Effects of Various Parameters on the Antioxidant Activities of the Synthesized Heterocyclic Pyrimidinium Betaines

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Abstract: *Betaine derivatives are widely used in cosmetic, industrial uses, biology and other scientific fields. Pyrimidinium betaine is a special class of bioactive heterocyclics. They have interesting antioxidant and free radical scavenging activities. This work aims to examine the influence of some parameters on the antioxidant activity of some synthesized betaines containing pyrimidine ring. Four pyrimidinium betaines: monocyclic, bicyclics, and one with a fatty alkyl chain were synthesized from condensation of 2-aminopyrimidine or amidine derivatives with malonic esters, and their antioxidant capacity was evaluated. The effects of concentration, reaction time and temperature on their antioxidant activities were investigated by three common methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferric reducing antioxidant power (FRAP) and β-carotene bleaching. The results showed that all pyrimidinium betaines exhibited antioxidant activities in different assays. In the DPPH and reducing power assays, antioxidant activity increased with concentration, whereas in the β-carotene/linoleic acid system, it increased with temperature. On the other hand, the DPPH assay showed an increase in antioxidant capacity over time, while the β-carotene bleaching assay showed a decrease. These results indicate that the antioxidant activity differs depending on the method used and that the various factors affect the antioxidant activity in a different order.*

Keywords: *antioxidant activity; DPPH; FRAP; β-carotene bleaching assay; pyrimidinium betaines*

■ INTRODUCTION

Molecules or molecular fragments with an unpaired electron are classified as free radicals. They are typically reactive and can have negative consequences [1]. Free radicals are one of the principal byproducts of lipid oxidation and have been linked to over a hundred disorders, including cancer, atherosclerosis, and arthritis [2]. Excessive free radical production leads to oxidative stress, which is thought to be a crucial factor in a variety of human diseases such as neurological, inflammatory, carcinogenesis, and psychiatric disorders [3].

Antioxidants are substances that can delay or prevent the oxidation process of free radicals [4]. Excess free radicals are neutralized by dietary antioxidants that transform them into non-radical products and/or scavenge the intermediates [5]. Due to their ability to scavenge reactive free radicals, antioxidants can protect the human body from free radicals and the consequences of reactive oxygen species (ROS). They slow the evolution of several chronic diseases while also lipid peroxidation [6-7]. A variety of antioxidant molecules have been added to foods to preserve their quality, and a variety of antioxidant molecules have been provided to humans and animals as food additives or pharmaceuticals [6]. Antioxidants have proven effective in treating many health problems, including neurodegeneration, systemic, and infectious [8]. Recently, many researchers have focused on research on

substances that can act as a free radical scavenger, and there is an increasing interest in substances that present antioxidant capacities. Concurrently, several methods for evaluating the antioxidant activity of natural and synthetic compounds have been developed [9-11].

Betaine is an interesting group of zwitterionic surfactants. The term betaine refers to the glycine betaine (*N*,*N*,*N*-trimethylglycine), as well as its derivatives and any organic compound with quaternary nitrogen [12]. Betaine is found in a variety of plants, animals, and microorganisms as it aids in the resistance to osmotic stress in these organisms. This molecule is a human nutrient and is used to treat a variety of ailments [13].

Betaine derivative is a class of compounds that are becoming increasingly important in cosmetic, domestic, and industrial uses [14]. They have a variety of applications in biological research, medicine, pharmacy, and other scientific fields [15]. Pyrimidine derivatives have been reported to have a wide spectrum of biological activity since the pyrimidine ring is a critical nucleus in DNA and RNA [16]. Betaines containing a pyrimidine ring have generated a lot of interest among synthetic chemists for their chemical stability [17]. These materials are strongly stabilized by π electron and charge delocalization [18]. Pyrimidine betaines continue to be an important group of bioactive heterocyclics [19] due to the presence of a biologically active pyrimidine ring [16] in their structure.

However, there are very few reports on the antioxidant activities of pyrimidinium betaines [20], and to our knowledge, no information is available on the antioxidant potency of pyrimidinium betaines having a fatty alkyl chain. For this reason, we decided in a recent study to evaluate the *in vitro* antioxidant capacity of various betaines containing a pyrimidine ring in their structure, including one with a long chain, as a type of alkyl pyrimidinium betaines used as amphoteric surfactants. The results showed that the tested compounds have variable and interesting antioxidant properties and free radical scavenging activities compared to the standard antioxidants [20-22]. However, without considering many factors that could affect the results, the measurement of antioxidant activities cannot be evaluated satisfactorily by a simple antioxidant test [23].

The goal of this study was to investigate how different factors affected the antioxidant activity of some synthesized pyrimidinium betaines. We used three distinct methods: DPPH free radical scavenging, ferric reducing antioxidant power (FRAP) and β-carotene bleaching, to examine the effect of concentration, reaction time, and temperature on the antioxidant activity of these compounds and on some conventional antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ascorbic acid (vitamin C).

■ EXPERIMENTAL SECTION

Materials

Chemicals and solvents were analytical grade and purchased from Sigma-Aldrich, Merck (Germany). They were supplied in purities ≥ 99. Triethylamine ($(C_2H_5)_3N$) was purified by distillation before use.

Instrumentation

Melting points were determined by using a Buchi 512 oil bath. UV-Visible absorption spectra were carried out using a Shimadzu 160 double-beam spectrophotometer. FTIR spectra were recorded as KBr pellets on a JASCO System 4100. ^{1}H-NMR and ^{13}C-NMR spectra were acquired on a Bruker AC 200 instrument at 300 and 75 MHz, respectively, using TMS as the internal standard. Electron impact mass spectra (EIMS) were performed on a Nermag R10-10 type apparatus.

Procedure

Synthesis

The selected pyrimidinium betaines: monocyclic BT1, bicyclics BT2, BT3 and one with fatty alkyl chain having 12 carbon atoms BT12 (Fig. 1), were synthesized from condensation of 2-aminopyrimidine or amidines derivatives with malonic acid derivatives according to the methods reported in previous publications [24-26]. Their structures were confirmed by spectroscopic analyses, including UV-visible, IR, ^{1}H-NMR, ^{13}C-NMR and MS.

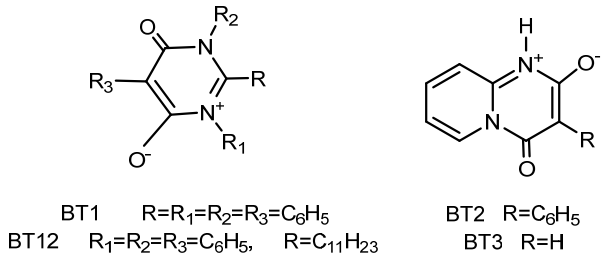

Fig 1. Chemical structure of studied pyrimidinium betaines

General procedure for the synthesis of bicyclic Betaines BT2, BT3. The bicyclic betaine BT3 was synthesized according to the method reported by Tschitschibabin [24] by condensation of 2-aminopyrimidine **C** with an excess of diethyl malonate **B** through heating the reaction mixture at 160–200 °C for 3 h with a continuous distillation of the resulting alcohol (Fig. 2). The corresponding betaine was obtained in 81% yield as an orange solid.

The bicyclic betaine BT2 was prepared according to the method reported by Dvortsák et al. [25] by condensation of 2-aminopyrimidine **C** with the bis pentachlorophenyl ester of phenyl malonic acid **A** (Fig. 2). The reaction was performed in acetone at room temperature in the presence of triethylamine. In a few minutes, the product precipitated and was isolated by filtration with a yield of 72%.

General procedure for the synthesis of monocyclic Betaines BT1 and fatty chain betaine BT12. Monocyclic betaine BT1 was prepared by condensation of *N*,*N*'-diphenyl benzamidine **D** instead of α-aminopyrimidine **C** with the bis pentachlorophenyl ester of phenyl malonic acid **A** in diethyl ether at room temperature [25-26] in the presence of triethylamine (Fig. 3). The product was isolated by filtration, purified by extraction or column procedure [27-28] and then obtained as yellow crystals in a yield of 66%.

Under the identical protocol, the fatty chain pyridinium betaine BT12 was prepared by reacting fatty *N*,*N*′-diphenyldodecamidine **E** with the same ester **A** in diethyl ether at room temperature [26] in the presence of triethylamine (Fig. 3). Due to the chemical reactivity of malonic ester **A**, the fatty betaine BT12 precipitated within minutes. The precipitate was then purified [29-30] and obtained as a white solid with a 37% yield.

Characterization of synthesized compounds

Pyrimidinium betaines, BT3, BT2, BT1, and BT12 were characterized by common spectroscopic analyses, including UV-visible, IR, ^{1}H-NMR, ^{13}C-NMR and MS [29-30]. Their physical and spectroscopic data are as follows:

BT3 (6-oxo-4*H*-pyrido[1,2-a]pyrimidin-3-ium-4-olate). Chemical formula: $C_8H_6N_2O_2$; Orange solid; Mol. Mass. 162 g/mol, m.p.: 306–317 °C (decomposed), UV-Visible (dioxane) λ_{max}: 268 nm; IR (KBr) cm^{-1}: 3093 (C-H, Ar.), 2553 (NH); 1693 (C=O); 1519 (C=C Ar.); ^{1}H-NMR (300 MHz, DMSO-d_6): insoluble; ^{13}C-NMR (75 MHz, DMSO-d_6): insoluble; EI-Mass *m/z*: 163 $[M+1]^+$.

BT2 (6-oxo-5-phenyl-4*H*-pyrido[1,2-a]pyrimidin-3-ium-4-olate). Chemical formula: $C_{14}H_{10}N_2O_2$; Yellow crystals; Mol. Mass. 238 g/mol, m.p.: 310–312 °C (decomposed); UV-Visible (dioxane) λ_{max}: 355 nm; IR (KBr) cm^{-1}: 3112 (C-H Ar), 2657 (NH), 1678 (C=O),

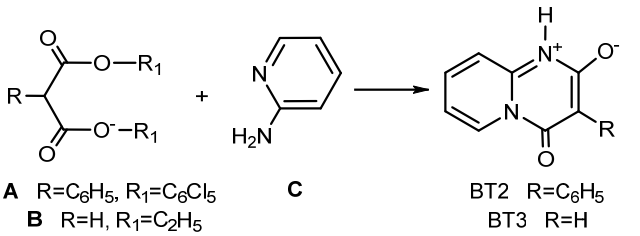

Fig 2. Synthetic pathway of bicyclic betaines BT2 and BT3

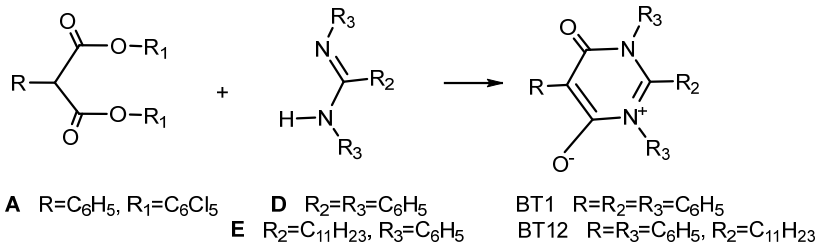

Fig 3. Synthetic pathway of bicyclic betaines BT1 and BT12

1527 (C=C, Ar.); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.42–12.27 (s, 1H, NH), 9.05–9.03 (d, 1H, 7), 8.14–8.10 (m, 1H, 9), 7.72–7.66 (d, 2H, 8, 10), 7.46–7.13 (m, 5H, Ar.); ^{13}C-NMR (DMSO-d_6): δ 160 (C-4, C-6), 155.15 (C-2); 146–135 (C-7, C-8, C-9, C-10), 130–125.69 (C, Ar.) 116 (C-5); EI-Mass *m/z*: 238.0742 $[M]^+$.

BT1 (6-oxo-1,2,3,5-tetraphenyl-1,6-dihydropyrimidin-3-ium-4-olate). Chemical formula: $C_{28}H_{20}N_2O_2$, Yellow crystals; Mol. Mass. 416 g/mol, m.p.: 317–319 (decomposed); UV-Visible (dioxane) λ_{max}: 357 nm; IR (KBr) cm^{-1}: 3051 (C-H, Ar.); 1651 (C=O), 1593 (C=C, Ar.); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.82–6.98 (m, 20H, Ar.); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 159.87 (C-4, C-6), 159.35 (C-2), 137.77 (C-7, C-19), 135.76–130.58 (C-13, C-16), 129.91/128.77 (C-8,12,20,24, C-9,11,21,23, C-10,22), 127.65/125.36 (C-14,18, C-15,17), 96.03 (C-5); EI-Mass *m/z:* 416.1527 [M].

BT12 (6-oxo-2-dodecyl,1,3,5-tripheny-1,6-dihydro pyrimidin-3-ium-4-olate. Chemical formula: $C_{33}H_{38}N_2O_2$; White solid; Mol. Mass. 494 g/mol; m.p.: 124–128; UV-Visible (dioxane) λ_{max}: 352 nm; IR (KBr) cm^{-1}: 3055 (C-H, Ar.), 1647 (C=O); 1597 (C=C, Ar.); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.91–7.14 (15H, Ar.), 2.41–0.78 (23H, aliph.), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 161.42 (C-14,16), 159.00 (C-12), 136.27–125,76 (C, Ar.) 32.77–14.11 (C, aliph.), EI-Mass *m/z*: 495.2 $[M+1]^+$.

Antioxidant activity

There are still no standardized procedures for determining a sample's antioxidant potential [31]. Since various trends in antioxidant activity assays have been noticed, it is useful to estimate and compare the antioxidant potential of synthetic compounds using several assays [32]. To assess the effects of concentration, temperature, and reaction time on the antioxidant activity of the pyrimidinium betaines under investigation, we used three methods: DPPH assay, reducing power measurement and β-carotene bleaching procedure. In parallel tests, the effects of parameters on the antioxidant activity of conventional antioxidants (BHT, BHA and ascorbic acid (vitamin C)) used for comparison have been studied. All samples were assayed in triplicate.

DPPH radical scavenging assay

The DPPH method is commonly used to evaluate antioxidants' ability to scavenge free radicals [27]. The reduction of an alcoholic solution of DPPH in the presence of a hydrogen or electron donor antioxidant is the basis of this method. The ability of the related compounds to donate a hydrogen atom or an electron was determined spectrophotometrically by bleaching the purple methanolic solution of DPPH [28]. In this study, the capacities of pyrimidinium betaines to quench the DPPH radical were measured according to the method of Blois [10] and Brand-Williams et al. [11] with some modifications.

A 0.004% DPPH solution in methanol was prepared, and 1 mL of this solution was mixed with 1 mL of various concentrations of betaines solution in ethanol. The reaction mixture was carefully agitated and then kept at room temperature in the dark. The absorbance was measured spectrophotometrically at 517 nm in comparison to a negative control containing only DPPH solution.

The radical scavenging activity of DPPH was calculated as a percentage inhibition using the following formula (Eq. (1)):

$$\text{DPHH inhibition } (\%) = \frac{Ac - As}{Ac} \times 100 \quad (1)$$

where Ac denotes the absorbance of the control reaction (DPPH solution devoid of the substance to be tested) and As indicates the absorbance of the test sample.

Reducing power assay

The reducing power of a substance is a measure of its antioxidant ability, and it is evaluated by converting Fe(III) to Fe(II) in the presence of the sample extract [6]. Singh et al. [33] ascribe the capacity to reduce Fe(III) to the hydrogen-donating ability of phenolic compounds. The yellow color of the test solution in this assay changes to green and blue depending on the reducing power of the test material. Based on the absorbance at 700 nm after incubation, the oxidant's ability to decrease the ferric ferricyanide complex to the ferrous one was measured. The greater absorbance indicates a greater

reducing power [34]. The Oyaizu method was used to evaluate the reducing power of the synthesized compounds [35].

Different concentrations of betaines in 1 mL of ethanol were combined 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%) before incubating at 50 °C for 20 min. Following that, 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 top layer solution was combined with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%), and absorbance at 700 nm was recorded.

β-Carotene bleaching assay

This method is based on the fact that linoleic acid generates a free radical, which β-carotene reduces. The inclusion of an antioxidant allows for a delay in the kinetics of β-carotene discoloration [36]. Several researchers [37] found the test of inhibition of linoleic acid oxidation in combination with β-carotene to be highly useful as a model of lipid peroxidation in biological.

β-Carotene bleaching assay was conducted by using the method suggested by Tepe and Moure [38-39] with some modifications. The absorbance of the samples was measured at 470 nm at regular time intervals using a spectrophotometer. The same procedure was followed with the positive controls, BHT, BHA, and vitamin C, as well as a blank. The following formula (Eq. (2)) was used to compute the Relative Antioxidant Activity (RAA) as a percentage:

$$\text{RAA } (\%) = \frac{\text{As}}{\text{Ac}} \times 100 \qquad (2)$$

where As is the absorbance of the sample) and Ac, the absorbance of BHT used as the positive control.

Statistical analysis

Data were analyzed with the statistical software statistica. Values were expressed as means ± standard deviations (SD). Differences were considered significant at $p < 0.05$.

■ RESULTS AND DISCUSSION

Pyrimidinium betaines were synthesized, as reported earlier [24-26]. The effects of various parameters (concentration, reaction time, and temperature) on their antioxidant activities were investigated using DPPH assay, ferric ions reducing antioxidant power (FRAP), and β-carotene bleaching procedure as three separate tests.

Concentration Effect

The antioxidant properties of the examined substances were studied *in vitro*, and the effect of the concentration on their antioxidant activity was examined by DPPH radical scavenging capacity and FRAP in the concentration range of 0–500 g/mL.

DPPH radical scavenging assay

Fig. 4 presents the inhibition percentage of the DPPH free radical in the presence of betaines at various concentrations for 180 min. As shown in Fig. 4, all betaines exhibited potent activity in a concentration-dependent manner. This activity can be attributed to the conjugate systems with nitrogen atoms in these molecules, which are known to stabilize free radicals [20]. The findings of this study show that the compounds examined have variable rates of scavenging DPPH radicals.

On the basis of the statistical analysis of our data, we noted that at low concentrations (0–100 µg/mL), bicyclic betaine BT3 exhibited a higher DPPH radical scavenging rate than the remaining betaines, whereas at high concentrations (200–500 µg/mL) both monocyclic BT1 and fatty betaine BT12 showed the highest DPPH radical scavenging rate, and that monocyclic betaine BT1 is more active than fatty betaine BT12 and betaine BT3, while the bicyclic betaine BT2 has the lowest radical

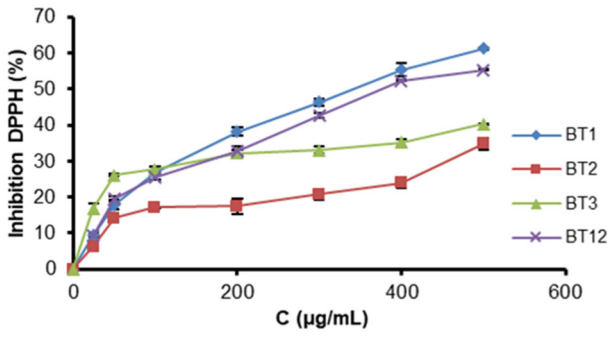

Fig 4. Inhibition of DPPH free radical in the presence of pyrimidinium betaines

scavenging activity over the entire concentrations range (0–500 µg/mL). Fig. 5 shows the impact of concentration on betaines' antioxidant activity, using different concentrations (25, 100, 500 µg/mL) as an example, maintaining the reaction time constant for 180 min.

Fig. 5 indicated that the antioxidant's efficiency increased as the concentration of the tested betaines increased. At 25 µg/mL, the antiradical activity of the betaines was very weak, and the percentages of inhibition of DPPH radical by the compounds BT1, BT2, and BT12 were very close and lower than that of BT3. At 100 µg/mL, their antioxidant capacity was moderate. At the maximum value (500 µg/mL), the antioxidant's power had increased, and monocyclic betaine BT1 had the highest activity that was comparable to long-chain betaine BT12 ($p < 0.05$).

Betaines' DPPH radical scavenging activity could be due to their reducing action by electron donation, converting a free radical to a nonreactive species [20]. The nitrogen-atom conjugated systems that are more abundant in monocyclic betaine BT1 and fatty betaine BT12 than in bicyclic betaines BT2 and BT3 may be responsible for the antioxidant activity of these two molecules. In betaines BT1 and BT12, the radical formed is strongly stabilized by resonance through the phenyl and carbonyl groups [22].

Reducing power assay

The reducing properties of tested betaines at 700 nm are illustrated in Fig. 6. As shown in Fig. 6, all betaines displayed potent reducing power in a concentration-dependent manner. With increasing concentrations, the reducing power of the tested betaines increased, reaching a plateau further than 200 g/mL.

To investigate the effect of concentration on betaines' reducing power, we used as an example three different concentrations (20, 100, 500 µg/mL), as shown in Fig. 7. By examining the results, we observed that the reducing power of each betaine examined increased somewhat with increasing concentration and that on the three proposed concentrations, monocyclic betaine BT1 exhibited the highest reducing power, while long chain betaine BT12 revealed the lowest one. Decreased reducing

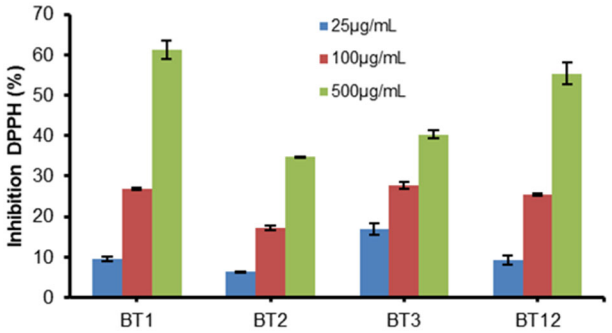

Fig 5. Effect of concentration on the antioxidant activity of tested betaines

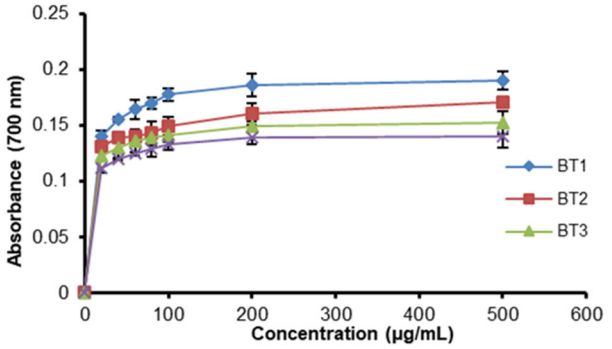

Fig 6. Reducing powers of pyrimidinium betaines

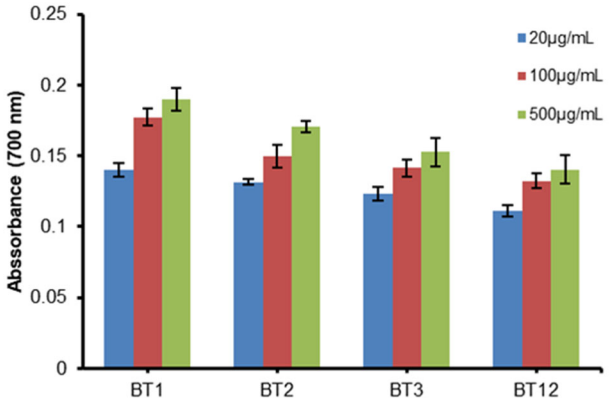

Fig 7. Effect of concentration on reducing the power of pyridinium betaines

power of BT12 appears to be related to a steric effect [22]. The presence of a long alkyl chain in fatty betaine could provide steric hindrance, which would decrease the contact with the Fe^{3+} ions. Based on the previous findings, we conclude that in both tests (DPPH and reducing power), the antioxidant efficiency of betaines increases with increasing concentration and that monocyclic betaine BT1 has the highest antioxidant activity.

The results of the effect of concentration on the reducing power of betaines and reference antioxidants (BHA and vitamin C) are shown in Fig. 8. As shown in Fig. 8, the evaluated betaines were less active than the reference antioxidants BHA, BHT, and Vitamin C ($p < 0.05$). The latter is strongly influenced by the concentration and showed the highest activity. With regard to the molecular structures, the reference antioxidants' higher reducing power could be due to the presence of hydroxyl groups in their structure [31], while the pyridinium betaines' lower reactivity was mainly due to steric hindrance in these molecules.

Effect of Time

We used two methods to study the effect of time on the antioxidant activity of selected betaines: the DPPH radical scavenging assay and the β-carotene bleaching assay.

Test for DPPH radical scavenging

To assess the scavenging activity as a function of time, kinetic studies of the DPPH-betaines reaction were conducted while keeping their concentration constant at 300 µg/mL. The percentages of inhibition of the DPPH radical as a function of time are represented in Fig. 9.

Fig. 9 illustrated that the antioxidant activity of the betaines increased as the reaction time increased. The results revealed that specific activity of the compounds was obtained depending on the reaction time used: during the 0–60 min period, bicyclic betaines BT2 and BT3 had low activity, as did monocyclic BT1 and fatty chain betaine BT12, which were more active. As the reaction time increased, the antioxidant activity of the betaines increased as well. They reached their highest activity at the maximum time of 240 min, at which monocyclic betaine BT1 showed the highest activity compared to other compounds ($p < 0.05$).

Fig. 10 depicts the influence of time on the antioxidant activity of studied betaines for 30, 120, and 240 min. As can be seen, the antioxidant activity was significantly improved from 30 to 240 min ($p < 0.05$). The results showed that the studied betaines were more effective at 240 min compared to 30 and 120 min. At 240 min, monocyclic betaine BT1 had the highest antioxidant activity, while bicyclic BT2 had the lowest one.

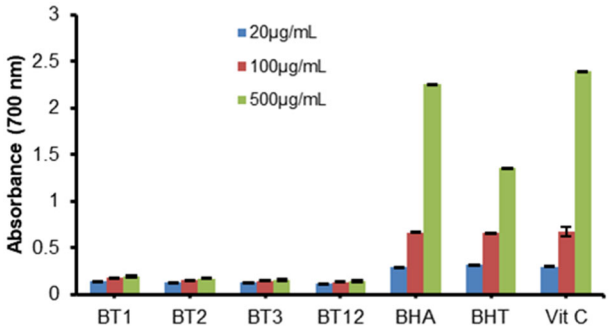

Fig 8. Effect of concentration on the reducing power of betaines and reference antioxidants

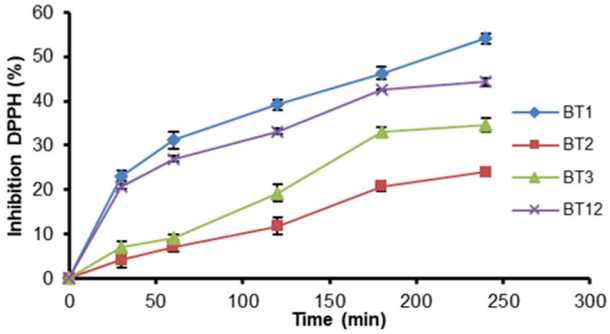

Fig 9. Betaines' DPPH radical scavenging activity as a function of time

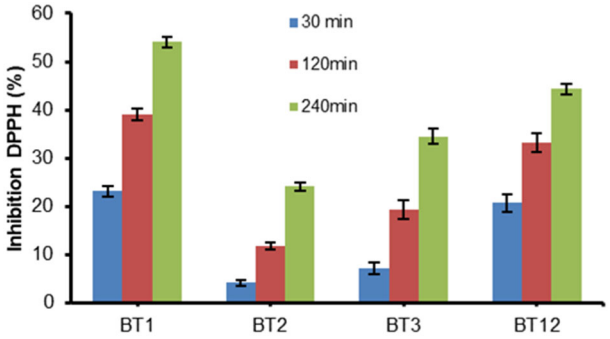

Fig 10. Effect of time on the antioxidant activity of betaines

From the above findings, we conclude that in the DPPH assay, the antioxidant activity of betaines is profoundly influenced by reaction time: the longer the reaction time, the greater the antioxidant activity.

β-Carotene bleaching test

Fig. 11 represents the bleaching kinetics of β-carotene in the presence of synthesized betaines and the reference antioxidant BHT as a positive control. It was obvious that all betaines efficiently inhibited linoleic acid

oxidation and prevented β-carotene bleaching. The findings demonstrated that betaine activity was reduced with increasing time. The relative rate of reduction in antioxidant activity with increasing time was not the same for all betaines investigated. Indeed, as compared to other betaines, bicyclic BT3 demonstrated a slower reduction in antioxidant activity with increasing time.

We calculated the antioxidant activities of betaines relative to that of positive control (BHT) for 2, 24, and 48 h. The results are shown in Fig. 12. According to the results, the highest oxidation inhibitory action of linoleic acid in the presence of tested betaines is observed at 2 h, and the effectiveness of the monocyclic BT1 and bicyclic BT2 reduced significantly with increasing time ($p < 0.05$). At 48 h, the highest activities were found for BT3 (RAA = 90.02 ± 1.01%) and BT12 (RAA = 76.22 ± 1.08%), whereas the activity of BT1 (RAA = 45.57 ± 0.47%) was lower than that of BT2 (RAA = 63.25 ± 1.9%).

A decrease in antioxidant activity with increasing reaction time could be caused by a decrease in the ability of antioxidants to react with free radicals (particularly with peroxyl radicals of fatty acids) and, consequently, a discoloration of β-carotene by radicals produced by fatty acid oxidation, since the assay is carried out in an aqueous emulsion of linoleic acid and β-carotene. Through the results above, we noted that bicyclic betaine BT3 exhibited the highest inhibition of linoleic acid oxidation at all times when compared to BHT. In contrast, long-chain betaine BT12 showed higher antioxidant activity in comparison to monocyclic BT1, especially at 24 and 48 h ($p < 0.05$). Increasing lipophilicity appears to have resulted in an increase in antioxidant activity. In the β-carotene discoloration test, steric effects can be very important. Indeed, the presence of four phenyl groups in monocyclic betaine BT1 may cause steric hindrance, resulting in low reactivity. The greater reactivity of bicyclic betaine BT3 may, on the other hand, can be attributed to its smaller molecule size when compared to the other betaines.

The results of the antioxidant activities of betaines and reference compounds (BHA and vitamin C) relative to that of the BHT at 2, 24, and 48 h are shown in Fig. 13. Compared to the tested compounds, BT3 and the reference antioxidant BHA exhibited the highest inhibition of linoleic acid oxidation at all times relative to BHT. Although the activity of BT1 was the lowest among the other betaines, it was much higher than that of ascorbic acid (Vitamin C) used as a positive control ($p < 0.05$), which was very poor in activity.

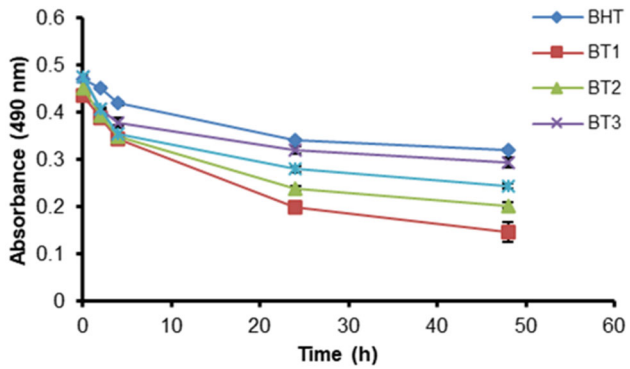

Fig 11. Antioxidant capacity of synthesized betaines in linoleic acid/β-carotene system

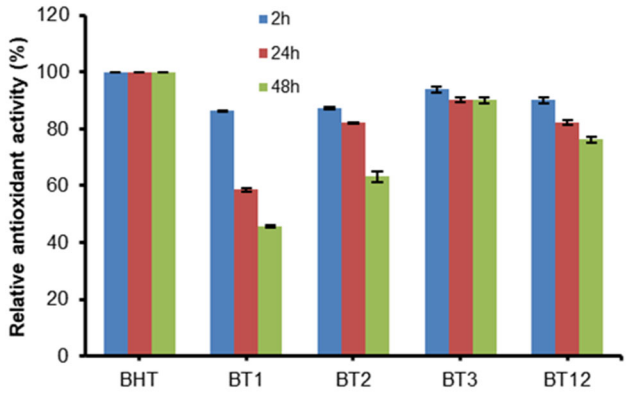

Fig 12. Effect of time on the antioxidant activity of betaines in β-carotene/linoleic acid system

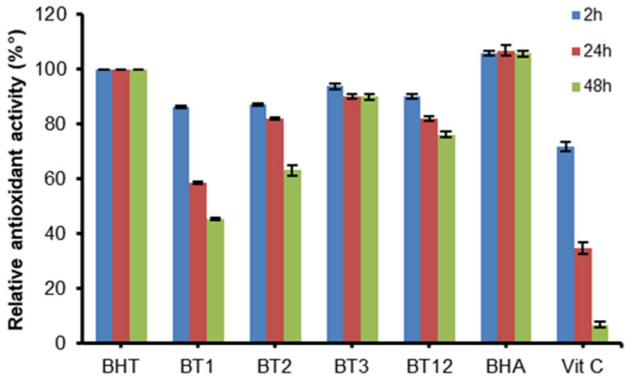

Fig 13. Effect of time on the antioxidant activity of betaines and reference antioxidants in β-carotene/linoleic acid system

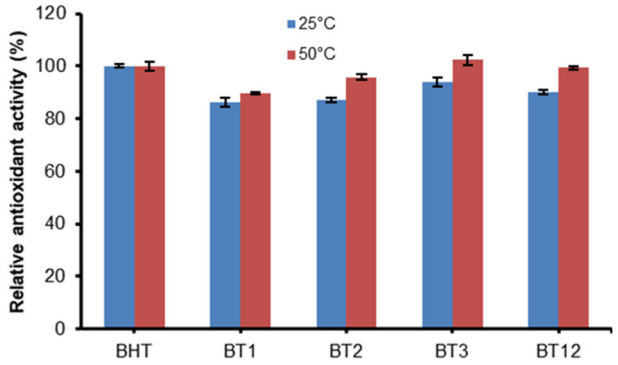

Fig 14. Effect of temperature on the antioxidant activity of betaines at 25 and 50 °C

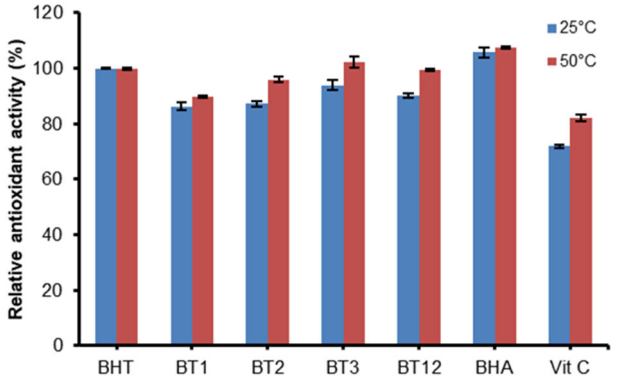

Fig 15. Effect of temperature on the antioxidant activity of betaines and reference antioxidants

Effect of Temperature

Temperature is one of the most significant factors influencing antioxidant activity [40]. The effect of temperature on the antioxidant power of betaines was investigated using a β-carotene assay. Fig. 14 represents the antioxidant activity of the betaines tested and BHT in the β-carotene/linoleic acid system at 25 and 50 °C, after 2 h of inhibition of the oxidation of linoleic acid. As can be seen, the antioxidant activity in the β-carotene/linoleic acid system increased as a function of temperature; increasing temperature caused an increase in antioxidant activity, indicating a high dependence of the antioxidant activity on the temperature.

According to the findings, heating betaines and reference antioxidants to 50 °C was found to be more effective, and as Fig. 15 showed, the pyrimidinium betaines exhibited considerable antioxidant activities compared to those of the reference antioxidants. The above revealed that the temperature has a favorable effect on the inhibition of the oxidation of linoleic acid.

■ CONCLUSION

In this study, four pyrimidinium betaines were evaluated for their antioxidant capacities by three methods, and the effects of some factors such as concentration, reaction time and temperature on their antioxidant activity were investigated. The results revealed that all the betaines tested had potent antioxidant and free radical scavenging properties in various assays. The findings indicated that, in both the DPPH and the reducing power assays, antioxidant activity increased as the concentration increased; however, increasing the temperature caused an increase in antioxidant activity in the β-carotene/linoleic acid system. On the other hand, as time increased, the antioxidant capacity increased in the DPPH assay, but it decreased in the β-carotene bleaching assay. By comparing the results obtained according to the three methods, it can be seen that the antioxidant activity varies depending on the method used and that the different factors affect the antioxidant activity in a different order. This could be due to differences in reaction conditions, molecular interactions in the reaction medium, specific free radicals being used as a reactant and radical kinetics. *In vitro* antioxidant assays suggest that pyrimidinium betaines may be important sources of synthetic antioxidants that could be helpful in preventing the progression of various oxidative stresses and could have significant and wide applications in the pharmaceutical and food industries.

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