Antioxidant Activity of some Amidine Derivatives

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Abstract: *Three amidines, N, N'-Diphénylbenzamidine (**1**), N-Cyclohexyl-N'-phényl-octamidine (**2**), N, N'-diphényldodécamidine (**3**), were synthesized and investigated for their antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP) and β-carotene/linoleic acid assays. The results indicated that amidines tested possess good antioxidant activity. Amidine **1** showed the highest radical scavenging activity, while the fatty amidine **3** showed the lowest one. Increasing the amidine concentration resulted in an increasing ferric reducing antioxidant power for all amidines tested. The reducing power of fatty amidine **3** was relatively more prominent than those of **1** and **2**. Oxidation of the linoleic acid was strongly inhibited by all amidines. The obtained results were comparable to antioxidant properties of the standard antioxidants: butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT), ascorbic acid (vitamin C) and α-tocopherol (vitamin E).*

Keywords: *Amidines, Antioxidants, β-Carotene bleaching, DPPH, FRAP.*

1. Introduction

Antioxidants are promising agents for management of oxidative stress related diseases [1]. They play a major role in the prevention of cardiovascular diseases, cancers and neurodegenerative diseases, as well as inflammation and aging [2]. For this reason, there is a growing interest in substances that exhibit antioxidant properties, which are supplied to humans and animals as food components or as specific pharmaceuticals [3]. Several methods to quantify the antioxidant activity of natural or synthetic compounds are at hand [4, 5]. Of the synthetic compounds, amidines and their derivatives stand as valuable substances in various biological and industrial fields, namely medicine [6-8]. In fact, they have been reported to have multiple biological effects, including anticancer, antitumor, antiviral, antifungal and antimicrobial activities [8, 9]. They are also important intermediates in the field of organic synthesis [10], especially for the preparation of various heterocyclic compounds [11].

The aim of this study was to evaluate the antioxidant activities of three synthesized disubstituted amidines [12] and compare them to those of standard antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT), ascorbic acid (vitamin C) and α-tocopherol (vitamin E). To this end, three tests were employed: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric ions reducing antioxidant power (FRAP) and β-carotene/linoleic acid assays.

2. Material and methods

2.1. Chemical

Chemicals were purchased from either Sigma-Aldrich or Merck and were purified when necessary.

2.2. Synthesis

The selected amidines in this study have been synthesized by the reported procedures [12, 13]. The amidines prepared are: *N*, *N'*-diphenylbenzamidine **1**, *N*-cyclohexyl-*N'*-phenyl-octamidine **2** (amidine C8), and *N*, *N'*-diphenyldodecamidine **3** (amidine C12) (Figure 1).

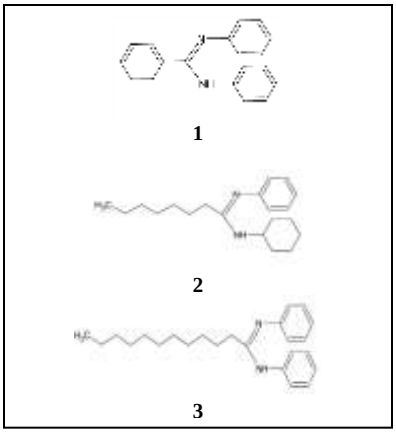

Fig. 1: Chemical structure of synthesized amidine derivatives : *N*, *N'*-diphenylbenzamidine 1, *N*-cyclohexyl-*N'*-phenyl-octamidine 2 (amidine C8), and *N*, *N'*-diphenyldodecamidine 3 (amidine C12)

2.3. Antioxidant assay

- **DPPH radical scavenging assay**

The free radical scavenging capacity of amidines was determined using the DPPH according to the method of Blois with some modifications [14]. A solution of DPPH (0.004%) in methanol was prepared and 1 mL of this solution was mixed with 1 mL of varying concentrations of amidine solution in ethanol. The reaction mixture was vortex thoroughly and left in the dark at room temperature for 3 h. The absorbance of the mixture was measured at λ_{max} = 517 nm using BHA, BHT, α-tocopherol (vitamin E) and ascorbic acid (vitamin C) as standards. The ability to scavenge DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_C - A_S)] / (A_C)] \text{ x100}$$

Where A_C is the absorbance of the control reaction (DPPH solution without the compound to be tested), A_S is the absorbance of sample.

The concentration of amidines providing 50% inhibition (IC_{50}) was calculated from the graph of the plot of inhibition percentage against amidine concentration (μg/mL) [14, 15].

- **Reducing power assay**

The ferric reducing potential of amidines was assayed as described by Oyaisu [16]. Different concentrations of amidines **1**, **2**, **3** in 1 mL of ethanol were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%), and then the mixture was incubated at 50 °C for 20 min. Afterwards, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at λ_{max} = 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

- ***β*-Carotene bleaching assay**

β-Carotene bleaching method is based on *β*-carotene oxidation (discoloring) induced by the products from linoleic acid oxidative degradation. *β*-Carotene bleaching assay was conducted by using the method suggested by Tepe [17] with some modifications. A solution of *β*-carotene was prepared by dissolving 0.5 mg of *β*-carotene in 1 mL of chloroform. 25 μL of linoleic acid and 200 mg of Tween 80 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL of distilled water saturated with oxygen were added with a vigorous shaking. 2.5 mL of this reaction mixture were dispersed in test tubes and 350 μL portions of the samples prepared at 2 mg/mL concentrations were added; the ensued emulsion system was incubated up to 48 hours at room temperature. The same procedure was repeated for positive control BHT and a blank. The absorbances of the mixtures were measured at λ_{max} = 490 nm at regular time intervals. The relative antioxidant activity (AA) was calculated according to the following equation:

$$AA\% = (A_{S\ 48h} / A_{C\ 48h}) \text{ x100}$$

Where $A_{S\ 48h}$ is the absorbance of the sample after 48h and $A_{C\ 48h}$ the absorbance of BHT used as the positive control.

3. Results and discussion

3.1. Synthesis

The derivative amidines synthesized were obtained in moderate yields (50-70%). Their structures were confirmed by spectroscopic analyses, including UV-visible, IR, ^{1}H-NMR and MS, as reported earlier [12].

3.2. Antioxidant activity

The antioxidant activity is the capability of a compound to inhibit the oxidative degradation, e.g. lipid peroxidation [18].

- **DPPH radical scavenging activity**

DPPH has been used extensively as a free radical to evaluate reducing substances [19] and is a useful reagent for investigating the free radical scavenging activities of compounds [20]. In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form, DPPH–H [14]. Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution [21].

In this study radical scavenging capacities of the amidines and standards (BHA and BHT) were measured by DPPH assay. Figure 2 shows free radical scavenging activity of amidines at different concentrations. The radical scavenging activity was observed to increase very slightly with increasing concentrations.

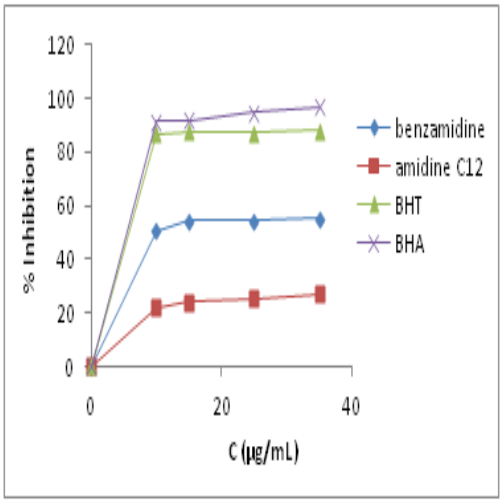

Fig. 2: Scavenging activity of the synthesized amidines compared with BHA and BHT as standards.

The IC_{50} values represent the concentration at which 50% of DPPH radical are inhibited. A lower IC_{50} value indicates a higher DPPH free radical scavenging activity. IC_{50} values (μg/mL) of amidines and the reference compounds are given in Table 1.

TABLE I: IC_{50} values (μg/mL) of amidines and reference antioxidants in the DPPH assay.

Antioxydant	IC_{50} (μg/mL)
BHT	3.85
BHA	3
Vitamin C	2.38
Benzamidine **1**	9.8
Dodecamidine **3**	5000

As shown in Table 1, amidine **1** with three phenyl groups exhibited higher activity (IC_{50} = 9.8 μg/mL), whereas amidine **3** with a long chain was of lower activity (IC_{50} = 5.10^3 μg/mL). This would indicate a potential

antioxidant activity of the amidine **1** and that the introduction of a bulky substituent on the amidine moiety like a dodecyl group as in **3** led to a considerable reduction in the radical scavenging activity as revealed by the high IC_{50} value.

The DPPH scavenging potential of amidines may be due to their reducing action by donating hydrogen atom to a free radical, reducing it to nonreactive species [22]. Higher DPPH scavenging potential of amidine 1 is likely due to its higher reducing potential.

From Table 1, the radical scavenging capacity of amidines and standards decreased in the following order: vitamin C > BHA > BHT > amidine **1** > amidine **3**.

- **Reducing power assay**

The reducing power of a substance is a measure of its reductive ability as antioxidant, and it is estimated by the transformation of ferric ion Fe^{3+} to ferrous one Fe^{2+} in the presence of the substance [23]. The reducing powers of amidines **1-3** are illustrated in Figure 3. From the figure, the reducing power increased with an increasing amidine concentration, particularly that of **3**. As observed, amidine **3** had the highest reducing capacity. The ability to reduce Fe^{3+} may be attributed to hydrogen donation from amidines. The reducing powers of amidines and of reference compounds at λ_{max} = 700 nm are shown in Figure 3and 4.

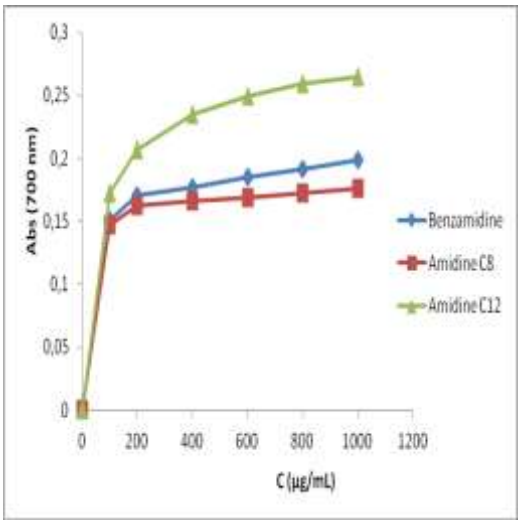

Fig. 3: Reducing powers of amidines at 700 nm.

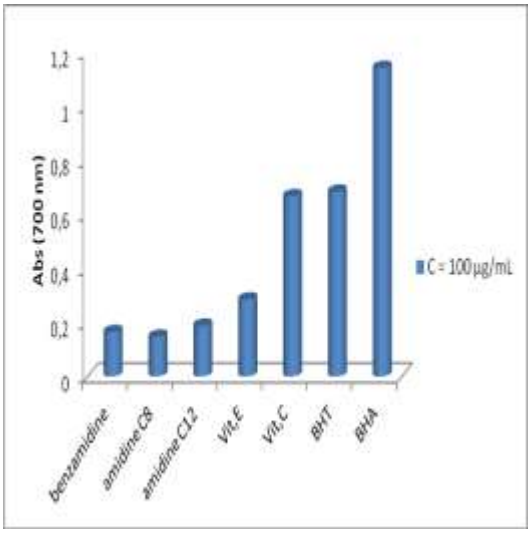

Fig. 4: Reducing power of amidines and reference antioxidants (C = 100 μg/mL).

As can be seen in Figure 4, the three amidines **1-3** demonstrated powerful Fe^{3+} reducing ability. However, these reducing powers were lower than those of the standard antioxidants. The order of reducing powers of amidines and the standard compounds at the same concentration was as follows: BHA > BHT > Vitamin C > vitamin E > **3** > 1 > **2**. The results cleary indicate a good antioxidant activity of the amidines.

- ***β*-Carotene bleaching assay**

The *β*-carotene bleaching method is widely used to measure the antioxidant activity of plant extracts. This method is based on the fact that linoleic acid produces a free radical which is reduced by *β*-carotene. The presence of an antioxidant prevents the reducing action of *β*-carotene which remains yellowish-orange in color [24]. The antioxidant activity of different amidines was assessed by following the discoloration of *β*-carotene at λ_{max} = 490 nm (Figure 5).

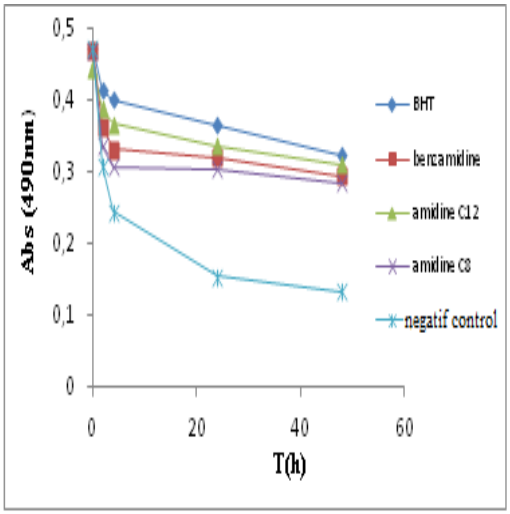

Fig. 5: Antioxidative potentials of various amidines and positive control BHT in *β*-carotene/linoleic acid system.

As seen in Figure 5, all the amidines studied inhibited the oxidation of *β*-carotene. This effect is due to either; the inhibition of linoleic acid peroxidation or the radical scavenging hydroperoxides formed during the peroxidation of linoleic acid (scavenger effect).

Figure 6 shows the percentage of the relative antioxidant activity (AA) of amidines and reference antioxidants in *β*-carotene / linoleic acid system.

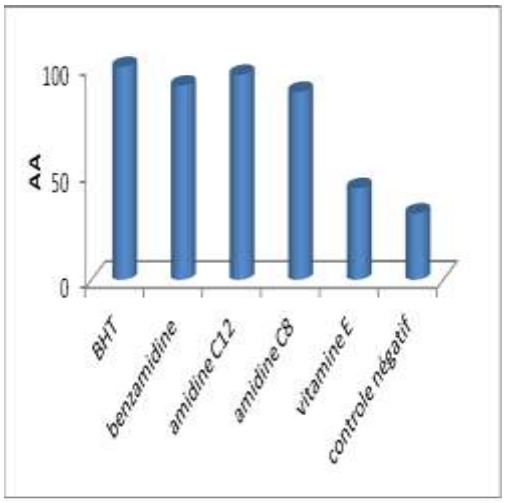

Fig. 6: Relative antioxidant activity (AA) of various amidines and positive control BHT in *β*-carotene/linoleic acid system.

The results indicate that fatty amidine **3** showed the highest antioxidant activity (96.30 %), followed by amidine **1** (91.23 %), then amidine **2** (88.28%). These values are higher than that of vitamin E and very similar to that of the antioxidant BHT.

4. Conclusion

On the basis of the above results, it can be concluded that the synthesized amidine derivatives exhibit variable and interesting antioxidant properties and free radical scavenging activities when compared to the standard antioxidants. Higher activity was observed in β-carotene bleaching assay.

Mechanistically, the antioxidant properties of the amidines may be attributed to their strong hydrogen donating ability and their effectiveness as scavengers of free radicals. According to the obtained data, it is clear that amidines have powerful antioxidant activity against various antioxidant systems. These *in vitro* assays indicate that amidines derivatives can be utilized as antioxidants in pharmaceutical applications and others such as food supplements.

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