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Mediterranean Journal of Biosciences **2016**, 1(2), 62-68

Antioxidant and antimicrobial activities of two amidine derivatives

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Abstract: Two substituted amidines, *N*, *N'*-diphenylbenzamidine 1 and *N*, *N'*-diphenyldodecamidine 2, were investigated *in vitro* for their antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay under *kinetic process*, as well as for their antimicrobial activity against some strains of bacteria and fungi. The antioxidant propriety was assessed by the effective concentration of the amidine (EC_{50}), the time required to reach the steady state of DPPH discoloration at EC_{50} (t_{EC50}), the antiradical efficiency (ARE) and the reduction kinetics. The highest value of radical scavenging activity was obtained for amidine 1. Overall, the two amidines showed a moderate activity against Gram-negative *Salmonela enteric* with amidine 2 being more effective. The latter amidine exhibited also a potent inhibition against Gram-negative *Pseudomonas aeruginosa* and an antifungal activity against *Mucor ramannianus*.

Keywords: Amidine; Antioxidant activity; Antimicrobial activity; Radical scavenging.

Introduction

Bacteria were reported to arouse a number of life-threatening infections all over the world [1, 2]. Therefore, the development of new antimicrobial drugs is indubitably crucial and an intensive ongoing research is being focused towards the design of new agents [3]. Free radicals are among the main products of lipid oxidation and have been involved in over hundred diseases including cancer, atherosclerosis and arthritis [4]. Antioxidants can protect the human body from free radicals and reactive oxygen species (ROS) effects and retard the progress of many chronic diseases as well as lipid peroxidation [5-7]. Thus, antioxidants may play an important role in disease prevention by virtue of their propensity to trap the reactive free radicals [8].

Amidines are chemical compounds, bis-nitrogen analogue of carboxylic acids and esters, containing an amino nitrogen atom with a free electron pair, conjugated with the π-electrons of the C=N double bond [9].These compounds are very interesting thanks to their basicity, biological activity and their use as intermediates in the synthesis of some metallocyclic as well as heterocyclic complexes [10]. Amidines are characteristic structural features of many natural substances and important pharmacophores in the active ingredients of drugs [11].

The pharmacological activity of the amidine functional group within a molecule is well established [12]. Indeed, several amidine derivatives have been associated with a range of biological activities [13] and medical potentials such as antidegenerative [14], antitumor [15], antiplatelet [16], anti-HIV [17], diuretic, anti-inflammatory, analgesic, antivirus, fungicidal and bactericidal activities [18] agents.

A broad range of nitrogen compounds containing an amidine moiety were synthesized and examined for their antioxidant properties. Lautre et al. [19] synthesized benzamidine derivatives to be used as ligands in metal complexes, screened then for their DPPH scavenging activity. Both ligands and complexes were observed to be active for scavenging the free radical and the highest activity was observed for metal complexes. Silva et al. [20, 21] also reported the potential antioxidant activity of amidine/phenol-containing compounds and of their modifications.

In a previous study [22], we evaluated the *in vitro* antioxidant activity of three amidine derivatives by three methods: DPPH radical scavenging *at a*

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DOI: http://dx.doi.org/

fixed reaction time, ferric reducing antioxidant power (FRAP), and β-carotene bleaching. The obtained results showed that the tested amidines have variable and interesting antioxidant properties and free radical scavenging activities when compared to the standard antioxidants and the higher activity was observed in *ß*-carotene bleaching assay.

In tune with these sought-for properties, we wish to report the results of biological activities of *N*, *N'*-diphenylbenzamidine **1** and *N*, *N'*-diphenyldodecamidine **2,** emphasizing on: 1) the time reaction effect, which seems to be a critical parameter [23] on the improvement of their DPPH radical scavenging activity, and 2) the evaluation of their antimicrobial and antifungal activities.

Material and methods

All chemicals used were analytical grade and purchased from Sigma-Aldrich, Schuchardt and Merck. Thin-layer chromatography analyses were performed on Merck silica gel 60F254 plates and the spots were visualized with UV light. The used UV-visible spectrophotometer was SHIMADZU-1605. *N*, *N'*-Diphenylbenzamidine **1** and *N*, *N'*-diphenyldodecamidine **2** (Fig. 1) were synthesized and obtained as white needles according to a literature procedure [24, 25].

Antioxidant activity

In this study, antioxidant activity of amidines **1** and **2** was assessed using DPPH assay [26]. DPPH is a common reagent used to quantify the free radical scavenging activity of antioxidants. By virtue of being a stable free radical, the delocalization of the spare electron gives rise to a deep violet color, characterized by an absorption band in ethanol solution at about 517 nm.

In the DPPH radical-scavenging assay, an antioxidant reacts with DPPH, leading to the reduced form of the latter; DPPH pulls out either an electron or hydrogen atom from the antioxidant. As a result, the color changes from violet to yellow. The color fading extent proves indirectly the radical-scavenging capacity of the antioxidant [27].

Several concentrations of test compounds **1** and **2** in ethanol were prepared. The ethanolic solution (1.0 mL) was added to the methanolic DPPH solution (1.0 mL, 0.004%). The mixture was vortexed thoroughly and kept in the dark for the time necessary to reach the plateau [26, 28]. At specified time interval, UV-visible absorbance of the mixture was measured at λ_{max} = 517 nm. The reduction kinetics of DPPH was followed over the time until plateaued to the final time (t_{eq}). The extent of unreacted DPPH, $\%[DPPH]_R$, for each concentration of the substrate was calculated from equation (1).

$$\%[DPPH]_R = 100\ (A_t/A_o) \quad (1)$$

where A_0 and A_t are the absorbance of DPPH solution at the start of reaction (t = 0) and at $t \neq 0$, respectively.

Vitamin C (ascorbic acid), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as positive controls.

The EC_{50} values (µg/mL) were determined by graphic interpolation from the curve plotting the percentage of $\%[DPPH]_R = f(C)$ at $t = t_{eq}$; C was the amidine concentration in µg/mL.

To characterize the behavior of a substance as an antioxidant, the antiradical efficiency parameter (ARE) was calculated, combining the two parameters (EC_{50} and t_{EC50}) according to equation (2) .

$$ARE = 1/(EC_{50} \times t_{EC50}) \quad (2)$$

where EC_{50} is the concentration of the sample required to scavenge 50% of DPPH-free radical and t_{EC50}, the time required by each compound to reach the steady state of DPPH discoloration at EC_{50} concentration [29].

Antimicrobial Activity

The synthesized amidines **1** and **2** were screened *in vitro* for their antimicrobial activity by the paper disk-diffusion method [30].

The tested microorganisms included two Gram-positive bacteria (*Staphylococcus aureus* CIP 7625, *Bacillus subtilis* ATCC 6633), four Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica, Klebsiella pneumoniae*), and four fungi; two molds (*Mucor ramannianus* NRRL 1829, *Fusarium culmorum*) and two yeasts (*Saccharomyces cerevisiae* ATCC 4226, *Candida albicans* IPA 200). All microorganisms were regenerated twice before use in the test. Microbial suspensions were prepared in sterile distilled water and adjusted as inoculum to a concentration equivalent to the McFarland 0.5 standard. Mueller-Hinton and the Sabouraud media were used to evaluate the antibacterial and the antifungal activities respectively. A volume of 20 mL of culture medium was inoculated with 20 µL of microbial suspension and then poured into a Petri dish.

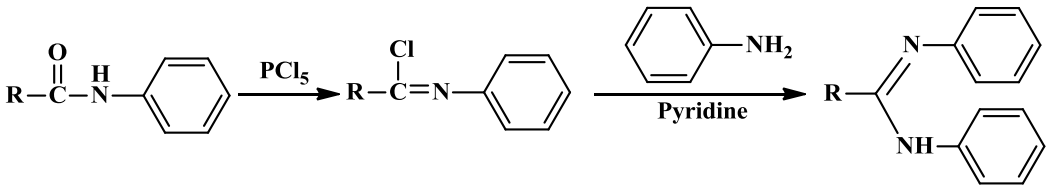
The amidines **1** and **2** were dissolved in methanol (at the concentration of 1 mg/mL). Sterile 6-mm diameter filter paper disks were impregnated with the solution of compound to be tested (10 to 60 µg/disk), then the paper disks were placed manually on the culture medium inoculated with an indicator microorganism. As positive control, gentamicin (10 µg/disk) was used to determine the sensitivity of Gram-positive and Gram-negative bacteria species while Fluconazole and amphotericin B (25 µg/disk) were used for yeasts and fungi species respectively. The Petri dishes were kept at 4 °C for 2 h to allow diffusion of amidines in the medium and then incubated for 24 h at 37 °C for bacteria, and 48 h at 30 °C for yeasts and fungi.

A positive antimicrobial activity appeared as a clear zone around the disks indicating an inhibition of growth [31]. The antimicrobial activity was evaluated by measuring with a ruler, the diameters of inhibition zones, including the disk diameter (6 mm).

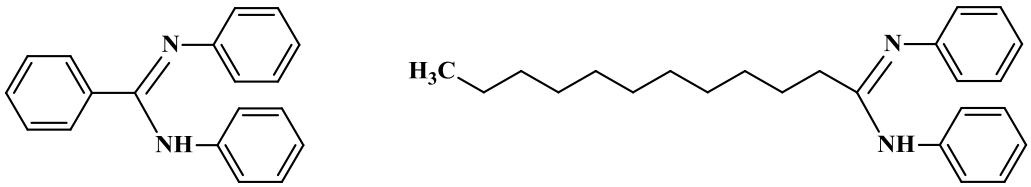
Results and Discussion

Substituted amidines **1** and **2** were synthesized in satisfactory yields (50-70%) and their structures were confirmed by the common spectroscopic analyses [25].

Their synthesis involves the conversion of the corresponding substituted anilide to imidoyl chloride using phosphorous pentachloride, and the formed imidoyl chloride was treated *in-situ* with aniline in the presence of pyridine (Scheme 1).



Scheme 1. Synthetic pathway of amidines derivatives.



1 **2**

Figure 1. Synthesized amidine derivatives: **1**, *N*, *N'*-diphenylbenzamidine; **2**, *N*, *N'*-diphenyldodecamidine.

Antioxidant activity

DPPH has been widely used for the evaluation of free radical scavenging effectiveness of various antioxidant substances [32]. This method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant [33]. The radical scavenging activities of amidines **1** and **2** were determined spectrophotometrically by monitoring the disappearance of DPPH at λ_{max} = 517 nm [27]. Figs. 2 and 3 illustrate the kinetic profiles of DPPH-scavenging of **1** and **2.**

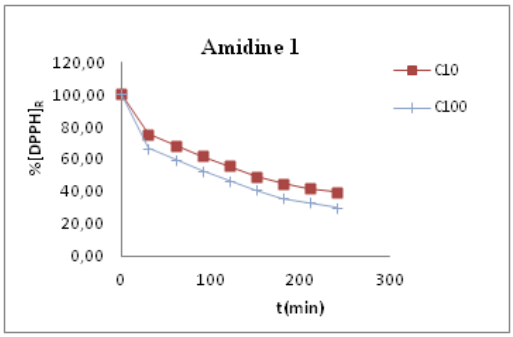


Figure 2. Kinetics of DPPH-scavenging of amidine **1**; C10: 10 µg/mL of amidine; C100: 100 µg/mL of amidine

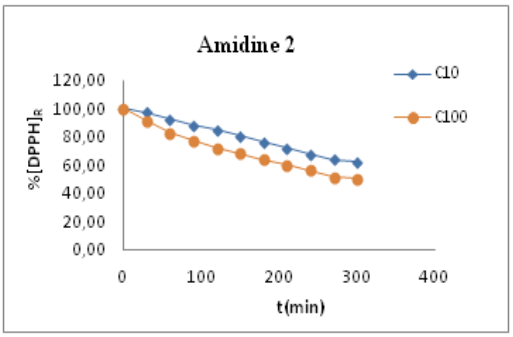


Figure 3. Kinetics of DPPH-scavenging of amidine **2**;C10: 10 µg/mL of amidine; C100: 100 µg/mL of amidine

From Figs. 2 and 3, one can clearly notice a significant decrease in the percentage of residual DPPH, $\%[DPPH]_R$, due to the scavenging ability of amidines **1** and **2**, and $\%[DPPH]_R$ decreased with increasing amidine concentration.

In Figs. 4 and 5 $\%[DPPH]_R$ is plotted as function of amidine concentration. From the curves of these figures, the EC_{50} values of amidines **1** and **2** were taken at 50% of residual [DPPH]. The results in EC_{50} and t_{EC50} of the DPPH scavenging activity of the two amidines and those for the positive controls (vitamin C, BHA, and BHT) are compiled in Table 1.

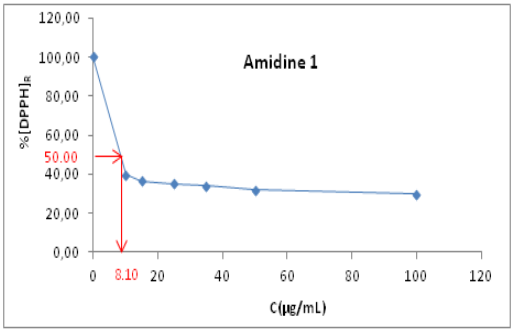


Figure 4. % $[DPPH]_R$ as function of concentration of amidine **1**

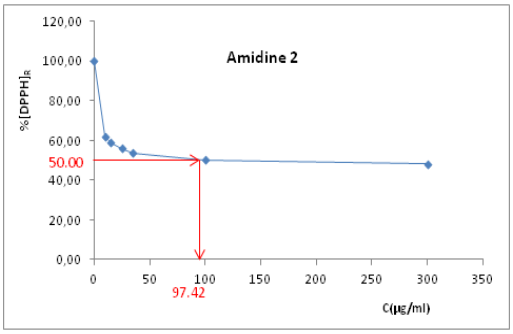


Figure 5. % $[DPPH]_R$ as function of concentration of amidine **2**

Table 1. EC_{50} and t_{EC50} from the kinetics of DPPH reduction by the tested compounds.

Compounds	EC_{50} ($\mu g.mL^{-1}$)	t_{EC50} (min)	ARE ($mL.\mu g^{-1}.min^{-1}$)
Amidine 1	8.10	210	5.88×10^{-4}
Amidine 2	97.42	300	3.46×10^{-5}
BHA	6.40	210	7.44×10^{-4}
BHT	4.80	255	8.17×10^{-4}
Vitamin C	2.38	60	7.00×10^{-3}

According to the results in Table 1, it is interesting to note that all the screened compounds displayed a radical scavenging activity by hydrogen donation. Amidine **1** with phenyl group, exhibited an antioxidant activity greater than that of amidine **2** with a fatty chain, as suggested by its low EC_{50} (8.10 $\mu g.mL^{-1}$) and high ARE (5.88×10^{-4} $mL.\mu g^{-1}.min^{-1}$).

The introduction of fatty alkyl group in the amidine **2** led to a considerable reduction of the radical scavenging activity as revealed by the significantly higher EC_{50} value (97.42 $\mu g.mL^{-1}$) and lower ARE value (3.46×10^{-5} $mL.\mu g^{-1}.min^{-1}$), suggesting the incidence of steric hindrance. These results indicate that the nature of substituent in amidine moiety is very pivotal and affects the related radical scavenging activity. We should also note that the reaction time profoundly influenced such activity. Indeed, in the case of amidine **2**, the amidine with a fatty alkyl chain, an improvement in the radical scavenging antioxidant activity (EC_{50} = 97.42 $\mu g.mL^{-1}$) was observed when the reaction time was prolonged to five hours, compared with that obtained after three hours ($EC_{50} = 5\times10^{3}$ $\mu g.mL^{-1}$) as reported in the literature [22]. The tested vitamin C displayed a radical scavenging activity significantly higher than BHT and BHA. The scavenging effect of amidine **1** and **2** and the standards on the DPPH radical decreased in this order: vitamin C >> BHT > BHA > amidine **1** > amidine **2**. With regard to the molecular structures, the efficacy of vitamin C, BHT and BHA was mainly due to the presence of hydroxyl group in their structures, vitamin C having the highest antiradical efficiency because of its two hydroxyl groups [34].

Antimicrobial Activity

The amidines **1** and **2** were screened for their *in vitro* antibacterial and antifungal activities against different bacterial and fungal species and the results are gathered in Tables 2 and 3.

Table 2. Results of antibacterial assay of amidines (diameters of inhibition zone in mm)

Indicator microorganisms	Amidines		Reference Standard Gentamicin
	1	**2**	
Gram-positive bacteria			
Staphylococcus aureus	0	0	21
Bacillus subtilis	0	0	21
Gram-negative bacteria			
Pseudeumonas aeruginosa	0	27	25
Escherichia coli	0	0	11
Klebsiella pneumoniae	0	0	8
Salmonella enterica	8	10	17

The data represent mean values of triplicate determinations. Values include the diameter of disk (6 mm).

Table 3. Results of antifungal assay of amidines (diameters of inhibition zone in mm)

Indicator microorganisms	Amidines		Reference Standards	
	1	**2**	Amphotericin B	Fluconazole
Fungi				
Mucor ramannianus	0	17	15	-
Fusarium culmorum	0	0	13	-
Yeasts				
Candida albicans	0	0	-	25
Saccharomyces cerevisiae	0	0	-	31

The data represent mean values of triplicate determinations. Values include the diameter of disk (6 mm).
-: Not determined.

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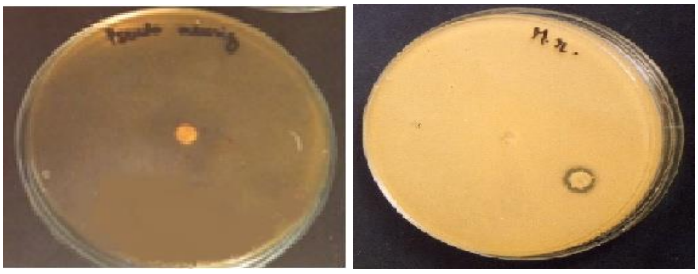
The results shown in Table 2 indicate that both amidines presented inhibition effects on the Gram-negative bacteria *Salmonella enterica* and that amidine **2** was more active than amidine **1**. On the other hand, only amidine **2**, with the fatty chain within the amidine moiety, exhibited potent inhibitory activity on the Gram-negative bacteria *Pseudomonas aeruginosa* as shown in Fig. 6. However, none of them manifested antibacterial activities against Gram-negative bacterium *Escherichia coli*, and *Klebsiella pneumonia*, or against Gram-positive bacterium *Staphylococcus aureus* and *Bacillus subtilis*. Also, with the exception of *Mucor ramannianus* towards which amidine **2** was active, the remaining fungi proved to be resistant to the amidines; this finding would imply the selective antimicrobial activity of amidine and the positive impact of fatty chain within it.

It should also be noted that the various inhibition assays were carried out at a charge of disk with an interval from 10 to 60 µg/disk . The maximums of inhibition were practically achieved at low charge not exceeding 10 to15 µg/disk for all of the strains tested.



A **B**

Figure 6. Antimicrobial activity of amidine **2** towards *Pseudomonas aeruginosa* (**A**) and *Mucor ramannianus* (**B**) .

Conclusion

Two amidine derivatives **1** and **2** were synthesized successfully and were subjected to investigation for their antioxidant activity *in vitro* (DPPH radical scavenging activity) as well as for their antimicrobial activity against some tested strains. The results indicated the potential antioxidant properties of amidines. The kinetic studies showed that the compound with phenyl group substituent on the amidine moiety exhibited a significant radical scavenging activity on DPPH assay compared with the standard antioxidants (BHT and BHA). The reaction time affects greatly the radical scavenging activity. On the other hand, an interesting antimicrobial activity against *Pseudomonas aeruginosa* and moderate antifungal one against *Mucor ramannianus* were observed only with amidine 2. The results showed also that both amidines tested had a weak antibacterial activity against *Salmonella enterica*.

These findings suggest that these molecules might serve as interesting compounds for the development of new antioxidant and antimicrobial agents and might be used in many diseases.

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