Original Article

Application of ABTS method for assessment of radical-binding effect of Creatine monohydrate

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ABSTRACT

Due to its antioxidant properties Creatine exhibits benefits on muscle, bone, and brain function, and can be of great importance for the prevention of the oxidative stress-related diseases. The aim of current study was the investigation of antiradical effect of Creatine monohydrate by the application of ABTS method. The radical-scavenging activity of Creatine monohydrate against methanol solution of ABTS radical was evaluated by measuring the decrease in the absorbance at $\lambda = 744$ nm. For the estimation of antiradical effect of the compound examined the following parameters were calculated: radical scavenging activity in [%], IC_{50} value; antioxidant power $1/IC_{50}$, Trolox equivalent activity. Linear relationship between the enhanced radical scavenging activity and decrease of absorbances and of not-bound ABTS-radical with the increase of concentration of Trolox (0.002 mM ÷ 0.75 mM) and Creatine monohydrate (20 mM ÷ 200 mM) has been established. Linearity was characterized by coefficients of linear regression, which were proven to be higher than 0.97. From the experimental results it was observed that Creatine monohydrate ($IC_{50} = 100.98$ mM) exerts antiradical effect, but is less active compared to Trolox ($IC_{50} = 0.2$ mM) due to higher IC_{50} value and lower antioxidant power ($1/IC_{50}$, = 0.01) than Trolox ($1/IC_{50} = 5$).

Keywords: ABTS, Creatine monohydrate, Trolox, Scavenging activity, Reactive oxygen species

Introduction

The disballance between the decreased activity of endogenous antioxidant protective enzymes and the overproduction of free radicals [1] leads to oxidative stress. Brain neurons are particularly sensitive to the effect of oxidative stress, caused by glutamate and ß-amyloid peptide, due to the increased oxygen consumption [2]. Low brain Creatine content has been associated with cognitive and movement disorders, epilepsy, and muscle myopathies [3]. In regulating the contractile ability of vascular smooth muscle, endothelial cells produce superoxide radicals [4]. Oxidative stress is associated with the development of neurodegenerative diseases such as Alzheimer, Parkinson, Huntington [5], Lou Gehrig disease (amiotrophic lateral sclerosis) [6]. Oxidative stress is a factor in pathogenesis of cancer [7], rheumatic diseases [8], and aging [4].

The consumption of antioxidants in form of dietary supplements [9] is of great importance for the prevention of the oxidative stress-related diseases [10]. Multiingredient nutritional supplements improve cognitive function [11].

The insufficient biosynthesis is a factor for Creatine deficiency syndromes which leads to the developmental of mental disorders [12]. Creatine supplementation has been investigated to be effective against different diseases [13]. The potential therapeutic role of Creatine in health has been proven as a result from its antioxidant properties [14].

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It has been reported that in adults Creatine exhibits beneficial properties on muscle, bone [15], and brain function [15, 16]. It has been investigated that dietary Creatine is essential for brain health [17] due to improves cognition [18] and enhances hippocampal-dependent spatial memory, and bioenergetics [19]. In several studies have been investigated therapeutic benefits of Creatine supplementation against neurodegenerative diseases such as Parkinson [20] Huntington [21], amyotrophic lateral sclerosis [22], and encephalomyopathies [23]. In different studies has been reported that Creatine supplementation are effective in muscular atrophy and sarcopenia [24], can improve reproductive perinatal outcomes [25, 26], and during pregnancy prevents acute deficits in skeletal muscle after birth asphyxia [27].

It has been described that in chronic heart failure Creatine supplementation are effective in combination with with Coenzyme Q_{10} (Ubiquinone, Vitamin Q_{10}) which is one of the most important lipid antioxidants, and prevents the generation of free radicals and modifications of proteins, lipids and DNA. The main biochemical action of Coenzyme Q_{10} is as a cofactor in the electron transport chain, in the series of redox reactions involved in the synthesis of adenosine triphosphate. In many diseases associated with increased generation and action of reactive oxygen species, the concentration of coenzyme Q_{10} in the body decreases and its deficiency leads to dysfunction of the respiratory chain. The potential use of Coenzyme Q_{10} in combination with Creatine may help prevent: cardiovascular disease, mitochondrial disorders, Parkinson's disease, muscular dystrophy and aging [28].

The aim of current study was the evaluation of the radical-scavenging activity of Creatine monohydrate against methanol solution of ABTS radical by measuring the decrease in the absorbance at $\lambda = 744$ nm.

Materials and Methods

Materials

I. Test compound: Creatine monohydrate

II. Reagents with pharmacopoeial purity

1. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, N:51796 PMV 291913)
2. ABTS: diammonium salt of (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma Aldrich, N:SLBH 2992 V)
3. potassium persulfate (Sigma Aldrich, N:BCBL 7396 V)
4. methanol (99.9 %) (Sigma-Aldrich, N:SZBD 063 AV UN 1230).
5. distilled water.

III. ABTS-radical-scavenging method for in-vitro study of radical-scavenging activity of Creatine monohydrate.

1. *Preparation of 7 mM ABTS stock solution*
   An accurarely measured amount of 0.3841 g ABTS diammonium salt (M = 548.7) was dissolved in phosphate buffer solution pH = 6.8 and diluted in volumetric flask of 100.0 ml with phosphate buffer solution pH = 6.8 to obtain a solution of concentration 7 mM.
2. *Preparation of 2.45 mM potassium persulfate stock solution*
   An accurarely measured quantity of 0.0662 g potassium persulfate (M = 270.322) was dissolved with phosphate buffer solution pH = 6.8 and diluted in volumetric flask of 100.0 ml with phosphate buffer solution pH = 6.8 to obtain a solution with a concentration of 2.45 mM.
3. *Preparation of stock mixed solution of 7 mM ABTS and 2.45 mM potassium persulfate*
   Equal aliquot parts of 100.0 ml 7 mM ABTS solution and 100.0 ml 2.45 mM potassium persulfate solution were mixed and the reagent was left in the dark for 17 h.
4. *Preparation of working solutions of Trolox in methanol for ABTS-assay*
   An accurarely measured amount of 0.0125 g of Trolox (M = 252.294) was dissolved and diluted in a 50.0 ml volumetric flask with methanol to obtain a solution of Trolox with a concentration of 1 mM Trolox (1000 μM). Aliquot parts of 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml, 4.0 ml, 4.5 ml, 5 ml, 5.5 ml, 6.0 ml, 6.5 ml, 7.0 ml, 7.5 ml were diluted in 10.0 ml volumetric flasks with methanol to obtain Trolox stock solutions with concentrations: 100 μM (0.1 mM), 150 μM (0.15 mM), 200 μM (0.2 mM), 250 μM (0.25 mM), 300 μM (0.3 mM), 350 μM (0.35 mM), 400 μM (0.4 mM), 450 μM (0.45 mM), 500 μM (0.5 mM), 550 μM (0.55 mM), 600 μM (0.6 mM), 650 μM (0.65 mM), 700 μM (0.7 mM), 750 μM (0.75 mM). An aliquot part of 2.0 ml 100 μM (0.1 mM) was diluted with methanol in volumetric flask of 10.0 ml to obtain Trolox working solution with concentration 20 μM (0.02 mM). An aliquot part of 1.0 ml 20 μM (0.02 mM) was diluted with methanol in volumetric flask of 10.0 ml to obtain Trolox working solution with concentration 2 μM (0.002 mM).
5. *Preparation of phosphate buffer solution pH = 6.8*
   For the preparation of phosphate buffer solution with pH = 6.8, an accurarely measured 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 volumetric flask of 100.0 ml with distilled water.
6. *Preparation of stock solution of Creatine monohydrate*
   An accurarely measured amount of 1.4916 g Creatine monohydrate (M = 149.15) was dissolved in phosphate buffer pH = 6.8 and was diluted in volumetric flask of 50.0 ml with phosphate buffer pH = 6.8 to obtain the stock solution with concentration 200 mM (0.2 M).
7. *Preparation of working solutions of Creatine monohydrate*
   From stock solution of 200 mM Creatine monohydrate an aliquot parts respectivively of 10.0 ml, 20.0 ml, 30.0 ml, 40.0 ml, 50.0 ml, 60.0 ml and 80.0 ml were diluted with

phosphate buffer pH = 6.8 separately in volumetric flasks of 100.0 ml to obtain final concentrations of working solutions of Creatine monohydrate: 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 120 mM, 160 mM.

8. *ABTS – procedure*

The ABTS assay was performed according to the following procedure: ABTS stock solution was diluted 1 : 10 v/v with methanol. 5.0 ml of the obtained ABTS working solutions were mixed separately respectively with 5.0 ml of working solutions of Trolox (0.002 mM ÷ 0.75 mM) and Creatine monohydrate (20 mM ÷ 200 mM) to obtain final concentrations of Trolox (0.001 mM ÷ 0.375 mM): 1 µM (0.001 mM), 10 µM (0.01 mM), 50 µM (0.05 mM), 75 µM (0.075 mM), 100 µM (0.1 mM), 125 µM (0.125 mM), 150 µM (0.15 mM), 175 µM (0.175 mM), 200 µM (0.2 mM), 225 µM (0.225 mM), 250 µM (0.25 mM), 275 µM (0.275 mM), 300 µM (0.3 mM), 325 µM (0.325 mM), 350 µM (0.35 mM), 375 µM (0.375 mM) and of Creatine monohydrate (10 mM ÷ 100 mM): 10 mM, 20 mM, 30 mM, 40 mM. 50 mM, 60 mM, 80 mM, 100 mM. Mixture of 5.0 ml of ABTS working solution and 5.0 ml of methanol was used as a control. The tubes are shaken vigorously and left to incubate in the dark for 10 min. at temperature: 25 °C ÷ 27 °C. After incubation, absorbances were measured against a blank: methanol at λ = 744 nm with a UV-vis spectrophotometer (Hewlett-Packard A Diode Array 8452).

Calculation methods

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

The results of ABTS-radical scavenging activity (RSA), and for not-scavenged radical (R, [%]), for a period of 10 min. reaction of methanol solution of ABTS with solutions of standard Trolox, and Creatine monohydrate, were calculated by the equation:

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

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

A_{ABTS} control – absorbance of the solution of ABTS-radical before interaction with the compound investigated

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

Absorbance of ABTS solution in control is measured against methanol.

2. Calculation of IC50 value (inhibitory concentration)

The concentration of the compound, at which the inhibition ot of ABTS-radical reaches 50 % is presented as IC_{50} value. From the ABTS radical-scavenging curves of standard Trolox and Creatine monohydrate at λ = 744 nm were calculated IC_{50} values (mM). The 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. Higher radical-scavenging activity of the compounds investigated corresponds to a lower IC_{50} value.

3. Calculation of antioxidant power: 1/IC_{50}

4. Calculation of Trolox equivalent antioxidant capacity

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

$$TEAC = \frac{IC50Trolox}{IC50sample} \quad (3)$$

The higher TEAC value means the higher ABTS radical scavenging activity.

Results and Discussion

The increase in the radical-scavenging effect of compounds is directly proportional to the decrease of the absorbance of a solution in electron transfer based methods as:

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 [29].

The oxidation of ABTS with potassium persulfate generates a green $ABTS^+$-radical which reduction in the presence of hydrogen donating antioxidants is measured [29].

DPPH and ABTS methods has been applied for the investigation of free radical scavenging effect of extracts from different plants as follows:

1. DPPH for *Curcuma xanthorrhiza Roxb* [30]
2. ABTS for *Thymelaea hirsute* [31].

Results for ABTS radical-scavenging activity

In spectra of methanol solutions of ABTS at λ = 744 nm the absorbance of control is 0.99425. Spectra of ABTS methanol solutions at λ = 744 nm after 10 min. interraction with Creatine monohydrate solutions (10 mM ÷ 100 mM) .are illustrated on **Figure 1**.

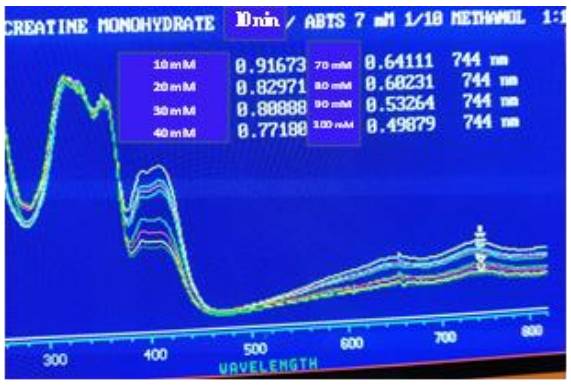


Figure 1. Spectra of ABTS methanol solutions at λ = 744 nm after 10 min. interraction with Creatine monohydrate solutions (10 mM ÷ 100 mM).

The experimental results for the values of absorbance at λ = 744 nm [A, AU], radical-binding activity [RSA, (%)] and for the unbound ABTS-radical [R, (%)] after 10 min. interaction with ABTS-radical solution of methanol solutions of standard Trolox (0.001 mM ÷ 0.375 mM) **(Table 1)** and with Creatine monohydrate solutions (10 mM ÷ 100 mM) **(Table 2)** are presented.

Table 1. Absorbance at λ = 744 nm, radical-binding activity [RSA] and unbound ABTS-radical [R] after 10 min. interaction of methanol solutions of standard Trolox (0.001 mM ÷ 0.375 mM) with ABTS-radical solution.

	C [mM]	A [AU]	RSA [%]	R [%]
1.	0.001	0.89273	10.21	89.79
2.	0.01	0.81888	17.64	82.36
3.	0.05	0.75548	24.02	75.98
4.	0.075	0.70116	29.48	70.52
5.	0.1	0.64565	35.06	64.94
6.	0.125	0.59406	40.25	59.75
7.	0.150	0.54890	44.79	55.21
8.	0.175	0.50122	49.59	50.41
9.	0.200	0.46295	53.44	46.56
10.	0.225	0.42628	57.13	42.87
11.	0.250	0.39632	60.14	39.86
12.	0.275	0.36690	63.10	36.90
13.	0.300	0.33975	65.83	34.17
14.	0.325	0.31114	68.71	31.29
15.	0.350	0.28517	71.32	28.68
16.	0.375	0.26250	73.60	26.40

Table 2. Absorbance at λ = 744 nm, radical-binding activity [RSA] and unbound ABTS-radical [R] after 10 min. interaction of methanol solutions of Creatine monobydrate (10 mM ÷ 100 mM) with ABTS-radical solution.

N:	C [mM]	A [AU]	RSA [%]	R ABTS [%]
1.	10	0.91673	7.80	92.20
2.	20	0.82971	16.55	83.45
3.	30	0.80888	18.64	81.36
4.	40	0.77180	22.37	77.63
5.	50	0.64111	35.52	64.48
6.	60	0.60231	39.42	60.58
7.	80	0.53264	46.43	53.,57
8.	100	0.49879	49.83	50.17

The results for the absorbance values of ABTS methanol solution at λ = 744 nm after 10 min. interraction with methanol solutions of standard Trolox (0.001 mM ÷ 0.375 mM) **(Figure 2)** and with Creatine monohydrate solutions (10 mM ÷ 100 mM) **(Figure 3)** were putted against the corresponding concentrations into linear regression analysis and the linear dependence between the decrease of absorbances with an increase of concentration in the investigated ranges was observed. In calibration curves linearity is characterized by coefficient of linear regression, which is $R^2 = 0.974$ for Trolox and $R^2 = 0.991$ for Creatine monohydrate.

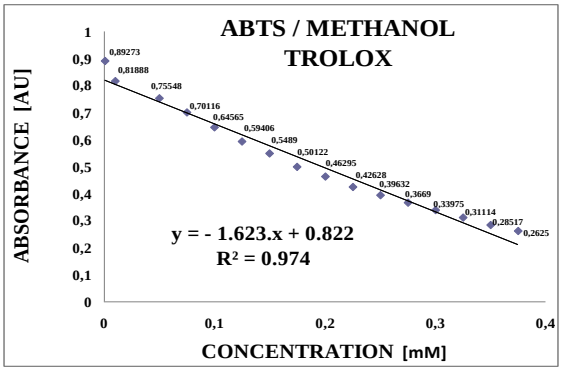


Figure 2. Calibration curve for absorbances of ABTS-radical after 10 min. reaction with methanol solutions of standard Trolox (0.001 mM ÷ 0.375 mM).

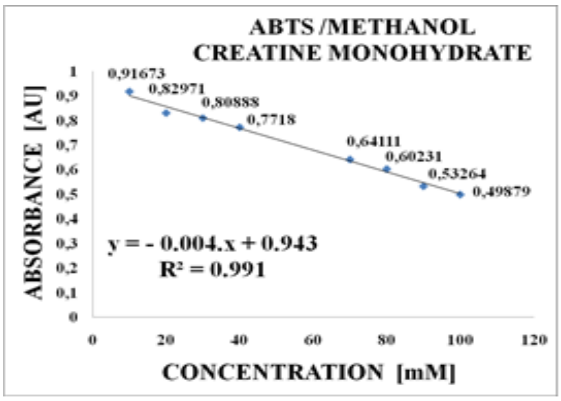
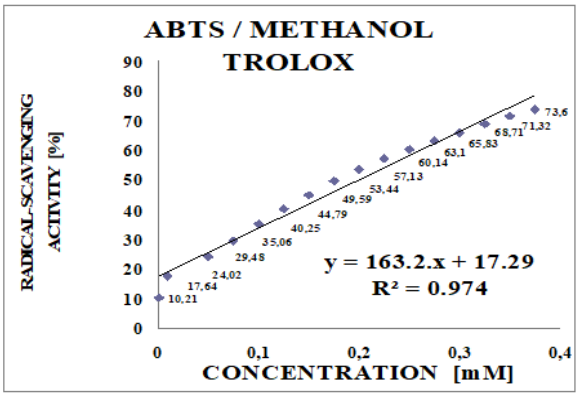
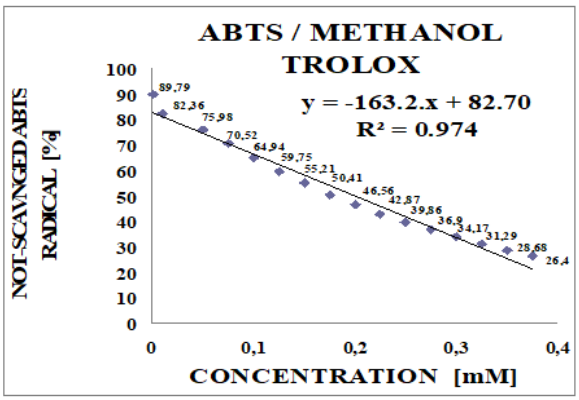


Figure 3. Calibration curve for absorbances of ABTS-radical after 10 min. reaction with Creatine monohydrate solutions (10 mM ÷ 100 mM).

Lower absorbance values indicate higher free radical scavenging activity. The results for scavenging activity and for not-scavenged ABTS-radical of Trolox from 0.001 mM to 0.325 mM are subjected to a linear regression analysis against the respective concentrations. On **Figure 4** is illustrated calibration curves for linear relationship between the enhanced radical binding activity with the increase of concentration of Trolox from 0.001 mM to 0.325 mM and for not-scavenged ABTS-radical.



a)

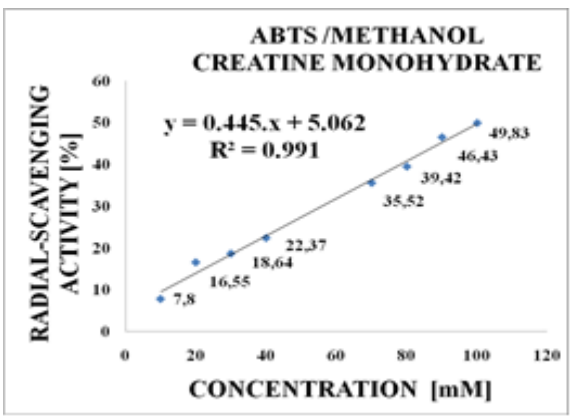


b)

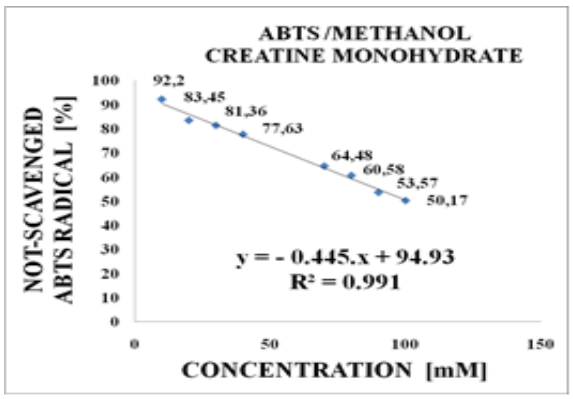
Figure 4. Calibration curves for scavenged and not-scavenged ABTS-radical after 10 min. reaction with methanol solutions of standard Trolox (0.001 mM ÷ 0.375 mM).

The high values for regression coefficients obtained from calibration curve after linear regression analysis prove the linear dependence between the increase of radical-scavenging activity with increase of concentration of standard Trolox **(Figure 4)**.

The data for ABTS-radical scavenging effect and for not-scavenged ABTS-radical from Creatine monohydrate (10 mM ÷ 100 mM) are subjected to a linear regression analysis. On **Figure 5** is shown calibration curve for linear relationship between the enhanced radical-binding activity and the decreased not-scavenged ABTS-radical with the increase of concentration of Creatine monohydrate from 10 mM to 100 mM.



a)



b)

Figure 5. Calibration curves for scavenged and not-scavenged ABTS-radical after 10 min. reaction with methanol solutions of Creatine monohydrate (10 mM ÷ 100 mM).

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

The results are expressed as: IC_{50} and antioxidant power: $1/IC_{50}$. High radical-scavenging activity is associated with low IC_{50} value, which defines that at less concentration the compounds exert high antiradical effect. The regression analysis method was employed and the obtained regression equations for standard Trolox and Creatine monohydrate were used to calculate the IC_{50} value which determines the amount of antioxidant needed for decreasing the radical concentration by 50 %.

Due to the lower IC_{50} = 0.2 mM standard Trolox possess the higher antioxidant activity than Creatine monohydrate (IC_{50} = 100.98 mM)

Calculation of Trolox equivalent antioxidant capacity

The ABTS-radical scavenging activity of Creatine monohydrate, expressed as Trolox equivalent antioxidant capacity is: TEAC = 0.002.

Conclusion

From the experimental results it was observed that the ABTS-radical scavenging effect of Creatine monohydrate (IC_{50} = 100.98 mM) is lower compared to the standard Trolox (IC_{50} = 0.2 mM), which antioxidant power ($1/IC_{50}$ = 5) is higher in comparison with Creatine monohydrate ($1/IC_{50}$ = 0.01).

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Conflict of interest: None

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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. Butterfield DA. Amyloid ß-peptide [1-42]-associated free radical-induced oxidative stress and neurodegeneration in Alzheimer's disease brain: mechanisms and consequences. Curr Med Chem. 2003;10(24):2651-9. doi:10.2174/0929867033456422
3. Salazar MD, Zelt NB, Saldivar R, Kuntz CP, Chen S, Penn WD, et al. Classification of the molecular defects associated with pathogenic variants of the SLC6A8 Creatine transporter. Biochemistry. 2020;59(13):1367-77. doi:10.1021/acs.biochem.9b00956
4. 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
5. 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
6. 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
7. 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):4084-01. doi:10.2174/0929867324666170711114336
8. 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):210-5. doi:10.1002/jcla.20243
9. Rawson ES, Miles MP, Larson-Meyer DE. Dietary supplements for health, adaptation, and recovery in athletes. Int J Sport Nutr Exerc Metab. 2018;28(2):188-99. doi:10.1123/ijsnem.2017-0340
10. 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/biomedicines/10123051
11. Bell KE, Fang H, Snijders T, Allison DJ, Zulyniak MA, Chabowski A, et al. A Multiingredient nutritional supplement in combination with resistance exercise and high-intensity interval training improves cognitive function and increases N-3 index in healthy older men: a randomized controlled trial. Front Aging Neurosci. 2019;11:107. doi:10.3389/fnagi.2019.00107
12. Stockler-Ipsiroglu S, van Karnebeek CDM. Cerebral Creatine deficiencies: a group of treatable intellectual developmental disorders. Semin Neurol. 2014;34(3):350-6. doi:10.1055/s-0034-1386772
13. Ricci T, Forbes SC, Candow DG. Creatine supplementation: practical strategies and considerations for mixed martial arts. J Exerc Nutr. 2020;3(1):s2. Available from: https://www.journalofexerciseandnutrition.com/index.php/JEN/article/view/58
14. Kreider RB, Stout JR. Creatine in health and disease. Nutrients. 2021;13(2):447. doi:10.3390/nu13020447
15. Forbes SC, Candow D, Ferreira L, Souza-Junior T. Effects of Creatine supplementation on properties of muscle, bone, and brain function in older adults: a narrative review. J Diet Suppl. 2022;19(3):318-35. doi:10.1080/19390211.2021.1877232
16. Forbes SC, Cordingley DM, Cornish SM, Gualano B, Roschel H, Ostojic, SM, et al. Effects of Creatine supplementation on brain function and health. Nutrients. 2022;14(5):921. doi:10.3390/nu14050921
17. Gualano B, Ostojic SM, Rawson ES. Creatine supplementation and brain health. Nutrients. 2021;13(2):586. doi:10.3390/nu13020586
18. Smolarek AC, McAnulty SR, Ferreira LH, Cordeiro GR, Alessi A, Rebesco DB, et al. Effect of 16 weeks of strength training and Creatine supplementation on strength and cognition in older adults: a pilot study. J Exerc Physiol Online. 2020;23(4):88-94.
19. Snow WM, Cadonic C, Cortes-Perez C, Chowdhury SKR, Djordjevic J, Thomson E, et al. Chronic dietary Creatine enhances hippocampal-dependent spatial memory, bioenergetics, and levels of plasticityrelated proteins associated with NF-B. Learn Mem. 2018;25(2):54-66. doi:10.1101/lm.046284.117
20. Bender A, Samtleben W, Elstner M, Klopstock T. Long-term creatine supplementation is safe in aged patients with Parkinson disease. Nutr Res. 2008;28(3):172-8. doi:10.1016/j.nutres.2008.01.001
21. Kumar A, Kumar V, Singh K, Kumar S, Kim YS, Lee YM, et al. Therapeutic Advances for Huntington's Disease. Brain Sci. 2020;10(1):43. doi:10.3390/brainsci10010043
22. Ellis AC, Rosenfeld J. The role of Creatine in the management of amyotrophic lateral sclerosis and other neurodegenerative disorders. CNS Drugs. 2004;18(14):967-80. doi:10.2165/00023210-200418140-00002
23. Komura K, Hobbiebrunken E, Wilichowski EKG, Hanefeld FA. Effectiveness of Creatine monohydrate in mitochondrial encephalomyopathies. Pediatr Neurol. 2003;28(1):53-8. doi:10.1016/s0887-8994(02)00469-1
24. 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

25. de Guingand DL, Ellery SJ, Davies-Tuck ML, Dickinson H. Creatine and pregnancy outcomes, a prospective cohort study in low-risk pregnant women: study protocol. BMJ Open. 2019;9(1):e026756. doi:10.1136/bmjopen-2018-026756
26. 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
27. LaRosa DA, Ellery SJ, Snow RJ, Walker DW, Dickinson H. Maternal creatine supplementation during pregnancy prevents acute and long-term deficits in skeletal muscle after birth asphyxia: a study of structure and function of hind limb muscle in the spiny mouse. Pediatr Res. 2016;80(6):852-60. doi:10.1038/pr.2016.153
28. Fumagalli S, Fattirolli F, Guarducci L, Cellai T, Baldasseroni S, Tarantini F, et al. Coenzyme Q10 terclatrate and Creatine in chronic heart failure: a randomized, placebo-controlled, double-blind study. Clin Cardiol. 2011;34(4):211-7. doi:10.1002/clc.20846
29. 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
30. Rosidi A. The difference of Curcumin and antioxidant activity in Curcuma xanthorrhiza at different regions. J Adv Pharm Edu Res. 2020;10(1):14-8.
31. Saida K, Sara H, Amel B, Samira M. Free radical scavenging and biological activity of leaves and roots of Thymelaea hirsuta, collected from Algeria. J Adv Pharm Educ Res. 2020;10(4):15-20.