H_3$—N—CH_3); 41.2 (s, CH_3—N—$\underline{C}H_3$); 49.4 (s, $\underline{C}H$—CH_2); 56.4 (s, CH_2—N); 209.1 (s, C=O).

Antioxidant, pro-oxidant and chelating investigation

Compounds **1–3** were tested for their *in vitro* antioxidant activities by DPPH, H_2O_2, hydroxyl radical and Ferric Reducing Power (FRP) methods. The ratio of pro-oxidant to the antioxidant activity (ProAntidex) was also examined and allowed us to evaluate the net antioxidant potential of these compounds. Ferrous, calcium and magnesium ion chelating abilities were also evaluated.

All the tests, for the determination of antioxidant activity, were carried out in triplicate and the results averaged. Absorbance was recorded using an UV–Vis spectrophotometer (Jinway UV–Vis 6405).

IC_{50} values were calculated by linear regression. Means ± SD were calculated. The data were analyzed for statistical significance using one-way ANOVA followed by Tukey post test.

DPPH radical scavenging activity

The most common methods to determine antioxidant activity in a practical, rapid and accurate manner are those that involve a radical chromophore, simulating the reactive oxygen species (ROS), and the free radical DPPH, of purple coloration that absorbs at 517 nm, is one of the most widely used radical chromophores for *in vitro* evaluation of antioxidant activity.

The DPPH radical scavenging assay employed here is as described by Braca et al. [21]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid (AA) is the reagent used as standard. The sample is able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. Experimentally, various dilutions of the methanolic solution of compounds **1–3** or standard (0.003–0.3 mg/mL, in triplicate) were added to DPPH solution (0.035 mg/mL). The absorbance of the mixture was taken at 517 nm with methanol as blank. A control sample with no added test compounds was also analyzed. Radical scavenging activity was expressed as a percentage and calculated using the formula: % Scavenging = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound **1–3**. The result was presented as IC_{50} (the concentration of test compound required for scavenging 50% of the DPPH radical).

The results of these experiments are summarized in (Fig. 4). It was found that bis-Mannich bases **2** have the highest DPPH radical scavenging activity with an IC_{50} at 1.73 mg/mL for **2b** followed by **2a** with an IC_{50} at 2.52 mg/mL when compared with other compounds. The remaining compounds exhibited DPPH radical scavenging activity in the following order: **1a** (IC_{50}: 3.83 mg/mL), **3a** (IC_{50}: 5.73 mg/mL), **3b** (IC_{50}: 7.03 mg/mL), **1b** (IC_{50}: 9.09 mg/mL), and compared with ascorbic acid (IC_{50}: 0.0396 mg/mL).

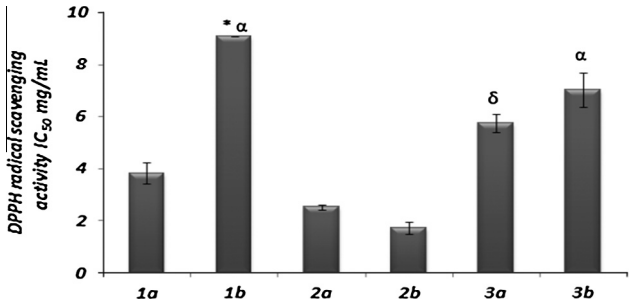

Fig. 4. DPPH radical scavenging activity (IC_{50} AA: 0.0396 mg/mL). Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2) itself is not very reactive, but it can be toxic to cell because it may give rise to the very harmful hydroxyl radical, through the Fenton or the Haber–Weiss reactions [22]. In organism, H_2O_2 can diffuse through the plasmic, mitochondrial or peroxysomale membranes inducing disruption [23,24]. Thus, the removing of H_2O_2 is very important for antioxidant defence in organism.

The ability of compounds **1–3** to scavenge hydrogen peroxide was determined according to the method of Yen et al. [25]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4), then a methanolic solution of the test compound at various concentrations (0.003–1 mg/mL) was added. Absorbance of hydrogen peroxide at 230 nm was determined 19 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated from the equation: % Scavenging = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound **1–3**.

The results of these experiments are summarized in (Fig. 5). It was found that bis-Mannich bases **2** have the highest H_2O_2 scavenging activity with an IC_{50} at 0.51 mg/mL for **2a** followed by **2b** with an IC_{50} at 0.52 mg/mL when compared with other compounds. The remaining compounds exhibited H_2O_2 scavenging activity in the following order: **1b** (IC_{50}: 1.11 mg/mL), **1a** (IC_{50}: 1.35 mg/mL), **3a** (IC_{50}: 2.32 mg/mL), **3b** (IC_{50}: 2.73 mg/mL), and compared with ascorbic acid (IC_{50}: 0.85 mg/mL).

Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive oxygen species that is capable of attacking and damaging almost every molecule found in living cells. It can hydroxylate purine and pyrimidine bases leading to DNA mutations. It can also initiate the peroxidation of cell membrane lipids and can increase the malondialdehyde (MDA) levels which is cytotoxic, mutagenic and carcinogenic [22].

The hydroxyl radical scavenging activity of compounds **1–3** was measured by the deoxyribose method [26] and compared with that of ascorbic acid. It was determined by measuring the competition between deoxyribose and the test compounds in scavenging hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radicals on deoxyribose led to the formation of thiobarbituric acid-reactive substances (TBARS) which were measured by the method of Ohkawa et al. [27]. The percentage of hydroxyl radical scavenging was calculated from the equation:% Scavenging = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound **1–3**.

The results of these experiments are summarized in (Fig. 6). It was found that bis-Mannich bases **2** have the highest hydroxyl radical scavenging activity with an IC_{50} at 1.20 mg/mL for **2b** followed by **2a** with an IC_{50} at 1.23 mg/mL when compared with other compounds. The remaining compounds exhibited hydroxyl radical scavenging activity in the following order: **3a** (IC_{50}: 1.60 mg/mL), **3b** (IC_{50}: 1.77 mg/mL), **1b** (IC_{50}: 1.79 mg/mL), **1a** (IC_{50}: 1.83 mg/mL), and compared with ascorbic acid (IC_{50}: 0.0062 mg/mL).

Ferric reducing power (FRP)

It has been observed a direct correlation between antioxidant activity and reducing power of certain compounds. The reducing power compounds **1–3** was determined according to the method of Oyaizu [28] and compared with ascorbic acid. Substances which have a reducing potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has a maximum absorption at 700 nm. Experimentally, a methanolic solution of the test compound (1 mL) at various concentrations (0.003–0.3 mg/mL) was mixed with phosphate buffer (0.2 M) and potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. Aliquots of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with distilled water and a freshly prepared ferric chloride solution (0.1%). The absorbance was measured at 700 nm. Ascorbic acid was used as standard. A control sample was prepared without adding standard or test compound. Increased absorbance of the reaction mixture indicates increase in reducing power. The percent increase in reducing power was calculated using the following formula: Increase in reducing power (%) = $[(A_{test} - A_{cont})/A_{cont}] \times 100$, where A_{test} is the absorbance of the sample in the presence of test compound and

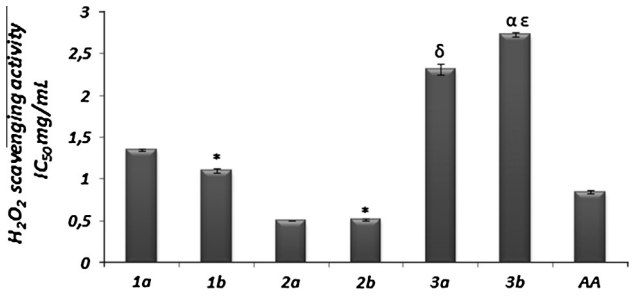

Fig. 5. H_2O_2 Scavenging activity. Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

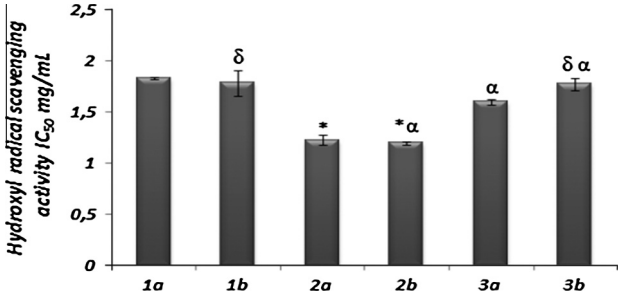

Fig. 6. Hydroxyl radical scavenging activity (IC_{50} AA: 0.0062 mg/mL). Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

A_{cont} is the absorbance of the control. The result was expressed as IC_{50} which corresponds to the concentration of test compound necessary to reduce 50% of ferric ferrous complex.

In the case of tested compounds **1–3** (Fig. 7), bis-Mannich bases **2** showed the highest ferric reducing power with an IC_{50} at 0.0075 mg/mL for **2a** followed by **2b** with an IC_{50} at 0.0076 mg/mL when compared with other compounds. The remaining compounds exhibited FRP activity in the following order: **3a** (IC_{50}: 0.0087 mg/mL), **3b** (IC_{50}: 0.0104 mg/mL), **1b** (IC_{50}: 0.01095 mg/mL), **1a** (IC_{50}: 0.01099 mg/mL), and compared with ascorbic acid (IC_{50}: 0.0031 mg/mL).

Ferrous ion chelating (FIC) activity

It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion [29].

The FIC ability of compounds **1–3** was determined according to the method of Singh and Rajini [30]. A methanolic solution of test compound **1–3** (1.0 mL) at various concentrations (0.003–0.3 mg/mL) was added to 1.0 mL of $FeSO_4$ (0.1 mM) and 1.0 mL of ferrozine (0.25 mM). The tubes were shaken well and left to stand for 10 min. The absorbancewas measured at 562 nm. The ability of each sample to chelate ferrous ions was calculated relative to the control consisting of only iron ferrozine, using the following formula: % FIC = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound.

In the case of tested compounds **1–3** (Fig. 8), derivative **3a** showed the highest FIC ability with IC_{50} at 0.068 mg/mL when compared with other compounds. The remaining compounds exhibited FIC activity in the following order: **3b** (IC_{50}: 0.06954 mg/mL), **2b** (IC_{50}: 0.06959 mg/mL), **2a** (IC_{50}: 0.070 mg/mL), **1a** (IC_{50}: 0.072 mg/mL), **1b** (IC_{50}: 0.100 mg/mL), and compared with ascorbic acid (IC_{50}: 0.006 mg/mL).

It is important to mention here that metal chelating capacity is significant as it contributes to reduce the concentration of the catalyzing transition metals in lipid peroxidation [31]. The data obtained from Fig. 8 reveal that all test compounds demonstrate an effective capacity for iron binding, suggesting that their action as antioxidants may be related to their iron-binding capacity.

Calcium and magnesium chelating activity

Metal ions can be very toxic through the activation of reduced forms of oxygen leading to increased formation of ROS [32]. These later react with macromolecules such as lipids, proteins and nucleic acids. ROS alter lipid composition of cell's plasmic membranes which is the origin of membrane peroxidation. They are responsible for activation or deactivation of several enzymes such as enzymes involved in the oxidative metabolism. All the

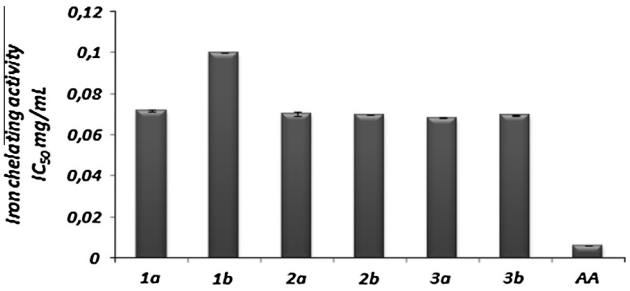

Fig. 8. Iron chelating activity. Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

ROS cause impairment of the vital functions of the cell, sometimes leading to his death [22]. In this context, compounds **1–3** were tested as chelating agents of calcium (Ca^{2+}) and magnesium (Mg^{2+}).

Calcium chelating activity assay was carried out, according to the method of Stern and Lewis [33], by measuring the competition between o-cresolphtalein complexone and the test compound to form a complex with calcium ion in alkaline medium. Experimentally, a methanolic solution of test compound was added to a mixture containing 2-amino-2-methylpropanol (500 mM) in alkaline buffer, chromogen solution of o-cresolphtalein complexone (0.62 mmol/L), quinolin-8-ol (69 mmol/L) and standard calcium ($CaCl_2 \cdot H_2O$, 2.5 mM). Absorbance was measured at 570 nm after 5 min at room temperature. The ability of each sample to chelate calcium ions was calculated relative to the control prepared without adding test compound, using the following formula: Calcium chelating activity (%) = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound.

Magnesium chelating activity assay was carried out in a similar way [33] by measuring the competition between EBT (Erichrome black T) and the test compound to form a complex with magnesium ion in alkaline medium. Experimentally, a methanolic solution of test compound was added to a mixture containing buffer 2-amino-2-methylpropanol (1 mM), EGTA (0.20 mM), EBT (0.30 mM) and standard magnesium $MgCl_2$ (1 M). Absorbance was measured at 520 nm after 5 min at room temperature. The ability of each sample to chelate magnesium ions was calculated relative to the control prepared without adding test compound, using the following formula: Magnesium chelating activity (%) = $[(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound.

The results of these experiments are summarized in Figs. 9 and 10. It was found that all test compounds **1–3** exhibit an effective chelating activity compared with ethylenediaminetetraacetic acid

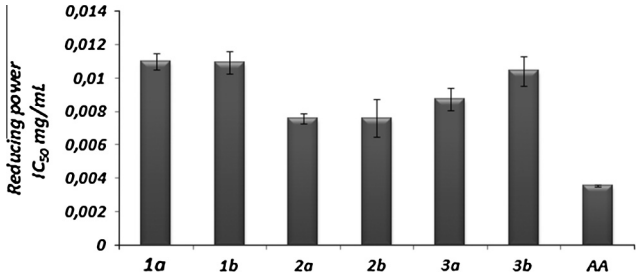

Fig. 7. Reducing power activity. Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

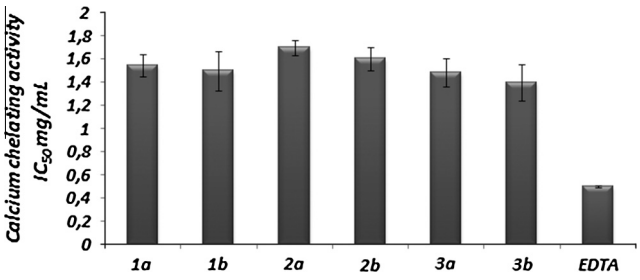

Fig. 9. Calcium chelating activity. Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

(EDTA) taken as standard reference. The calcium ion chelating ability of compounds **1–3** follows the order: **3b** (IC_{50}: 1.40 mg/mL), **3a** (IC_{50}: 1.48 mg/mL), **1b** (IC_{50}: 1.50 mg/mL), **1a** (IC_{50}: 1.54 mg/mL), **2b** (IC_{50}: 1.60 mg/mL), **2a** (IC_{50}: 1.70 mg/mL), and compared with EDTA (IC_{50}: 0.50 mg/mL). Whereas, the magnesium ion chelating activity of these compounds is in the following order: **3a** (IC_{50}: 1.24 mg/mL), **3b** (IC_{50}: 1.28 mg/mL), **2a** (IC_{50}: 1.30 mg/mL), **2b** (IC_{50}: 1.32 mg/mL), **1a** (IC_{50}: 1.41 mg/mL), **1b** (IC_{50}: 1.50 mg/mL), and compared with EDTA (IC_{50}: 1.03 mg/mL).

The pro-oxidant activity and ProAntidex

Antioxidants that are reducing agents can be pro-oxidants. They can reduce ferric ions leading to the generation of hydroxyl radicals through the Fenton reaction [34,35]. Thus, the pro-oxidant activity of a compound can be measured by its ferric reducing power (FRP).

The FRP of all the synthesized compounds were measured according to the method of Oyaizu described above. The pro-oxidant activities, deduced from the FRP measurements, were calculated by linear regression of plots where *x*-axis represented the various concentrations (0.01–1 mg/mL) of test compounds, while the *y*-axis represented the absorbance of the test compounds.

The pro-oxidant capacities of the test compounds, expressed as IC_{50} FRP (mg/mL), were compared to the IC_{50} (mg/mL) of the antioxidant scavenging activity of DPPH, $OH^{\cdot}$ and H_2O_2. This ratio of pro-oxidant/antioxidant activity (ProAntidex) [36] represents a useful index enabling us to evaluate the net antioxidant potential of the synthesized compounds as this index includes not only the effective free radical scavenging ability, but also the pro-oxidant capacity of the compounds. A better antioxidant agent should have a higher radical scavenging ability with lower pro-oxidant capacity.

The ProAntidex was calculated from the equation:

$$\text{ProAntidex} = IC_{50}\text{pro} - \text{oxidant activity} \; / IC_{50}\text{DPPH or } OH^{\cdot} \text{or} H_2O_2 \text{scavenging activity}$$

with IC_{50} pro-oxidant activity = IC_{50} FRP.

The higher the ProAntidex parameter of the test compound, the better its net antioxidant activity. Each of the measurements described was carried out in triplicate. Results are reported as mean ± standard deviation (Tables 1–3).

Conclusions

According to the results obtained, compounds **1–3** exhibited significant *in vitro* antioxidant activity compared to the standard ascorbic acid. Bis-Mannich bases **2** showed the highest antioxidant activity in DPPH, H_2O_2, hydroxyl radical and reducing power methods. This can be attributed to the well known tendency of keto function to reduce free radical levels and to inhibit the production of reactive oxygen species (ROS) [37]. Furthermore, the two ammonium groups present in these molecules could contribute to this activity. Indeed, it has been reported that molecules with double ammonium salt structure exhibit potent radical scavenging activity which increases with the increase of the positive charge density on nitrogen atoms [38].

The conversion of bis-Mannich bases **2** into bisphosphonates **3** increases the metal ion chelating activity. This may be due to the bisphosphonate affinity for bivalent cations. Indeed, bisphosphonates are known to possess a strong capacity for complexing metal-ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} or Fe^{2+}, by coordination of both phosphoryl groups with the cation [39]. With regard to the mechanism of antioxidant activity of compounds **3**, one can speculate that 3-keto-1,5-bisphosphonates, as iron chelators, exert a secondary antioxidant effect by chelating the Fe^{2+} ions necessary for the formation of hydroxyl radicals in the Fenton reaction [40].

The antioxidant activity of the synthesized compounds was analyzed simultaneously with their pro-oxidant capacity. The ratio of pro-oxidant to the antioxidant activity (ProAntidex) allowed us to evaluate the net antioxidant potential of these compounds. We found that bis-Mannich bases **2** exhibit the highest ProAntidex relative to DPPH, H_2O_2 and hydroxyl radical, indicating that they have the better net antioxidant activity.

Table 1
DPPH scavenging activity, pro-oxidant activity and ProAntidex (DPPH) of test compounds and standard.

	DPPH scavenging IC_{50} (mg/mL)	Pro-oxidant IC_{50} (mg/mL)	ProAntidex
1a	3.8286 ± 0.4086	0.0110 ± 0.0004	0.0028 ± 0.0001
1b	9.0909 ± 0.0000	0.0109 ± 0.0006	0.0012 ± 0.0000
2a	2.5204 ± 0.0904	0.0075 ± 0.0002	0.0030 ± 0.0002
2b	1.7259 ± 0.2479	0.0076 ± 0.0011	0.0043 ± 0.0001
3a	5.7332 ± 0.3483	0.0087 ± 0.0006	0.0015 ± 0.0001
3b	7.0349 ± 0.6573	0.0104 ± 0.0008	0.0016 ± 0.0001
AA	0.0396 ± 0.0020	0.0031 ± 0.0000	0.0028 ± 0.0001

All values represent means ± SEM, $n = 3$.

Table 2
Hydroxyl radical scavenging activity, pro-oxidant activity and ProAntidex (Hydroxyl radical) of test compounds and standard.

	Hydroxyl radical scavenging IC_{50} (mg/mL)	Pro-oxidant IC_{50} (mg/mL)	ProAntidex
1a	1.8303 ± 0.0104	0.0110 ± 0.0004	0.006036 ± 0.0001
1b	1.7897 ± 0.1254	0.0109 ± 0.0006	0.006037 ± 0.0004
2a	1.2280 ± 0.0506	0.0075 ± 0.0002	0.0066 ± 0.0004
2b	1.1987 ± 0.0148	0.0076 ± 0.0011	0.0065 ± 0.0009
3a	1.6012 ± 0.0283	0.0087 ± 0.0006	0.0053 ± 0.0002
3b	1.7751 ± 0.0626	0.0104 ± 0.0008	0.0058 ± 0.0003
AA	0.0062 ± 0.0003	0.0031 ± 0.0000	0.5000 ± 0.0152

All values represent means ± SEM, $n = 3$.

Table 3
H_2O_2 scavenging activity, pro-oxidant activity and ProAntidex (H_2O_2) of test compounds and standard.

	H_2O_2 scavenging IC_{50} (mg/mL)	Pro-oxidant IC_{50} (mg/mL)	ProAntidex
1a	1.3532 ± 0.0156	0.0110 ± 0.0004	0.0081 ± 0.0004
1b	1.1090 ± 0.0274	0.0109 ± 0.0006	0.0098 ± 0.0004
2a	0.5086 ± 0.0012	0.0075 ± 0.0002	0.0148 ± 0.0006
2b	0.5204 ± 0.0110	0.0076 ± 0.0011	0.0144 ± 0.0023
3a	2.3203 ± 0.0652	0.0087 ± 0.0006	0.0037 ± 0.0002
3b	2.7348 ± 0.0284	0.0104 ± 0.0008	0.0038 ± 0.0003
AA	0.8492 ± 0.0168	0.0031 ± 0.0000	0.0041 ± 0.0000

All values represent means ± SEM, $n = 3$.

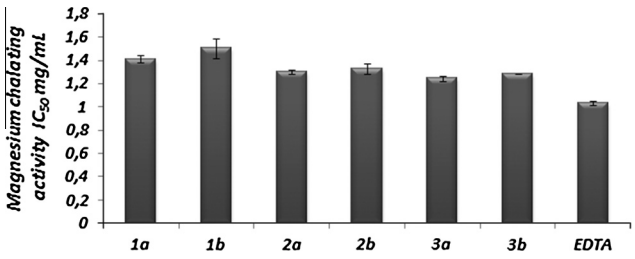

Fig. 10. Magnesium chelating activity. Results are expressed as mean ± SEM ($n = 3$). $p < 0.05$ was considered significant. *: $p < 0.05$ versus 1a, δ: $p < 0.05$ versus 2a, α: $p < 0.05$ versus 2b, ε: $p < 0.05$ versus 3a.

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