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Estimation of DPPH-radical scavenging activity of Creatine monohydrate

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ABSTRACT:

Oxidative stress is a result of overproduction of free radicals and the decreased activity of endogenous antioxidant protective enzymes. Oxidative stress plays an important role in the pathological changes in neurodegenerative diseases as Alzheimer, Huntington, Parkinson, amiotrophic lateral sclerosis, psoriatic arthritis. Creatine exhibits direct antioxidant properties and protects DNA and RNA from oxidative damage.

The aim of the current study was the evaluation of the radical-scavenging activity of Creatine monohydrate towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) by spectrophotometry at λ = 516 nm by using standard of Trolox solution.

KEYWORDS: Creatine monohydrate, DPPH assay, radical-scavenging activity.

I, INTRODUCTION.

Oxidative stress [1] is a result of the disbalance between the decreased activity of endogenous antioxidant protective enzymes and the overproduction of free radicals. The exogenous sources of free radicals are heavy metals, UV-ligth, pesticides, and tobacco smoke [2].

The endogenous free radicals are forming in the following biochemical reactions:
1) oxidation of fatty acids; 2) autooxidation of catecholamines, pterins and thiols. Through uncontrolled oxidation processes, free radicals cause irreversible damage to ribonucleic acid (RNA), deoxyribonucleic acid (DNA), proteins, carbohydrates, lipids. These processes unlock chain self-sustaining reactions, during which new radicals are formed, their quantity is increased, and their destructiveness action is intensified [1].

Oxidative stress plays an importamt role in in pathogenesis of neuronal degeneration, and in the progression of Alzheimer disease [3,4]. The result from the oxidative stress is the beginning of the development of the earlier pathological processes in amiotrophic lateral sclirosis (Lou Gehrig disease) [5], Down sindrome [6], and inflammatory diseases as psoriatic arthritis [7[.

Antioxidants protect from oxidative stress [8]. Antioxidant properties possess different medicinal plants as Amaranthus paniculatus L., Asparagus racemosus Willd., Coriandrum sativum L,, Moringa oleifera Lam., Zingiber officinales Roscoe [9], Cidoscolus phyllacanthus Pohl [10], Jasmine grandiflorum L. [11], Michelia champaca (L.) Baill ex Pierre [12], Punica granatum L. [13], Talinum triangulare (L.) Juss. [14], Trigonella foenum graecum L. [15].

Creatine is an antioxidant, isolated from skeletal muscle in 1832 by Michel Eugène Chevreul and is synthesized from aminoacids glycine, L-arginine and L-methionine [16]. Creatine exhibits direct antioxidant properties by binding superoxide anions and peroxitrile [17], and decreases the markers of lipid peroxidation [18].

The aim of the current study was the evaluation of the radical-scavenging activity of Creatine monohydrate against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by measuring the decrease of the absorbance of 0.05 mM methanol solution of DPPH radical at wavelength λ = 516 nm by using standard of Trolox methanol solution.

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II. EXPERIMENTATION.

II,1, Materials.

I. Test compound: Creatine monohydrate (Biogame)
II. Reagents with pharmacopoeial purity:
1) 1.1′-diphenyl-2-picrylhydrazyl (DPPH) (99 %), (Sigma Aldrich, N: STBD 4145 V 99122)
2) 6-hydroxy-2,5,7,8-tetramethylchroman-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.

II,2. Methods.

I) Determination of radical scavenging activity by the DPPH assay.

1) Preparation of 0.1 mM methanol solution of DPPH.

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

2) Preparation of stock solution of Trolox.

An accurarely quantity of 0.0125 g 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (M = 252.294) was dissolved in distilled water and was diluted in volumetric flask of 50 ml with distilled water to obtain concentration of 1 mM Trolox (1000 μM). An aliquot part of 1.0 ml was diluted in volumetric flask of 10.0 ml with distilled water to obtain the stock solution of Trolox with concentration 0.1 mM (10 μM).

3) Preparation of working solutions of Trolox.

From stock solution of 0.1 mM Trolox an 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, 0.04 mM, 0.06 mM, 0.08 mM.

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

4) Prepartation of phosphate buffer pH = 7.

For the prepartation of phosphate buffer pH = 7 solution, an accurarely quantities of 0.1 g potassium dihydrogenphosphate (KH_2PO_4), 0.2 g dipotassium hydrogenphosphate (K_2HPO_4), and 0.85 g sodium chloride (NaCl) were dissolved in volumetric flask of 100.0 ml with distilled water.

5) Preparation of stock solutions of Creatine monohydrate.

An accurarely quantity of 0.7458 g Creatine monohydrate (M = 149.15) was diluted in volumetric flask of 50.0 ml with phosphate buffer pH = 7 to obtain the stock solution of Creatine monohydrate with concentration 100 mM (0.1 M).

6) Prepartation of working solutions of Creatine monohydrate.

From stock solution of 100 mM Creatine monohydrate an aliquot parts respectivively 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 Creatine monohydrate: 20 mM, 40 mM, 60 mM, 80 mM.

7) DPPH – assay.

DPPH assay was performed according to the following procedure:
1) 5 ml 0.1 mM DPPH methanol solution were mixed separatelly with 5 ml of Creatine monohydrate in concentrations 20 mM, 40 mM, 60 mM, 80 mM to obtain final concentrations respectively of 0.05 mM DPPH methanol solution, and 10 mM, 20 mM, 30 mM, 40 mM of Creatine monohydrate.
2) 5 ml 0.1 mM DPPH methanol solution were mixed separatelly with 5 ml Trolox in concentrations: 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM to obtain final concentrations: 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM
3) 5 ml 0.1 mM DPPH methanol solution were mixed separatelly with 5 ml 0.002 mM Trolox to obtain final concentrations 0.001 mM.

As control was recorded the micture of 5 ml 0.1 mM DPPH methanol solution added to 5 ml methanol. The mixtures were shaken vigorously and allowed to stand for incubation in dark for 1 h at temperature: 25 ^oC ÷ 27 ^oC After incubation, the absorbances were measurement against a blank methanol at wavelength λ = 516 nm using a UV-VIS spectrophotometer Hewlett-Packard A Diode Array 8452. All the tests were performed in triplicates and the results were averaged.

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III, RESULTS AND DISCUSSION.

DPPH assay is based on the ability and capacity of an antioxidant to inactivate the stable DPPH cation radical by donating a hydrogen atom or electron. During this process, the violet colored DPPH molecule becomes colorless to pale yellow, which can spectroscopically be monitored at a wavelength λ = 517 nm [19].
The degree of discoloration indicates the scavenging potential of the compound present through hydrogen donating ability [20].

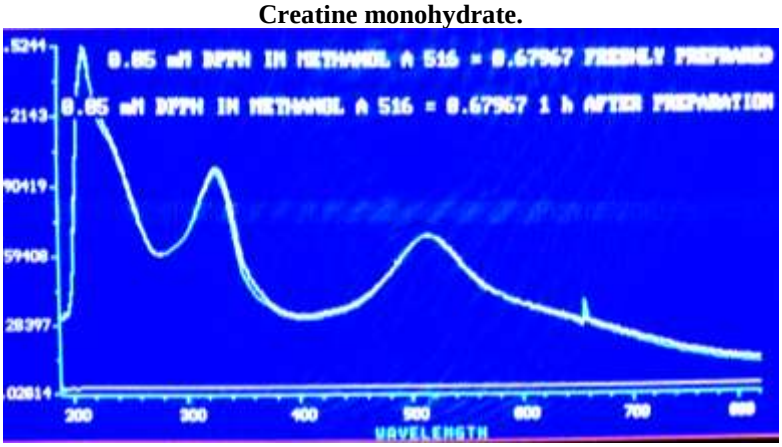
The reducing ability of Creatine monohydrate, and standard Trolox presented as percentage of antiradical activity (RSA %), was assessed by DPPH free radical scavenging assay. The measurement of the DPPH radical binding effect was performed according to methodology described by original methods of Blois and Brand-Williams with the following paramerets modified:

1) 0.05 mM DPPH [21]
2) Trolox solution as standard [22]
3) methanol as solvent [20]
4) temperature: 25 ^{o}C ÷ 27 ^{o}C [20]
5) ratio sample/DPPH solution: 1:1 [23]
6 duration of reaction: 1 h [24]
7) measurment of the decrease of DPPH absorbance at λ = 516 nm wavelength.

Spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after preparation and after 1 h reaction with solutions of Creatine monohydrate at in dark at temperature: 25 ^{o}C ÷ 27 ^{o}C is presented on Figure 1.

I. Spectra of 0.05 mM DPPH methanol solution.

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



The decrease of the absorbanses was registered and spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions are illustrated on Figure 2. (10 mM and 20 mM Creatine monohydrate), Figure 3. (30 mM and 40 mM Creatine monohydrate), Figure 4. (0.001 mM, 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM Trolox).

Figure 2. Spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions of 10 mM and 20 mM Creatine monohydrate.

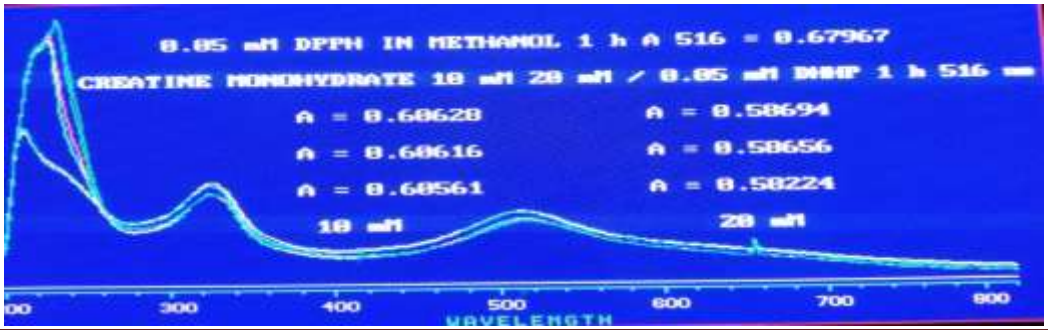


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Figure 3. Spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions of 30 mM and 40 mM Creatine monohydrate.

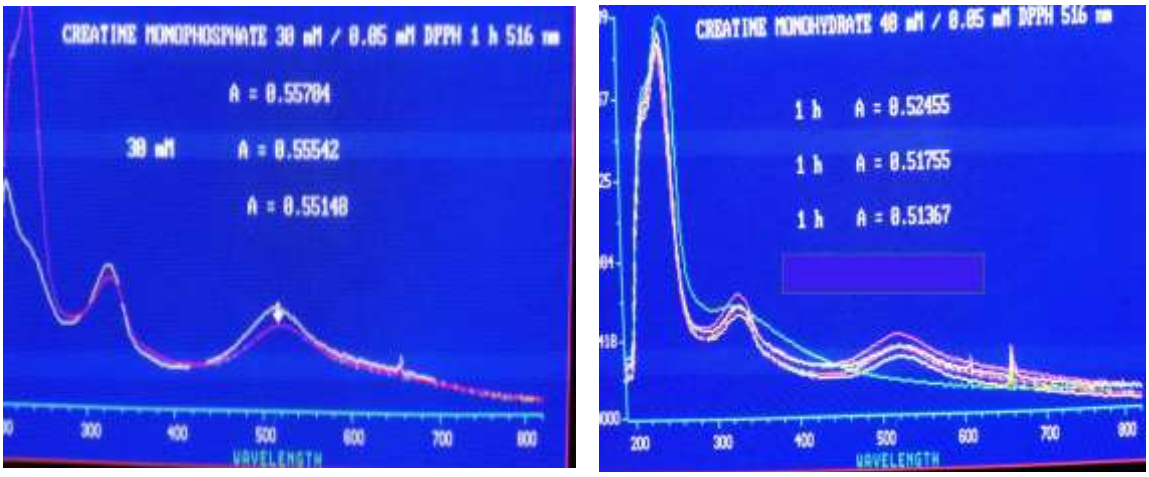
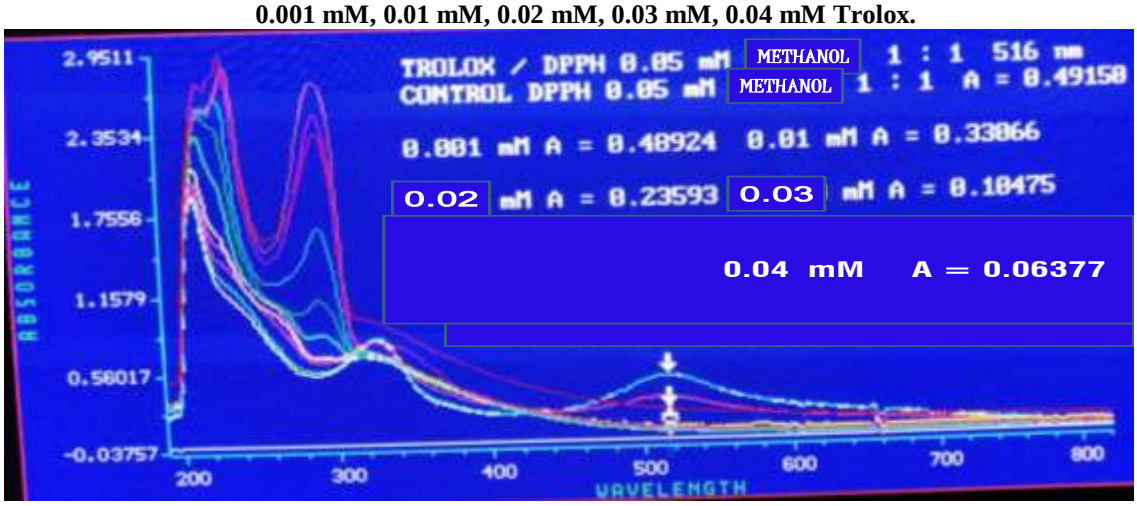


Figure 4. Spectra of 0.05 mM DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions of 0.001 mM, 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM Trolox.



II. Absorbances at λ = 516 nm of 0.05 mM methanol solution of DPPH.

On Table 1. are presented experimental results of absorbances of 0.05 mM methanol solution of DPPH after 1 h reaction with the Creatine monohydrate.

On Table 2. are summarized data of absorbances of 0.05 mM methanol solution of DPPH after 1 h reaction with methanol solutions of standard Trolox.

The results for the absorbance values of 0.05 mMl DPPH methanol solution at λ = 516 nm after 1 h reaction with solutions of Creatune monohydrate, and standard Trolox are putted against the corresponding concentrations into linear regression analysis.

The calibration curves are shown on Figure. 5. Linearity is characterized by linear regression coefficients, which are R^2 > 0.96. Parameters of regression equations are included in Table 3. The high values of the regression coefficients obtained from the calibration curves, after applying the regression analysis, prove the linear dependences between the absobances of the compounds and the concentration. Lower absorbance values indicate higher free radical scavenging activity.

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Table 1. Absorbances at λ = 516 nm of 0.05 mM methanol solution of DPPH after 1 h reaction with Creatine monohydrate.

Concentration	10 mM	20 mM	30 mM	40 mM
N:	A [AU]	A [AU]	A [AU]	A [AU]
1	0.60628	0.58694	0.55704	0.52455
2	0.60616	0.58656	0.55542	0.51755
3	0.60561	0.58224	0.55148	0.51367
$\overline{X}$	0.60602	0.58525	0.55465	0.51859
SD	3.57	2.61	2.86	5.51

Table 2. Absorbances at λ = 516 nm of 0.05 mM methanol solution of DPPH after 1 h reaction with Trolox.

N:	**Concentration** [mM]	**Absorbance** [AU]
1.	0.001	0.48924
2.	0.01	0.33066
3.	0.02	0.23593
4.	0.03	0.10475
5	0.04	0.06377

Figure 5. Absorption calibration curves for 0.05 mM methanolic solution of DPPH-radical after 1 h interaction with 10 mM ÷ 40 mM Creatine monohydrate and 0.001 mM ÷ 0.04 mM Trolox.

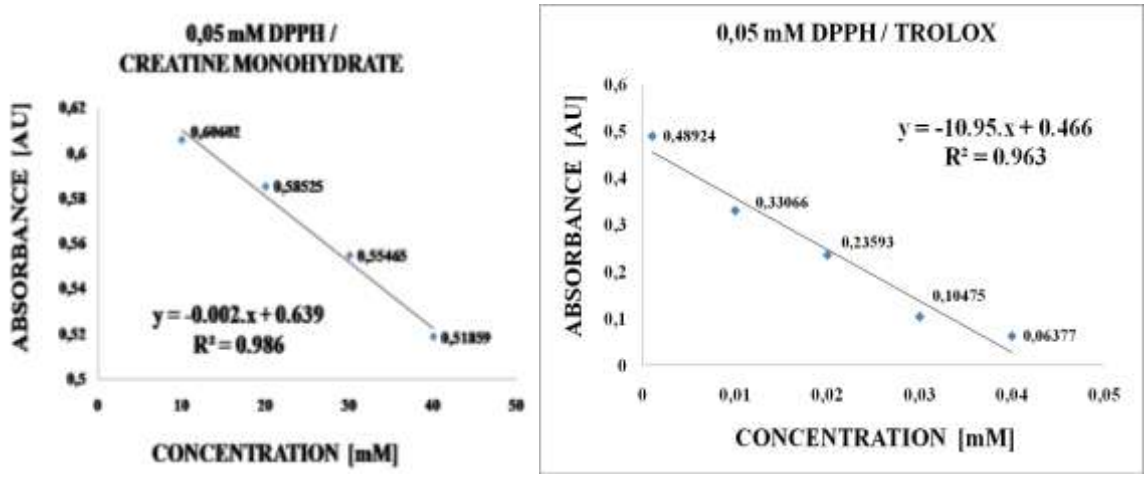

Table 3. Parameters of regression equations for absotbances of Creatine monohydrate and standard Trolox.

N:	**Parameters**	**Creatine monohydrate**	**Trolox**
1.	Range [mM]	10 mM ÷ 40 mM	0.001 mM ÷ 0.04 mM
2.	Regression equation	y = - 0.002.x + 0.639	y = - 10.95.x + 0.466
3.	Slope	- 0.002	- 10.95
4.	Intersept	0.639	0.466
5.	R^2	0.986	0.963

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

The results of DPPH-radical scavenging activity (RSA), for a period of 1 h reaction of 0.05 mM methanol solution of DPPH with 10 ÷ 50 mM standard Trolox, and 10 ÷ 40 mM Creatine monohydrate, were calculated by the equation:

$$\text{RSA [\%]} = \frac{ADPPHcontrol - Asample}{ADPPHcontrol}.100$$

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

Absorbance of DPPH solution in control is measured against methanol.

On Table 4. are presented radical-binding effect of 10 mM ÷ 40 mM Creatine monohydrate, and at 0.001 mM ÷ 0.04 mM Trolox after 1 h interaction with 0.05 mM methanol solution of DPPH-radical.

Table 4. Radical-scavenging activity of 10 mM ÷ 40 mM Creatine monohydrate and of 0.001 mM ÷ 0.04 mM Trolox after 1 h interaction with 0.05 mM methanol solution of DPPH-radical.

N:	Creatune monohydrate				Trolox	
	10 mM	20 mM	30 mM	40 mM		
	RSA [%]	RSA [%]	RSA [%]	RSA [%]	C [mM]	RSA [%]
1	10.80	13.64	18.04	22.82	0.001	28.02
2	10.82	13.70	18.28	23.85	0.01	51.35
3	10.90	14.33	18.86	24.42	0.02	65.29
$\overline{X}$	10.84	13.89	18.39	23.70	0.03	84.59
SD	0.05	0.38	0.42	0.81	0.04	90.62
RSD	0.46	2.74	2.28	3.42		

The results for the radical scavenging activity values of Creatine monohydrate and standard Trolox 0.05 mM methanol solution in the respective concentration intervals at λ = 516 nm after 1 h reaction with 0.05 mM DPPH methanol solution were subjected to linear regression analysis and the resulting calibration curves are illustrated on Figure 6.

The high values of the regression coefficients obtained from the calibration curves, after applying the regression analysis, prove the linear dependences between the radical scavenging activity of the compounds and the concentration. The experimental results show that with an increase in the concentration from 10 mM to 40 mM and for Creatine monohydrate, and for the Trolox standard (0.001 mM ÷ 0.04 mM), an increase in the antiradical effect is observed (Figure 6.)

Figure 6. Calibration curves for radical scavenging activity of 10 mM ÷ 40 mM Creatine monohydrate and 0.001 mM ÷ 0.04 mM Trolox standard.

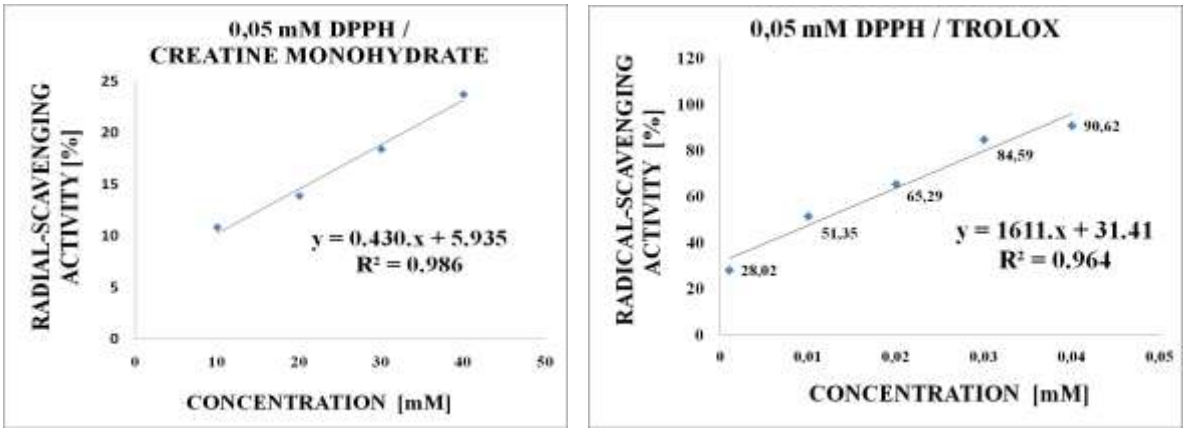

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Table 5. presents the parameters of the regression equations for the radical-binding effect of Creatine monohydrate and standard Trolox.

Table 5. Parameters of regression equations for radical-scavenging activity of Creatine monohydrate and standard Trolox.

N:	Parameters	Creatine monohydrate	Trolox
1.	Range [mM]	10 mM ÷ 40 mM	0.001 mM ÷ 0.04 mM
2.	Regression equation	y = 0.430.x + 5.935	y = 1611.x + 31.41
3.	Slope	0.430	1611
4.	Intersept	5.935	31.41
5.	R^2	0.986	0.964

III. Calculation of not-scavenged radical (R [%]).

The results for not-scavenged radical (R, [%]), for a period of 1 h reaction of 0.05 mM methanol solution of DPPH with 10 ÷ 40 mM Creatine monohydrate, and 0.001 mM ÷ 0.04 mM Trolox were calculated by the equation:

$$R\,[\%] = \frac{Asample}{ADPPHcontrol}.100$$

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
Absorbance of DPPH solution in control is measured against methanol.

The experimental results for the amount of free DPPH-radical (R) are described in Table 6.

Table 6. Uncoupled DPPH-radical from 10 mM ÷ 40 mM Creatine monohydrate, and 0.001 mM ÷ 0.04 mM Trolox after 1 h interaction with 0.05 mM methanol solution of DPPH-radical.

N:	Creatune monohydrate				Trolox	
	10 mM	20 mM	30 mM	40 mM		
	R [%]	R [%]	R [%]	R [%]	C [mM]	R [%]
1	89.20	86.36	81,96	77.18	0,001	71.98
2	89.18	86.30	81.72	76.15	0.01	48.65
3	89.10	85.67	81.14	75.58	0,02	34.71
$\overline{X}$	89.16	86.11	81.61	76.30	0,03	15.41
SD	0.05	0.38	0.42	0.81	0.04	9.38

The results for the values of unbound DPPH-radical from Creatine monohydrate and methanol solution of standard Trolox in the respective concentration intervals at λ = 516 nm after 1 h reaction with 0.05 mM DPPH methanol solution were subjected to linear regression analysis, and the obtained calibration curves are shown on Figure 7., and the parameters of the regression equations are summarized in Table 7.

Figure 7. Calibration curves for unscavenged DPPH-radical from 10 mM ÷ 40 mM Creatine

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monohydrate and 0.001 mM ÷ 0.04 mM Trolox.

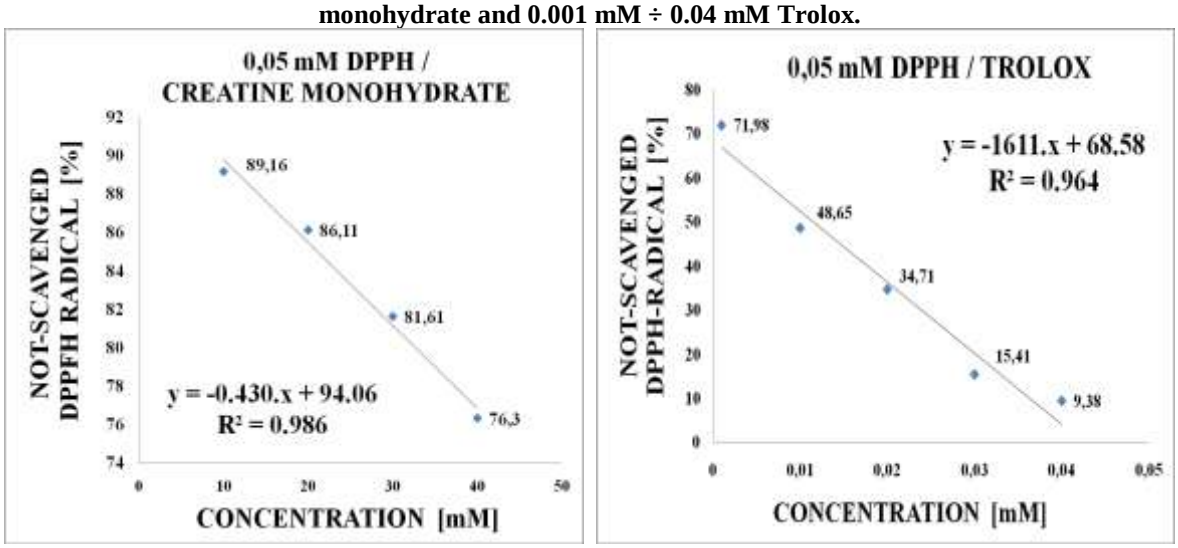

Table 7. Parameters of regression equations for unscavenged 0.05 mM methanol solution of DPPH-radical from 10 mM ÷ 40 mM Creatine monohydrateand 0.001 mM ÷ 0.04 mM Trolox.

N:	Parameters	Creatine monohydrate	Trolox
1.	Range [mM]	10 mM ÷ 40 mM	10 mM ÷ 50 mM
2.	Regression equation	y = 0.430.x + 94.06	y = - 1611.x + 68.58
3.	Slope	0.430	- 1611
4.	Intersept	94.06	68.58
5.	R^2	0.986	0.964

IV. Calculation of the IC50 value (inhibitory concentration) and the antioxidant power 1/IC50.

For the evaluation of the antiradical activity of the compounds, the results were expressed as: IC50 values, which determine the amount of compound needed to reduce the concentration of the radical by 50%, and as antioxidant power: 1/IC50. IC_{50} value is the concentration of the test samples at which the inhibition percentage reaches 50 %. 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 monohydrate and of standard Trolox at λ = 516 nm. The regression analysis method was used to calculate the IC50 values at which 50% of the DPPH-radical was inhibited, according to the following procedure: inhibition ratios (y) were plotted against the sample concentrations (x), and the respective regression line (y = ax + b) was drawn. X (sample concentration) was calculated by substituting the value of Y with 50 in the regression equation of Y = AX + B.

The equation obtained from the regression analysis for Trolox was used to calculate the IC50, and the resulting concentrations at which 50% scavenging of the DPPH-radical was achieved were:

Creatine monohydrate: IC50 = 102.48 mM
1/IC50 = 0.01
Trolox: IC50 = 0.01154 mM
1/IC50 = 8.67

The lower IC50 value determines that at a lower concentration the compounds show a higher antiradical effect. Due to the lower IC50 value, standard Trolox has a higher antioxidant activity, which expressed in antioxidant power (0.04) is 8.67 times higher than Creatine monohydrate (0.01).

V. Calculation of Trolox equivalent antioxidant capacity.

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

$$TEAC = \frac{IC50 Trolox}{IC50 sample}.$$

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The higher TEAC value means the higher DPPH radical scavenging activity

Anti-DPPH effect as Trolox equivalent antioxidant capacity is:TEAC = 0.00011.

VI. Calculation of relative radical scavenging activity (RRSA, [%]) and relative decrease in radical scavenging activity (RDRSA, [%]).of Creatine monohydrate against Trolox standards.

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

$$\text{RRSA [\%]} = \frac{RSA\,sample}{RSA\,Trolox}.100$$

$$\text{RDRSA [\%]} = \frac{RSA\,Trolox - RSA\,sample}{RSA\,Trolox}.100$$

RSA_{sample} - radical binding activity of the test compound

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

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, 0.04 mM solutions of Creatine monohydrate were calculated (Table 8.). Results for (RRSA, [%]) and (RDRSA, [%]) of Creatine monohydrate against standard Trolox are shown on Table 8.

Table 8. Relative radical scavenging activity (RRSA, [%]) and relative decrease in radical scavenging activity (RDRSA, [%]).of Creatine monohydrate against standard Trolox.

	Trolox	**Creatine monohydrate**		
C [mM]	RSA [%]	RRA [%]	RRSA [%])	RDRSA [%]
0.01	51.35	5.9393	11.57	88.43
0.02	65.29	5.9436	9.10	90.90
0.03	84.59	5.9479	7.03	92.97
0.04	90.62	5.9522	6.57	93.43

CONCLUSION

The obtained results for inhibitory concentration are: IC50 Creatine monohydrate = 102.48 mM and IC50 Trolox = 0.01154 mM. Trolox posssess higher antioxidant power (1/IC50 = 0.01) than Creatine monohydrate. (1/IC50 = 8.67).
Anti-DPPH effect as Trolox equivalent antioxidant capacity is: TEAC = 0.00011.

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Conflict of interest: All of the authors declare that they do not have any conflicts of interest.
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Ethics statement: None

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