Original Article

Application of DPPH assay for the evaluation of the antiradical activity of Creatine Lysinate

Dobrina Tsvetkova[1*], Ivanka Kostadinova[2], Boycho Landzhov[3], Lyubomir Vezenkov[4], Lyubomir Marinov[2], Ivalina Ivanova[2]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Sofia, Sofia 1000, Bulgaria.[2]Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, Sofia 1000, Bulgaria.[3]Department of Anatomy, Histology and Embryology, Faculty of Medicine, Medical University of Sofia, Sofia 1431, Bulgaria.[4]Department of Organic Chemistry, University of Chemical Technology and Metallurgy, Sofia 1756, Bulgaria.

Correspondence: Dobrina Tsvetkova, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Sofia, Sofia 1000, Bulgaria. dtsvetkova@pharmfac.mu-sofia.bg, dobrinka30@mail.bg

ABSTRACT

The aim of the current study was the estimation of the radical-scavenging activity of Creatine lysinate against 0.05 mM methanol solution of DPPH radical by measuring the decrease in the absorbance at $\lambda = 516$ in methanol. The antiradical effect of the compound examined is presented with the following parameters calculated: radical scavenging activity in [%], IC_{50} value; antioxidant power $1/IC_{50}$, and Trolox equivalent activity. relative radical scavenging activity (RRSA, [%]), and a relative decrease in radical scavenging activity (RDRSA, [%]).From the experimental results, it was observed that the DPPH binding ability of Creatine lysinate ($IC_{50} = 73.75$ mM) is lower compared to the standard Trolox ($IC_{50} = 0.001154$ mM), which antioxidant power ($1/IC_{50} = 8.67$) is higher in comparison with Creatine lysinate ($1/IC_{50} = 0.014$). The experimental data show that Creatine lysinate ($IC_{50} = 73.75$ mM) is more active compared to Creatine monohydrate ($IC_{50} = 102.48$ mM) due to lower IC_{50} and higher scavenging activity.

In comparison with mono application of alone antioxidant, the beneficial effect of the combination of Creatine monohydrate and Creatine lysinate with other antioxidants in form of food supplements could be an important strategy for a synergistic effect in the reduction of free radicals in the treatment of disorders resulted from oxidative stress.

Keywords: Creatine lysinate, DPPH, Radical-scavenging activity, Reactive oxygen species, Trolox

Introduction

Reactive oxygen species include charged and neutral species such as superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radicals [1]. Oxidative stress is as a result of the overrun of free radicals and the decreased activity of endogenous antioxidant protective enzymes [2]. The increased generation of reactive oxygen species leads to the disruption of multiple metabolic processes which results at the beginning of the development of the earlier pathological processes in neurodegenerative diseases such as Alzheimer, Parkinson, Huntington's [3], amyotrophic lateral sclerosis (Lou Gehrig disease) [4], cancer [5], rheumatic diseases [6], and aging [7].

Creatine as an antioxidant is important for human health and can provide benefits for different diseases [8]. Pre-exercise and post-exercise Creatine supplementation prevents bone mineral content and density [9] in the aging population [10], and supports bone health in older women [11, 12]. Creatine exerts a protective effect in pathological conditions of the brain and muscle [13]. Creatine supplementation shows effectiveness on aging muscle [14, 15] as on muscle function in childhood myositis [15], muscle function in cancer [16], muscular atrophy [17], sarcopenia [18], myopathy [19], and in spinal and bulbar

Access this article online	
Website:www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Tsvetkova D, Kostadinova I, Landzhov B, Vezenkov L, Marinov L, Ivanova I. Application of DPPH assay for the evaluation of the antiradicalactivity of Creatine Lysinate. J Adv Pharm Educ Res. 2023;13(1):162-9. https://doi.org/10.51847/GUliMKn4FKf

muscular atrophy [20]. Creatine or vitamin D supplementation can help in individuals with a spinal cord injury [21].

Creatine supplementation can provide a beneficial effect on the cognitive function of healthy individuals [22, 23], traumatic brain injury [24, 25], and the mental-associated decrease in visuomotor skills [26, 27]. Dietary supplementation with Creatine is important for bipolar depression [28, 29], pregnancy [30, 31], vascular health [32], and cancer therapy [33-35].

Antioxidants from natural sources and foods [36] are important for protection against age-related diseases [37, 38]. The investigation of antioxidant nutraceuticals [39] is an important therapeutic approach for the effective decrease of reactive oxygen species [40, 41].

The aim of the current study was the comparative evaluation of the radical-scavenging activity (RSA) of Creatine lysinate against 0.05 mM methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by measuring the decrease in the absorbance at λ = 516 nm. Based on the eventually experimentally confirmed antiradical properties of Creatine lysinate, the study would help to justify the advantages of choosing both antiradical compounds in the more active combination over monotherapy, which would contribute to increasing the effectiveness against oxidative stress-related diseases.

Materials and Methods

Materials

I. Test compounds: Creatine lysinate (synthesized from L. Vezenkov)

II. Reagents with pharmacopoeial purity

1. 1.1′-diphenyl-2-picrylhydrazyl (DPPH) (99 %), (Sigma Aldrich, N: STBD 4145 V)
2. 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, N:51796 PMV 291913)
3. methanol (99.9 %) (Sigma-Aldrich, N: SZBD 063 AV UN 1230)
4. distilled water.

Methods

I. Determination of radical scavenging activity by DPPH assay.

1. Preparation of 0.1 mM methanol solution of DPPH

An accurately measured quantity of 0.0039 g DPPH (M = 394.32) was dissolved with methanol and diluted in a volumetric flask of 100.0 ml with methanol to obtain 0.1 mM DPPH solution.

2. Preparation of stock solution of Trolox

An accurately measured quantity of 0.0125 g 6-hyd-roxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (M = 252.294) was dissolved in distilled water and was diluted in a volumetric flask of 50.0 ml with distilled water to obtain a concentration of 1 mM Trolox (1000 μM). An aliquot part of 1.0 ml was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.1 mM (100 μM).

3. Preparation of working solutions of Trolox

From the stock solution of 0.1 mM Trolox and aliquot parts respectively of 10.0 ml, 20.0 ml, 30.0 ml, and 40.0 ml were diluted with distilled water separately in volumetric flasks of 50.0 ml to obtain final concentrations of working solutions of Trolox: 0.02 mM (20 μM), 0.04 mM (40 μM), 0.06 mM (60 μM), 0.08 mM (80 μM).

An aliquot part of 1.0 ml of 0.1 mM was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.01 mM (10 μM). An aliquot part of 2.0 ml of 0.01 mM was diluted in a volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with a concentration of 0.002 mM (2 μM).

4. Preparation of phosphate buffer solution pH = 7

For the preparation of phosphate buffer solution with pH = 7,accurate quantities of 0.1 g KH_2PO_4, 0.2 g K_2HPO_4, and 0.85 g NaCl were dissolved in distilled water and diluted in a volumetric flask of 100.0 ml with distilled water.

5. Preparation of stock solution Creatine lysinate

An accurately measured quantity of 1.3866 g Creatine lysinate (M = 277.32) was dissolved in phosphate buffer pH = 7 and was diluted in a volumetric flask of 50.0 ml with phosphate buffer pH = 7 to obtain the stock solution with a concentration of 100 mM (0.1 M).

6. Preparation of working solutions of Creatine lysinate

From the stock solution of 100 mM, Creatine lysinate and aliquot parts respectively of 10.0 ml, 20,0 ml, 30.0 ml, and 40.0 ml were diluted with phosphate buffer pH = 7 separately in volumetric flasks of 50.0 ml to obtain final concentrations of working solutions of 20 mM, 40 mM, 60 mM, 80 mM.

7. DPPH assay procedure

DPPH assay was performed according to the following procedure: 5 ml 0.1 mM methanol solution DPPH was mixed separately with 5 ml of Creatine lysinate in concentrations 20 mM, 40 mM, 60 mM, and 80 mM to obtain final concentrations respectively of 0.05 mM methanol solution DPPH and 10 mM, 20 mM, 30 mM, 40 mM of Creatine lysinate. As control recorded a mixture of 5 ml 0.1 mM methanolic DPPH solution and 5 mlof methanol.The mixtures were shaken vigorously and allowed to stand for incubation in dark for 1 h at temperature: 25 °C ÷ 27 °C After incubation, the absorbances were measured against blank methanol at λ= 516 nm using a UV-VIS spectrophotometer Hullett Packard N: 8452 A. All the tests were performed in triplicates and the results were averaged.

II. Calculation methods

1. Calculation of radical scavenging activity(RSA, [%])

The results of DPPH-radical scavenging activity (RSA), and for not-scavenged radical (R, [%]), for a period of 1 h reaction of 0.05 mM methanol solution of DPPH with solutions of standard Trolox, and 10 mM ÷ 40 mM Creatine lysinate, were calculated by the equation:

$$\text{RSA [\%]} = \frac{ADPPHcontrol - sample}{ADPPHcontrol} \times 100 \quad (1)$$

$$\text{R [\%]} = \frac{Asample}{ADPPHcontrol} \times 100 \quad (2)$$

A DPPH control – absorbance of the solution of DPPH-radical before interaction with the compound investigated

Asample – absorbance of the solution of DPPH-radical after reacting with the compound investigated

the absorbance of the DPPH solution in control is measured against methanol.

2. Calculation of IC50 value (inhibitory concentration)

The IC_{50} value is the concentration of the test samples at which the inhibition percentage reaches 50%. A lower IC_{50} value corresponds to a higher antiradical activity of the tested sample. IC_{50} values (mM), were calculated from the DPPH radical–scavenging curve of Creatine lysinate and standard Trolox at λ = 516 nm according to the following procedure: inhibition ratios (*y*) were plotted against the sample concentrations (*x*), and the respective regression line ($y = a.x + b$) was drawn. The sample concentration) (*x*), was calculated by substituting the value of (*y*) with 50 in the regression equation.

3. Calculation of antioxidant power: 1/IC50

4. Calculation of Trolox equivalent antioxidant capacity

The DPPH radical scavenging activity of the sample was expressed as Trolox equivalent antioxidant capacity (TEAC) calculated as follows:

$$\text{TEAC} = \frac{IC50Trolox}{AIC50sample} \quad (3)$$

The higher TEAC value means a higher DPPH radical scavenging activity.

5. Calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%])

The relative radical scavenging activity (RRSA, [%]) and the relative decrease in radical scavenging activity (RDRSA, [%]) for Creatine lysinate with concentrations 10 ÷ 40 mM, were compared to the activity of standard Trolox with the same concentrations, and were calculated by the following equations:

$$\text{RRSA [\%]} = \frac{RSAsample}{RSATrolox} \times 100 \quad (4)$$

$$\text{RDRSA [\%]} = \frac{RSATrolox - RSAsample}{RSATrolox} \times 100 \quad (5)$$

RSA_{sample} - radical binding activity of Creatine lysinate

RSA_{Trolox} - radical binding activity of the standard Trolox.

Results and Discussion

Antioxidant methods are classified as hydrogen atom transfer (HAT)-based, and electron transfer (ET)-based assays. In spectrophotometric ET-based methods is measured the capacity of a redox-potential compound in the reduction of a colored oxidizing agent. The degree of color change in the reduction of an oxidant (either an increase or decrease of absorbance at a specific wavelength) is correlated to the concentration of the antiradical compound. In electron transfer-based methods, the increase in the radical-scavenging effect of the test compounds is directly proportional to the decrease of the absorbance of a solution of:

1. 1,1-diphenyl-2-(picrylhydrazyl) (λ = 516 nm): DPPH free radical scavenging assay
2. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (λ = 734 nm): ABTS method [42].

The DPPH (1,1-diphenyl-2-picrylhydrazyl; α,α-diphenyl-β-picrylhydrazyl) is a stable free organic radical with dark purple color. The DPPH radical scavenging method is a decolorization assay, which mechanism is based on the reaction between the DPPH solution and compounds by measuring its capacity to directly scavenge the DPPH radicals. In this interaction, DPPH is reduced to its nonradical form 1,1′-diphenyl-2-(2,4,6-trinitrophenol) hydrazine (DPPHH), and antioxidants are oxidized from DPPH. The determination of the capacity of compounds to directly scavenge the DPPH radicals is based on the monitoring of the decrease of absorbance of the DPPH radical most commonly at λ = 517 nm [43-45].

The method is applicable for the study of the radical scavenging activity of the test compounds because they do not possess a measurable absorbance at the absorption maximum λ = 517 nm, where the DPPH-test is carried out.

DPPH method has been applied for the investigation of the antioxidant activity and of free radical scavenging effect of extracts from different plants such as *Piper longum L.* [46, 47], *Zingiber officinale var. rubrum* [48, 49], milk thistle [50, 51].

Marinova *et al*. [52, 53] have reported that the literature review described different modifications of the original methods of Blois [43] and Brand-Williams *et al*. [44, 54] for the determination of the DPPH free radical scavenging activity. Following Blois for the DPPH method, are mixed 1 ml 0.1 mM methanol solution of DPPH solution is added with 3 ml of various concentrations of compounds in methanol or with 3 ml of reference standard Butylhydroxytoluene. After 30 min. at 25 °C in dark, the absorbance is measured at λ = 517 nm [43, 55].

The modification methods include the differences in the following parameters [53, 56]:

1. wavelength of absorbance measurements: between 492 nmand 540 nm: 515 nm, 516 nm, 517 nm, 518 nm, 520 nm, and 525 nm, with the most utilized at λ = 517 nm
2. concentration of DPPH solutions in the range from 0.05 mM to 1.5 M: 0.05 mM, 0.06 mM, 0.09 mM, 0.10 mM
3. the most applied solvents: methanol and ethanol
4. ratio between volumes of sample/DPPH solution: 1:1, 1:7,5,1:600, 3:1
5. duration of reaction: varies from 1 min. to 240 min.: 5 min., 10 min., 15 min., 20 min., 30 min.. 60 min., 90 min., 120 min
6. standard solutions used for expression of the results. Vitamin C, Vitamin E. BHT, BHA, Trolox
7. temperature (from 25 °C to 27 °C).

The reducing ability of Creatine lysinate and standard Trolox presented as a percentage of antiradical activity (RSA %), was assessed by DPPH free radical scavenging assay. For the investigation of the radical scavenging activity was applied methodology described by the original DPPH methods of Blois [43, 57] and Brand-Williams [44, 58, 59] with the following parameters modified in our previous work [60-63]:

1. 0.05 mM DPPH
2. Trolox solution as standard
3. methanol as solvent
4. temperature: 25 °C ÷ 27 °C
5. ratio sample/DPPH solution: 1:1
6. duration of reaction: 1 h
7. measurement of the decrease of DPPH absorbance at λ = 516 nm wavelength.

The reaction mixture consisted of 5 ml of 0.1 mM methanol solution DPPH and 5 ml respectively of 20 mM, 40 mM, 60 mM, and 80 mM of Creatine lysinate, to obtain final concentrations correspondingly of 0.05 mM methanol solution DPPH and 10 mM, 20 mM, 30 mM, 40 mM of Creatine lysinate.

The changes in color from deep violet to light yellow were observed. The decrease of the absorbance was registered and spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions of Creatine lysinate is illustrated in **Figure 1**.

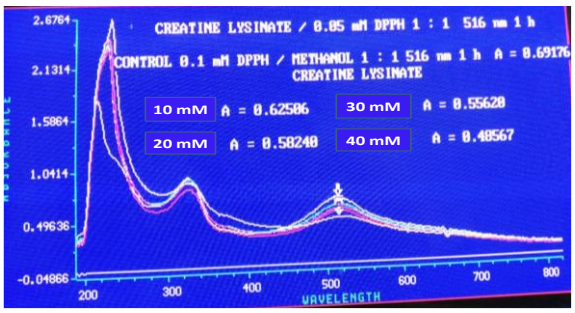

Figure 1. Spectra of 0.05 mM methanol solution of DPPH at λ = 516 nm after 1 h reaction with solutions of Creatine lysinate.

Table 1 are presented experimental results of absorbances of 0.05 mM methanol solution of DPPH after 1 h reaction with Creatine lysinate and Trolox. **Table 2** are summarized data for radical scavenging activity RSA (%), and for not scavenged radical (R).

Table 1. Absorbance at λ = 516 nm of 0.05 mM methanol solution of DPPH after 1 h reaction with solutions of Creatine lysinate and standard Trolox.

	Creatine lysinate		Trolox	
N:	**C [mM]**	**A [AU]**	**C [mM]**	**A [AU]**
1.	10	0.62506	0.001	0.48924
2.	20	0.58240	0.01	0.33066
3.	30	0.55620	0.02	0.23593
4.	40	0.48567	0.03	0.10475

Table 2. Radical–scavenging activity RSA [%]of Creatine lysinate and standard Trolox and not scavenged radical (R) at λ = 516 nm after 1 h reaction with 0.05 mM methanol solution of DPPH.

	Creatine lysinate C [mM]	**Creatine lysinate RSA [%]**	**Trolox C [mM]**	**Trolox RSA [%]**
1.	10	9.64	0.001	28.02
2.	20	15.81	0.01	51.35
3.	30	19.60	0.02	65.29
4.	40	29.79	0.03	84.59
			0.04	90.62
	Creatine lysinate C [mM]	**Creatine lysinate R [%]**	**Trolox C [mM]**	**Trolox R [%]**
N:	10	90.36	0.001	71.98
2.	20	84.20	0.01	48.65
3.	30	80.40	0.02	34.71
4.	40	70.21	0.03	15.41

In our previous work, the following results forstandard Trolox have been obtained:

1. parameters of regression equations for absorbances: $y = -10.95.x + 0.466$ ($R^2 = 0.963$)

radical-scavenging activity: $y = 1611.x + 31.41$ ($R^2 = 0.964$)
unscavenged DPPH-radical: $y = - 1611.x + 68.58$ ($R^2 = 0.964$)

2. the concentration at which the inhibition of radicals reaches 50 %: $IC_{50} = 0.01154$ mM

3. antioxidant power :1/IC50 = 8.67 [60]

The results for the absorbance values of 0.05 mM DPPH methanol solution at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate were put against the corresponding concentrations into linear regression analysis and the linear dependence between the absorbances and concentration in the tested range was observed. The calibration curve for Creatine lysinate is shown in **Figure 2**. Linearity is characterized by the coefficient of linear regression, which is $R^2 > 0.963$.

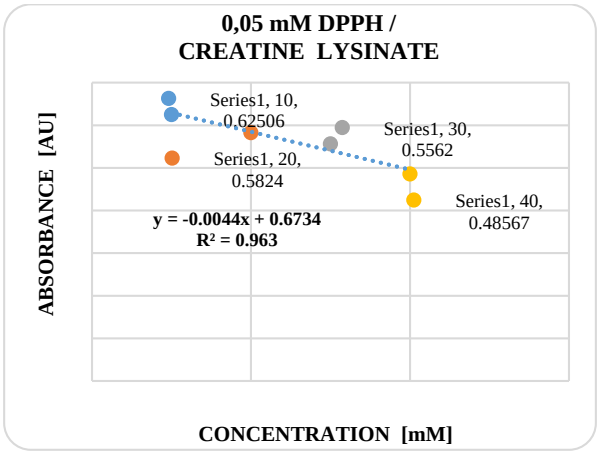

Figure 2. The absorbance of 0.05 mM DPPH methanol solution at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

The scavenging activity percentages are subjected to a linear regression analysis against the respective concentrations. **Figure 3**. is illustrated the calibration curve which presents the linear relationship between the enhanced radical binding activity with the increase of concentration from 10 mM to 40 mM. **Figure 4**. presents results for not-scavenged DPPH-radical at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

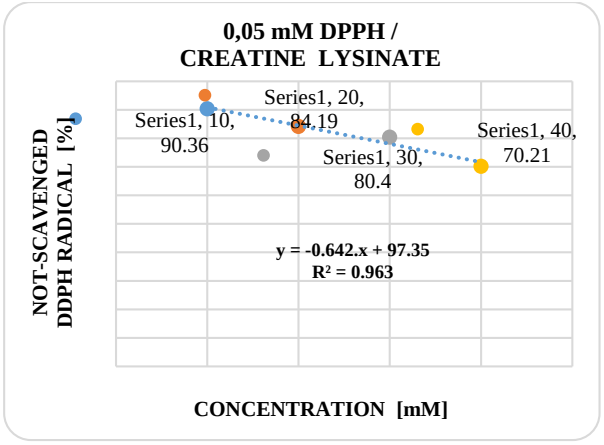

Figure 3. Radical-scavenging activity of 0.05 mM methanol solution of DPPH at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

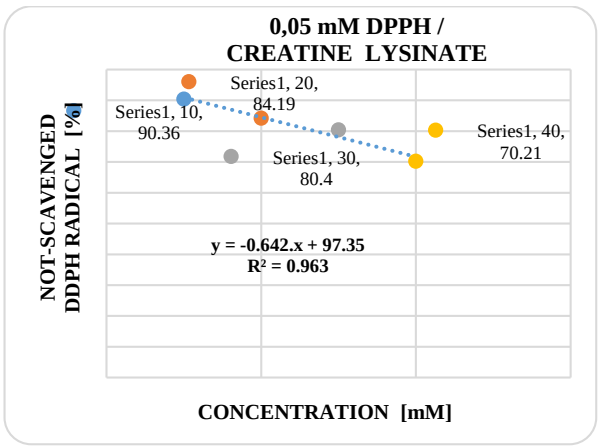

Figure 4. Not-scavenged DPPH-radical at $\lambda = 516$ nm after 1 h reaction with solutions of Creatine lysinate.

Calculation of IC_{50} value (inhibitory concentration) and antioxidant power $1/IC_{50}$

The results are expressed as IC_{50} values whichdetermine the number of antioxidants needed for decreasing the radical concentration by 50 % and antioxidant power: $1/IC_{50}$. The regression analysis method was employed and the obtained regression equations for standard Trolox and Creatine lysinate were used to calculate the IC_{50} values that provide 50 % inhibition of the DPPH radical.

A lower IC_{50} value defines that at less concentration the compounds exert higher scavenging activity and antiradical effect. Due to the lower $IC_{50} = 0.001154$ mM, standard Trolox possesses a higher antioxidant power $1/IC_{50} = 8.67$ than Creatine lysinate ($1/IC_{50} = 0.014$). The experimental results show that Creatine lysinate ($IC_{50} = 73.75$ mM) is more active compared to Creatine monohydrate ($IC_{50} = 102.48$ mM) due to lower IC_{50}.

Calculation of Trolox equivalent antioxidant capacity

The DPPH radical scavenging activity of Creatine lysinate expressed as Trolox equivalent antioxidant capacity is: TEAC = 0.00016.

Calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%])

From the regression equations for the radical-binding effect, the values for the radical-scavenging activity of 0.01 mM, 0.02 mM, 0.03 mM, and 0.04 mM solutions of Creatine lysinate were calculated.

The obtained data were used for the calculation of relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%]) shown in **Table 3**.

Table 3. Relative radical scavenging activity (RRSA, [%]) and a relative decrease in radical scavenging activity (RDRSA, [%]).

	C [mM]	RSA [%]	RRSA [%]	RDRSA [%]
1.	0.01	2.6564	5.17	94.83
2.	0.02	2.6628	4.08	95.92
3.	0.03	2.6693	3.16	96.84
4.	0.04	2.6757	2.95	97.05

Conclusion

From the experimental results, it was observed that the DPPH binding ability of Creatine lysinate (IC_{50} = 73.75 mM) is lower compared to the standard Trolox (IC_{50} = 0.001154 mM), which antioxidant power ($1/IC_{50}$ = 8.67) is higher in comparison with Creatine lysinate ($1/IC_{50}$ = 0.014). The experimental data show that Creatine lysinate (IC_{50} = 73.75 mM) is more active compared to Creatine monohydrate (IC_{50} = 102.48 mM) due to lower IC_{50} and higher scavenging activity.

The beneficial effect of combinations in the reduction of free radicals could be more effective in comparison with the mono application. Due to radical-scavenging activity, the application of Creatine monohydrate and Creatine lysinate in combination with other kinds of antioxidants such as Vitamin C or Coenzyme Q in form of food supplements could be an important strategy for obtaining of synergistic effect in an additive treatment of disorders resulting from oxidative stress-related diseases.

Acknowledgments: to prof. Nikolai Danchev.

Conflict of interest: None

Financial support: This article was prepared with financial support from Grant 2021 Project N: D-106/04.06.2021, Contract N:7892/19.11.2020, Medical University-Sofia, Bulgaria.

Ethics statement: None

References

1. Losada-Barreiro S, Bravo-Díaz C. Free radicals and polyphenols: The redox chemistry of neurodegenerative diseases. Eur J Med Chem. 2017;133(1):379-402. doi:10.1016/j.ejmech.2017.03.061
2. Kozakiewicz M, Kornatowski M, Krzywińska O, Kudziora-Kornatowska K. Changes in the blood antioxidant defense of advanced age people. Clin Interv Aging. 2019;14(1):763-71. doi:10.2147/CIA.S201250
3. Singh A, Kukreti R, Saso L, Kukreti S. Oxidative stress: A key modulator in neurodegenerative diseases. Molecules. 2019;24(8):1583. doi:10.3390/ molecules24081583
4. Cunha-Oliveira T, Montezinho L, Mendes C, Firuzi O, Saso L, Oliveira PJ, et al. Oxidative stress in amyotrophic lateral sclerosis: Pathophysiology and opportunities for pharmacological intervention. Oxid Med Cell Longev. 2020;2020(1):5021694. doi:10.1155/2020/5021694
5. Gurer-Orhan H, Ince E, Konyar D, Saso L, Suzen S. The role of oxidative stress modulators in breast cancer. Curr Med Chem. 2018;25(33):4084-101. doi:10.2174/ 0929867324666170711114336
6. Firuzi O, Spadaro A, Spadaro C, Riccieri V, Petrucci R, Marrosu G, et al. Protein oxidation markers in the serum and synovial fluid of psoriatic arthritis patients. J Clin Lab Anal. 2008;22(3):210-15. doi:10.1002/jcla.20243
7. Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, et al. Oxidative stress, aging, and diseases. Clin Interv Aging. 2018;13(1):757-72. doi:10.2147/CIA.S158513
8. Kreider RB, Stout JR. Creatine in health and disease. Nutrients. 2021;13(2):447. doi:10.3390/ nu13020447
9. Candow DG, Forbes SC, Vogt E. Effect of pre-exercise and post-exercise creatine supplementation on bone mineral content and density in healthy aging adults. Exp Gerontol. 2019;119(1):89-92. doi:10.1016/j.exger. 2019.01.025
10. Stares A, Bains M. The additive effects of Creatine supplementation and exercise training in an aging population: A systematic review of randomized controlled trials. J Geriatr Phys Ther. 2020;43(2):99-112. doi:10.1519/JPT.0000000000000222
11. Sales LP, Pinto AJ, Rodrigues SF, Alvarenga JC, Goncalves N, Sampaio-Barros MM, et al. Creatine supplementation (3 g/d) and bone health in older women: A 2-year, randomized, placebo-controlled trial. J Gerontol A Biol Sci Med Sci. 2020;75(5):931-8. doi:10.1093/ gerona/glz162
12. Sheth TR, Gokhe AA, Kumar PN, Chitnis KS. Recycling flower and kitchen waste to make biodegradable paper. World J Environ Biosci. 2021;10(1):35-8. doi:10.51847/tkMmywD2fA
13. Balestrino M, Adriano E. Beyond sports: Efficacy and safety of creatine supplementation in pathological or para-physiological conditions of brain and muscle. Med Res Rev. 2019;39(6):2427-59. doi:10.1002/med.21590
14. Candow DG, Forbes SC, Chilibeck PD, Cornish SM, Antonio J, Kreider RB. Effectiveness of creatine supplementation on aging muscle and bone: Focus on falls prevention and inflammation. J Clin Med. 2019;8(4):488. doi:10.3390/jcm8040488
15. Dover S, Stephens S, Schneiderman JE, Pullenayegum E, Wells GD, Levy DM, et al. The effect of creatine supplementation on muscle function in childhood myositis: A randomized, double-blind, placebo-controlled feasibility study. J Rheumatol. 2021;48(3):434-41. doi:10.3899/jrheum.191375
16. Fairman CM, Kendall KL, Hart NH, Taaffe DR, Galvao DA, Newton RU. The potential therapeutic effects of Creatine supplementation on body composition and

muscle function in cancer. Crit Rev Oncol Hematol. 2019;133:46-57. doi:10.1016/j.critrevonc.2018.11.003
17. Dolan E, Artioli GG, Pereira RMR, Gualano B. Muscular atrophy and sarcopenia in the elderly: Is there a role for Creatine supplementation? Biomolecules. 2019;9(11):642. doi:10.3390/biom9110642
18. Candow DG, Forbes SC, Chilibeck PD, Cornish SM, Antonio J, Kreider RB. Variables Influencing the effectiveness of Creatine supplementation as a therapeutic intervention for sarcopenia. Front Nutr. 2019;6(1):124. doi:10.3389/fnut.2019.00124
19. Balestrino M, Adriano E. Creatine as a candidate to prevent statin myopathy. Biomolecules. 2019;9(9):496. doi:10.3390/biom9090496
20. Hijikata Y, Katsuno M, Suzuki K, Hashizume A, Araki A, Yamada S, et al. Treatment with creatine monohydrate in spinal and bulbar muscular atrophy: Protocol for a randomized, double-blind, placebo-controlled trial. JMIR Res Protoc. 2018;7(3):e69. doi:10.2196/resprot.8655
21. Amorim S, Teixeira VH, Corredeira R, Cunha M, Maia B, Margalho P, et al. Creatine or vitamin D supplementation in individuals with a spinal cord injury undergoing resistance training: A double-blinded, randomized pilot trial. J Spinal Cord Med. 2018;41(4):471-8. doi:10.1080/10790268.2017.1372058
22. Avgerinos KI, Spyrou N, Bougioukas KI, Kapogiannis D. Effects of creatine supplementation on cognitive function of healthy individuals: A systematic review of randomized controlled trials. Exp Gerontol. 2018;108:166-73. doi:10.1016/j.exger.2018.04.013
23. Altowigri A, Mirghani HO. Blount disease, Vitamin D deficiency, and associated comorbidities: A review and Meta-analysis. World J Environ Biosci. 2021;10(1):5-8. doi:10.51847/j96vx6DHPC
24. Kumar R. Toxic algae and effects of algal poisoning in animals and human beings. World J Environ Biosci. 2021;10(1):9-12. doi:10.51847/8iu9ylaNgi
25. Dolan E, Gualano B, Rawson ES. Beyond muscle: The effects of creatine supplementation on brain creatine, cognitive processing, and traumatic brain injury. Eur J Sports Sci. 2019;19(1):1-14. doi:10.1080/17461391.2018.1500644
26. Van Cutsem J, Roelands B, Pluym B, Tassignon B, Verschueren JO, Pauw KDE, et al. Can creatine combat the mental fatigue-associated decrease in visuomotor skills? Med Sci Sports Exerc. 2020;52(1):120-30. doi:10.1249/MSS.0000000000002122
27. Alghamdi S, Alhazmi K. Appendectomy impact on inflammatory bowel diseases: A meta-analysis. World J Environ Biosci. 2021;10(1):13-8. doi:10.51847/Dz8IEE5R1x
28. Alhazmi K, Alghamdi S. Appendectomy and Parkinson's disease risk: A meta-analysis. World J Environ Biosci. 2021;10(1):19-23. doi:10.51847/zAvuw7jQXD
29. Toniolo RA, Silva M, Fernandes FBF, Amaral JAMS, Dias RS, Lafer B. A randomized, double-blind, placebo-controlled, proof-of-concept trial of Creatine monohydrate as an adjunctive treatment for bipolar depression. J Neural Transm. 2018;125(2):247-57. doi:10.1007/s00702-017-1817-5
30. de Guingand DL, Palmer KR, Bilardi JE, Ellery SJ. Acceptability of dietary or nutritional supplementation in pregnancy (ADONS) - Exploring the consumer's perspective on introducing Creatine monohydrate as a pregnancy supplement. Midwifery. 2020;82:102599. doi:10.1016/ j.midw.2019.102599
31. Alraddadi M, Alhazmi K. The twins-hit hypothesis of atopic dermatitis and autoimmune diseases: A review and meta-analysis. World J Environ Biosci. 2021;10(1):24-8. doi:10.51847/i74xNeqIdK
32. Van Clarke H, Kim DHM, Meza CAM, Ormsbee MJ, Hickner RC. The evolving applications of creatine supplementation: Could. Creatine improves vascular health? Nutrients. 2020;12(9):2834. doi:10.3390/nu12092834
33. Di Biase S, Ma X, Wang X, Yu J, Wang YC, Smith DJ, et al. Creatine uptake regulates CD8 T cell antitumor immunity. J Exp Med. 2019;216(12):2869-82. doi:10.1084/jem.20182044
34. Fairman CM, Kendall KL, Newton RU, Hart NH, Taaffe DR, Chee R, et al. Examining the effects of creatine supplementation in augmenting adaptations to resistance training in patients with prostate cancer undergoing androgen deprivation therapy: A randomized, double-blind, placebo-controlled trial. BMJ Open. 2019;9(9):e030080. doi:10.1136/bmjopen-2019-030080
35. Cella PS, Marinello PC, Borges FH, Ribeiro DF, Chimin P, Testa MTJ, et al. Creatine supplementation in walker-256 tumor-bearing rats prevent skeletal muscle atrophy by attenuating systemic inflammation and protein degradation signaling. Eur J Nutr. 2020;59(2):661-9. doi:10.1007/s00394-019-01933-6/
36. Anwar H, Hussain G, Mustafa I. Antioxidants from natural sources, antioxidants in foods and its applications. Editors: E Shalaby et GM Azzam. Intech Open, 2018, ISBN: 978-1-78923-379-7; Print ISBN: 978-1-78923-378-0, eBook (PDF) ISBN: 978-1-83881-640-7
37. Simioni C, Zauli G, Martelli AM, Vitale M, Sacchetti G, Gonelli A, et al. Oxidative stress: Role of physical exercise and antioxidant nutraceuticals in adulthood and aging. Oncotarget. 2018;9(24);17181-98. doi:10.18632/oncotarget.24729
38. Tan BL, Norhaizan ME, Liew WPP, Rahman HS. Antioxidant and oxidative stress: A mutual interplay in age-related diseases. Front Pharm. 2018;9(1):1162. doi:10.3389/fphar.2018.01162
39. Losada-Barreiro S, Sezgin-Bayindir Z, Paiva-Martins F, Bravo-Díaz C. Biochemistry of antioxidants: Mechanisms and pharmaceutical applications. Biomedicines. 2022;10(12):3051. doi:10.3390/biomedicines10123051
40. Liu Z, Ren Z, Zhang J, Chuang CC, Kandaswamy E, Zhou T, et al. Role of ROS and nutritional antioxidants in human

diseases. Front Physiol. 2018;9(1):477. doi:10.3389/fphys.2018.00477
41. Madhukar CV. Antimicrobial and antioxidant potentials of carotenoid pigment produced by indigenous novel soil isolate rhodococcus kroppenstedtii. World J Environ Biosci. 2021;10(1):29-34. doi:10.51847/9QrSrJyTN2
42. Xiao F, Xu T, Lu B, Liu R. Guidelines for antioxidant assays for food components. Food Front. 2020;(1):60-9. doi:10.1002/fft2.10
43. Blois MS. Antioxidant determination by the use of a stable free radical. Nature. 1958;181:1199-200. doi:10.1038/1811199a0
44. Brand-Williams W, Cuvelier M, Berset C. Use of the free radical method to evaluate antioxidant activity, LWT-Food Sci Technol. 1995;28(1):25-30. doi:10.1016/S0023-6438(95)80008-5
45. Al Hemly M, Alghamdi AAM, Alqarni SSA, Alzughaibi TKM, Al-mogamsy AHM, Aljohani YSH, et al. Bleeding per rectum: Is awareness of the general population essential? World J Environ Biosci. 2021;10(1):39-47. doi:10.51847/5uTOcit2wB
46. Pal RS, Pal Y, Wal P, Wal A, Saraswat N. Condensed tannins: Its various perspectives as a vital bio-metabolite. World J Environ Biosci. 2021;10(2):18-23. doi:10.51847/eLss4HKahC
47. Banerjee S, Mallick MA, Pathade GR. Comparison of antioxidant activity of in vivo and in vitro leaf explants of Piper longum. J Adv Pharm Educ Res. 2017;7(3):323-5.
48. Sulastri T, Sunyoto M, Suwitono RM, Levita J. The effect of red ginger bread consumption on the physiological parameters of healthy subjects. J Adv Pharm Educ Res. 2022;12(3):28-35. doi:10.51847/mznq1HW7vK
49. Sen K, Sanyal T, Karmakar SR. COVID-19 forced lockdown: Nature's strategy to rejuvenate itself. World J Environ Biosci. 2021;10(2):9-17. doi:10.51847/mhLv0Gijx5
50. Viktorova J, Stranska-Zachariasova M, Fenclova M, Vitek L, Hajslova J, Kren V, et al. Complex evaluation of the antioxidant capacity of Milk thistle dietary supplements. Antioxidants. 2019;8(8):317. doi:10.3390/antiox8080317
51. Allbban AM. Tribulus and ashwagandha diets to combat infertility of cadmium chloride injected male albino rats. World J Environ Biosci. 2021;10(2):24-9. doi:10.51847/ky98tlOd7L
52. Samir D, El-houda HN, Aicha Z. Hematological and oxidative stress markers analysis for detection and prediction of osteoporosis in post-menopausal women. World J Environ Biosci. 2021;10(2):30-6. doi:10.51847/movrtjOMuP
53. Marinova G, Batchvarov V. Evaluation of the methods for determination of the free radical scavenging activity by DPPH. Bulg J Agric Sci. 2011;17(1):11-24.
54. Pal RS, Wal P, Kumar P, Pal Y, Sheetal S. Herbal solid perfume: A Turkish concept-based synthesis and quality valuation. World J Environ Biosci. 2021;10(2):37-41. doi:10.51847/aIXaFnrrir
55. Jana B, Mondal AK. Tropical severe super cyclone amphan effects on coastal plant diversity of East Midnapore district, West Bengal. World J Environ Biosci. 2021;10(3):1-4. doi:10.51847/vUdlyaCyH3
56. Thobity AFA, Alghamdi TZA, Alqurashi AMA, Althobaiti MAM, Jawmin SAH, Alharthi MFM, et al. Awareness of smoking as a risk factor for bladder cancer in Taif city. World J Environ Biosci. 2021;10(3):5-9. doi:10.51847/SITMXmYKtq
57. Hazra A. A glimpse of world water scenario to apprehend the emergence of water laws. World J Environ Biosci. 2021;10(3):10-3. doi:10.51847/i5sBUo3jsu
58. Alqahtani MS, Alshaks NM, Alshahrani NFM, Naghi WM, Alharbi WM, Mahmood ST, et al. Review on anterior crossbite diagnosis and management approach in paediatric age, literature review. World J Environ Biosci. 2021;10(3):14-7. doi:10.51847/vrbgfj7OVY
59. Badauod AA, Sufta AA, Alabbadi AM, Alzahrani AA, Allahiani WK, Alzahrani YM, et al. An overview on the role of family physician in diagnosis and management of back pain. World J Environ Biosci. 2021;10(4):20-2. doi:10.51847/TotWQ27k5x
60. Kostadinova I, Landzhov B, Vezenkov L, Marinov L, Ivanova I, Tsvetkova D. Estimation of DPPH-radical scavenging activity of creatine monohydrate. Int J Pharm Res Appl. 2023;8(1):2374-83. doi:10.35629/7781-080123742383
61. Aljabri YA, Alghamdi FS, Almehmadi KA, Babkoor AA, Bahalaq AM, Althaqfi AA, et al. An overview on pheochromocytoma diagnosis and management approach, review article. World J Environ Biosci. 2021;10(3):18-22. doi:10.51847/tUlqbd3ivR
62. Taba H, Manivel N, Durairaj D. Indigenous agricultural practices among tribal farmers on lower subansiri district of arunachal pradesh. World J Environ Biosci. 2021;10(3):51-3. doi:10.51847/5PYG9V9Yd1
63. Alrusayyis NS, Alghamdi KM, Alahmari BM, Barnawi RM, Alfuraydan AYA, Alharbi BA, et al. Multiple sclerosis flare-ups diagnostic and management approach in emergency department, review article. World J Environ Biosci. 2021;10(4):9-12. doi:10.51847/NTYL4XWgmv