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Antioxidant Activity of Two Mesomeric Heterocyclic Betaines Containing a Pyrimidine Moiety

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ABSTRACT

Two mesomeric heterocyclic betaines, phenyl pyrimidinium betaine (BT_1) and undecyl pyrimidinium betaine (BT_2), were investigated for their antioxidant activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant propriety was assessed by the effective concentration of the betaine (EC_{50}), the time required to reach the steady state of DPPH discoloration at EC_{50} concentration (TEC_{50}), the antiradical efficiency (AE), and the reduction kinetics . Vitamin E (α-tocopherol) and di-*tert*-butylhydroxytoluene (BHT) were used as the standard antioxidants in *in vitro* assays. Kinetic studies showed that BT_1 was more effective than vitamin E and BT_2.

Keywords: Betaine, pyrimidine, antioxidant, DPPH assay, reduction kinetic

INTRODUCTION

Antioxidant substances play an important role in scavenging the deleterious oxygenated radical species, thus providing protection to humans against infectious and degenerative diseases (Thirunavukarasu *et al*., 2010); under an aerobic medium, oxidation of natural substances such as lipids may occur via the reaction of formed radicals ($R^{\cdot}$) with oxygen, resulting in hydroperoxides (ROOH) which may degrade and causes damages. Therefore, consumption of food rich in a natural or synthetic antioxidant, would annihilate the endogenous oxidative deterioration. In the last few years, much attention has been focused on the synthesis of compounds and the extraction of natural ones that can potentially act as antioxidants (Pereira *et al*., 1999; Lebeau *et al*., 2000; Du Toit *et al*., 2001). In addition, new methods

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of estimating the antioxidant efficiency were developed (Sanchez-Moreno, 2002; Schwartz *et al*., 2001). Among these, the 2,2-diphenylpicrylhydrazyl (DPPH) trapping technique has been the most common one.

DPPH is a stable free radical and becomes a stable diamagnetic molecule by pulling out an electron or a hydrogen atom. In the DPPH radical-scavenging assay, antioxidants react with DPPH, and convert it to yellow- colored diphenylpicrylhydrazine. The color fading extent proves indirectly the radical-scavenging capacity of the antioxidant (Blois, 1958). The DPPH tests provided in the literature are based on the same principle as described by Brand-Williams *et al*. (1995), but the analytical protocols differ in several parameters.

The term "betaine" was originally coined for *N*, *N*, *N*-trimethylglycine. Being a polar amphoteric compound, betaine acts as an osmoprotectant in plants and as a protective agent for the liver, the heart and the vessels in humans. It is present in several plants (beet, wheat, spinach), and animals micro-organisms (*Propionibacterium shermanii*, *Denitrifying Pseudomonas*) (Huang et al., 2008). Today, the chemistry of betaines has become a subject of particular interest due to their applications in biological research, especially with regard to their metabolic roles in the living organism (Myers & Jibril, 1957). Indeed, alkylbetaines nowadays have a variety of uses in medicine, pharmacy, biology and other scientific fields (Domingo, 1996; Gonzaleza & Mesab, 2009). Some glycine betaine derivatives are employed as surfactants in cosmetics (Nsimba *et al*., 2010).

In the aim at investigating the antioxidant property of betaines, the previously synthesized mesomeric heterocyclic betaines (Malki *et al.*, 2011): phenyl pyrimidinium betaine (BT_1)* and undecyl pyrimidinium betaine (BT_2)**; the latter one having a fatty alkyl chain as a typical example of alkyl pyrimidinium betaines used as amphoteric surfactants (Fig.1). However, reports on biological activities of pyrimidinium betaines are very limited (Lindner *et al*., 2009; Gonzalez *et al*., 2009). To our knowledge, there was only one work unraveling the antioxidant potency of betaines, namely $(CH_3)_3N^+(CH_2)_nCOO^-$ with n =1-5, using hydroxyl radical scavenging (Kalvinsh *et al*., 1999;. M.M. Islam *et al*. 2009).

The antioxidant activity was ascertained by the radical scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH), a radical widely used in the reactivity studies of phenolic antioxidants (Blois, 1958; Molyneux *et al*., 2004).

Aside from its biological activity (Naik & Chikhalia, 2007), pyrimidine ring in the herein chosen betaines would favor the formation of a nitrogen-containing conjugated system ($>N^+=C-^{\cdot\cdot}N<$) that stabilizes free radicals (Wentrup, 1984).

*4-*H*-4-oxo-1,2,3,5 -tetraphenyl-1-pyrimidinium-6-olate

**4-*H*-4-oxo-1,3, 5-triphenyl-2-undecyl-1-pyrimidinium-6-olate

MATERIALS AND METHODS

Synthesis of pyriminium betaines

Betaines BT_1 and BT_2 were prepared as described previously (Malki *et al*., 2011) and as illustrated by the reaction shown in Fig.1 (2).

A typical synthetic protocol of BT_1 is as follows: Into a 10 mL round-bottomed flask, 5 mL of acetone was added and sequential addition of the following reactants was made under stirring at room temperature : 10^{-3} mole of dipentachlorophenyl phenylmalonate, and 10^{-3} mole of *N, N'*-diphenylbenzamidine. To the milky suspension obtained , 2×10^{-3} mole of triethylamine was added. Within one minute after the latter addition, a yellow solid precipitated at the bottom of the flask, leaving a yellow solution. The whole system was then stirred for 30 min at room temperature. Afterwards, the yellow precipitate was filtered off and recrystallized from chlorobenzene, yielding betaine BT_1 as yellow bright crystals.

By using the same procedure, undecylpyrimidinium betaine was obtained as white solid by the reaction of *N, N'*-diphenylundecamidine and *bis*pentachlorophenyl ester of phenyl malonic acid in diethyl ether for 1 h.

The betaines BT_1 and BT_2 were characterized by spectroscopic analyses, including UV-visible, IR, ^{1}H ^{13}C NMR and MS (see Malki et al., 2011). Their structures, along with those of vitamin E (*α*-tocopherol) and di-*tert*-butylhydroxytoluene (BHT), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), are illustrated in Fig.2 (1). And, their physical characteristics are gathered in Table 1.

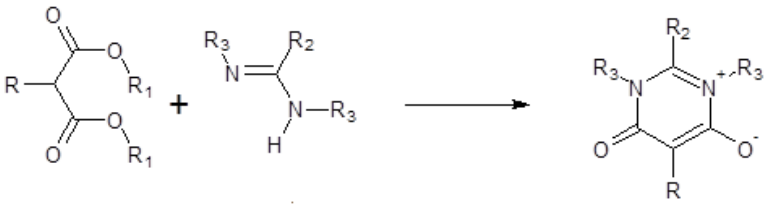
Protocol of DPPH radical scavenging assay

Methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was prepared and was added to the sample in ethanol at different concentrations (50, 100, 300, 500, 1000 µg/ml).

The first step was to determine the stabilization time for the DPPH methanolic solution discoloration. Then, the inhibition kinetics was studied for the two pyrimidinium betaines at each concentration. The reduction kinetics of DPPH at different concentrations in the tested antioxidants was followed until a plateau was reached, corresponding to the stabilization time (T_{eq}). At this stage, the UV-visible analysis was taken, and the percentage of unreacted DPPH, $(DPPH)_{unr}$ was estimated for each betaine concentration and at different times (T), from absorbance

TABLE 1
Physical characteristics of BT_1, BT_2, vitamin E (α-tocopherol), BHT, and DPPH.

Compound	Appearance	Molecular weight (g/mol)	Melting point °C
BT_1	Yellow crystals	416	317-319
BT_2	White needles	494	134-138
Vitamin E	Oily colorless liquid or yellow-brown liquid	430	2-3
BHT	White powder	220	70-73
DPPH	Black to green powder (purple in solution)	394	135



BT_1: $R = R_2 = R_3 = C_6H_5$
BT_2: $R = R_3 = C_6H_5$; $R_2 = C_{11}H_{23}$

Fig.1: Synthetic pathway of BT_1 and BT_2

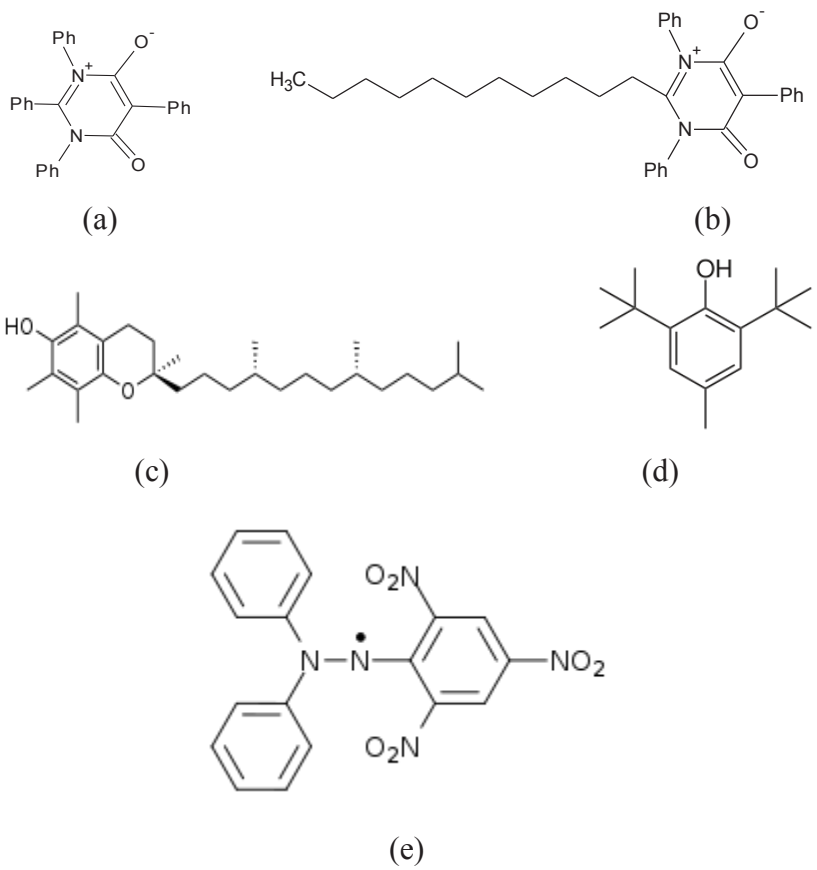


Fig.2: Structures of phenyl pyrimidinium betaine BT_1 (a), undecyl pyrimidinium betaine BT_2 (b), vitamin E (α-tocopherol) (c), di-*tert*-butylhydroxytoluene (BHT) (d), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (e).

at λ_{max} = 517 nm via equation Eq.1.

$$\% (DPPH)_{unr} = 100 \times (DPPH)_{T = Teq}/(DPPH)_{T = o} \quad [Eq.1]$$

where $(DPPH)_{T = Teq}$ and $(DPPH)_{T = 0}$ are the absorbances of DPPH solution at the start of reaction with sample (T = 0) and at T = T_{eq}, respectively.

The well-known antioxidants, vitamin E (α-tocopherol) and di-*tert*-butylhydroxytoluene (BHT), were employed as positive controls.

Effective concentration of sample required to scavenge 50% of DPPH radicals (EC_{50}) was computed from the plot of the percentage of unreacted DPPH curve versus sample concentrations. In order to easily characterize the behavior of a substance as an antioxidant, the antiradical efficiency parameter (AE) was also calculated. The latter parameter combines the two parameters, EC_{50} and T_{EC50}. Antiradical efficiency (AE) was then determined according to equation Eq.2:

$$AE = 1 / (EC_{50} \times T_{EC50}) \quad [Eq.2]$$

Where EC_{50} is the concentration required to reduce the DPPH discoloration by 50%, and T_{EC50} is the time required by each compound to reach the steady state of DPPH discoloration at EC_{50} concentration (Sanchez-Moreno *et al*., 1998).

RESULTS AND DISCUSSION

DPPH has been used extensively as a free radical to evaluate reducing substances (Motlhanka *et al*., 2008) and a reagent for investigating the free radical scavenging activities of compounds (Duan *et al*., 2006). As the electrons become paired off, the solution color faded stoichiometrically depending on the number of electron taken up (Blois, 1958). Hence, this assay provides an insight into the reactivity of the tested samples towards a stable free radical (Senthilkumar *et al*., 2010).

The kinetic effects of scavenging BT_1 and BT_2 by DPPH at their different concentrations are illustrated in Fig.3, showing the extent of DPPH inhibition obtained at each concentration for each betaine at the steady-state time (Teq). From this figure, the DPPH radical scavenging activity towards pyrimidinium betaines is clearly demonstrated, and the DPPH drop was concentration-dependent. The decrease in the UV-visible absorbances of the DPPH radical engendered by test samples was due to the radical scavenging by electron donation.

The kinetic effects of DPPH scavenging of vitamin E and BHT used as standards are presented in Fig.4.

The effective concentrations (EC_{50}) of BT_1 and BT_2 were obtained from the curve plotting the percentage of unreacted DPPH and its concentration at T = T_{eq}, using graphic interpolation.

The different antioxidant parameters EC_{50}, T_{EC50} and AE obtained are gathered in Table 2. It can be easily noticed that the synthesized betaines BT_1 and BT_2 revealed a radical scavenging activity. The kinetic studies showed that the obtained results for the betaines were closer to those of vitamin E and drastically lower when compared

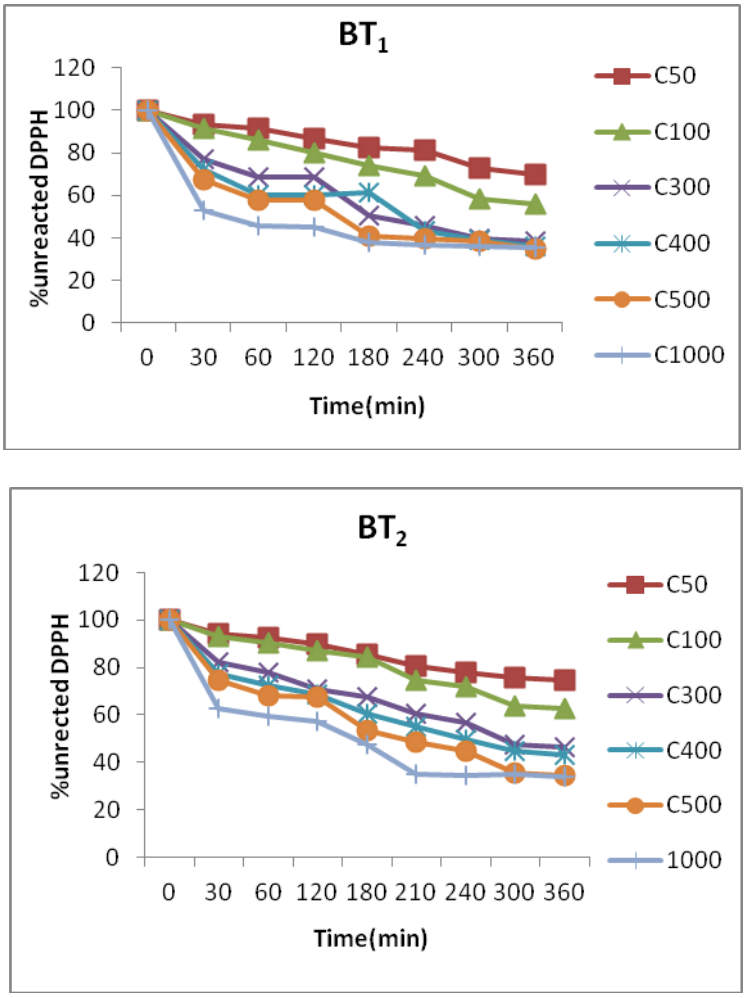


Fig.3: Kinetics of DPPH scavenging effects of BT_1 and BT_2

TABLE 2
Results of the kinetics of reduction of DPPH.

Compound	EC_{50}(μg/mL)	T_{EC50}(min)	AE(μg/mL.min)$^{-1}$
BT_1	150	180	3.70×10^{-5}
BT_2	250	240	1.66×10^{-5}
Vitamin E	152	210	3.13×10^{-5}
BHT	1.5	150	4.44×10^{-3}

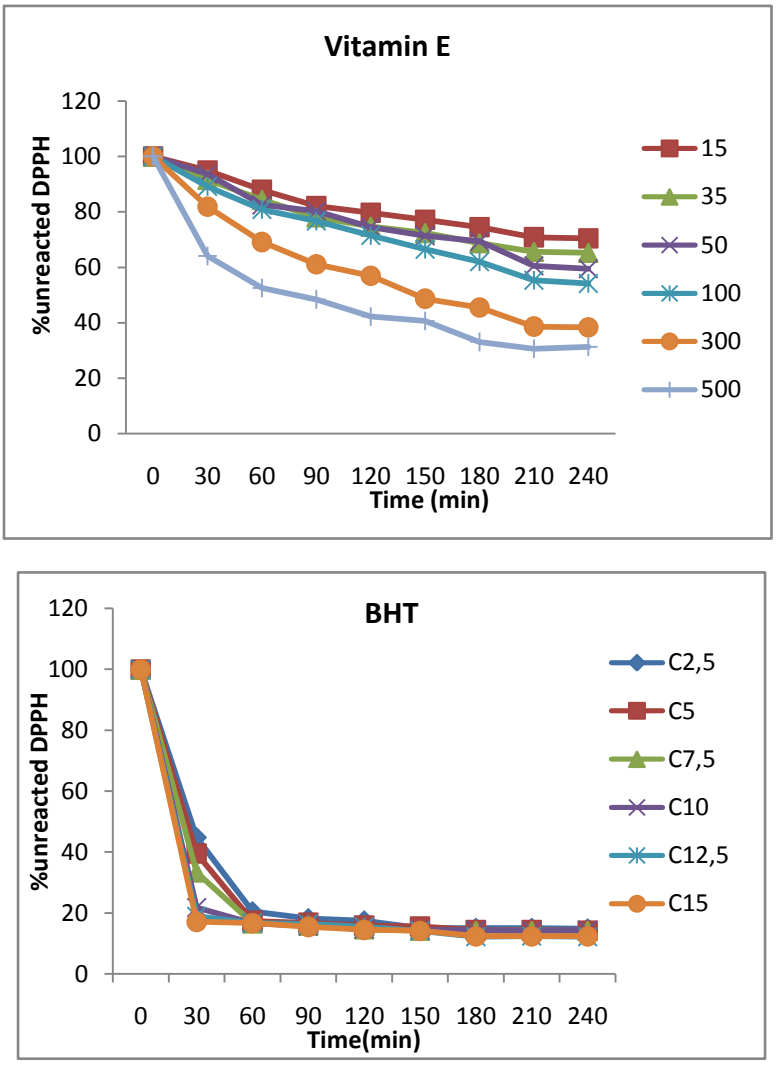
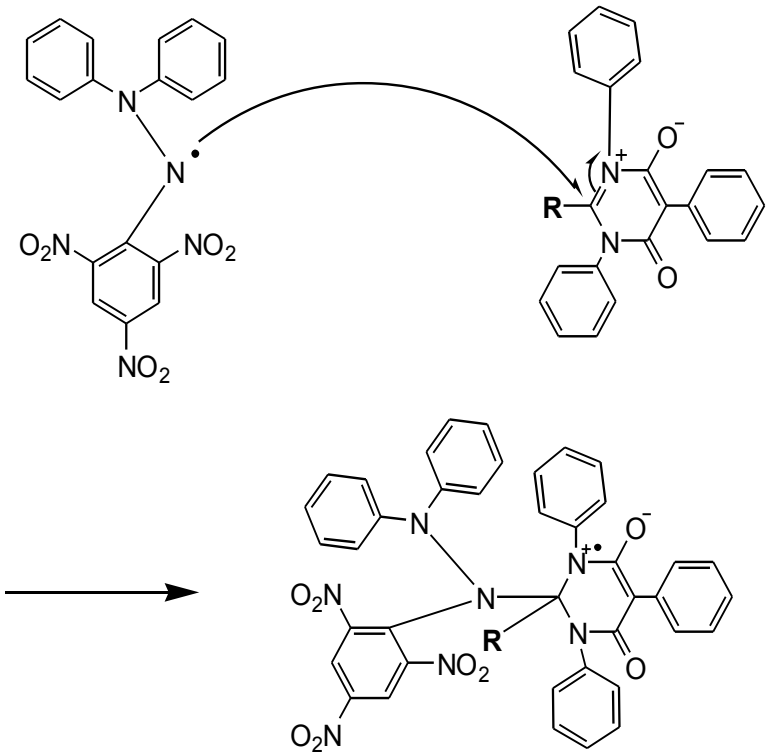


Fig.4: Kinetics of DPPH scavenging effects of vitamin E and BHT

with those of BHT. Considering the two pyrimidinium betaines and vitamin E, it is interesting to note that BT_1 was the most potent antiradical reactivity, because it has higher AE than BT_2 and vitamin E, and took a short time to reach the plateau. Whereas a lower antioxidant capacity was obtained for the BT_2. Steric effects might play a fundamental role in the reaction with DPPH. Hence, the antioxidant efficacy is probably related not only to its reducing properties, but also to steric factors that might influence its ability to approach the electron deficient reactive sites. Indeed, the low reactivity of BT_2 towards the DPPH radical can be ascribed to the presence of the fatty alkyl chain, being a long one, hence providing a steric hindrance; with a shorter alkyl chain



R = C_6H_5; $C_{11}H_{23}$

Fig.5: Proposed mechanism of the attack of betaine by DPPH

or a small group, such steric would be greatly insignificant. The higher reactivity of BT_1 could be associated with the size of the phenyl group. Fig.5 depicts the plausible mechanism of the reaction of DPPH with pyrimidinium betaines. As can be seen, the hindrance factor definitely aroused, not only from the betaines but also from the DPPH molecule. With regard to the mechanism of the action of DDPH towards vitamin E and BHT, as known, it takes place by pulling out the hydrogen atom of the phenolic hydroxyl group, being very labile (Patt & Hudson, 1990; Bondet *et al*., 1997). However, that for the action towards pyrimidinium betaines occurred differently; DPPH may attack the betaine and forms a covalent bond as shown in Fig.5; the radical on the nitrogen atom is greatly stabilized by the two phenyl and carbonyl groups of the pyrimidinium betaine.

By comparing the values of the antiradical efficiency (AE), the scavenging effect on the DPPH radical decreased in the order of: BHT >> BT_1 > Vitamin E > BT_2.

Although BHT has an antiradical efficiency higher than that of vitamin E, the lower antioxidant activity of the latter

could be attributed to steric effect due to the presence of the long alkyl chain in this molecule. On the other hand, the antioxidant activity of pyrimidinium betaines could be attributed to the conjugated systems with nitrogen atoms which are known to stabilize free radicals (Wentrup, 1984). In the case of $BT_{2,}$ the low antioxidant activity could be also imputed to the steric effect of fatty alkyl chain.

CONCLUSION

DPPH radical scavenging method has ascertained the oxidant potency of pyrimidinium betaines. The above results suggested the effects of the nature of the substituent and the steric hindrance of length of the alkyl chain affixed on the hydrophilic head of the betaine. The kinetic studies showed that phenyl pyrimidinium betaine BT_1 is more active than undecyl pyrimidinium betaine BT_2 and standard vitamin E and less active than BHT. Further investigation will decipher the exact mode of action of these compounds at molecular level.

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