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 λ = 744 nm. For the estimation of antiradical effect of the compound examined the following parameters were calculated: radical scavenging activity in [%], IC50 value; antioxidant power 1/IC50, 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 (IC50 = 100.98 mM) exerts antiradical effect, but is less active compared to Trolox (IC50 = 0.2 mM) due to higher IC50 value and lower antioxidant power (1/IC50 = 0.01) than Trolox (1/IC50 = 5).

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

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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 encephalopathies [23]. It 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 Q₁₀ (Ubiquinone, Vitamin Q₁₀) 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 Q₁₀ 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 Q₁₀ in the body decreases and its deficiency leads to dysfunction of the respiratory chain. The potential use of Coenzyme Q₁₀ 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 \( \lambda = 744 \text{ nm} \).

### Materials and Methods

#### Materials

1. **Test compound: Creatine monohydrate**
2. **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.


1. **Preparation of 7 mM ABTS stock solution**
   An accurately 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 accurately 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 accurately 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 100μM (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 accurately measured quantities of 0.1 g KH₂PO₄, 0.2 g K₂HPO₄, 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 accurately 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 respectively 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
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:

\[
\text{RSA} \% = \frac{\text{AABTS control} - \text{Asample}}{\text{AABTS control}} \times 100
\]  

\[
\text{R} \% = \frac{\text{Asample}}{\text{AABTS control}} \times 100
\]

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

\( \text{Asample} \) – 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 of ABTS-radical reaches 50% is presented as IC50 value. From the ABTS radical-scavenging curves of standard Trolox and Creatine monohydrate at \( \lambda = 744 \text{ nm} \) were calculated IC50 values (mM). The inhibition ratios (\( y \)) were plotted against the sample concentrations (\( x \)), and the respective regression line (\( y = ax + b \)) was drawn. The sample concentration (\( x \)), was 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 IC50 value.

3. Calculation of antioxidant power: 1/IC50

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:

\[
\text{TEAC} = \frac{1/\text{IC50 sample}}{1/\text{IC50 Trolox}}
\]  

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) (\( \lambda = 516 \text{ nm} \)): DPPH free radical scavenging assay

2. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (\( \lambda = 734 \text{ nm} \)): 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 Carcuma xanthorrhiza Roxb [30]

2. ABTS for Thymelaea hirsute [31].

Results for ABTS radical-scavenging activity

In spectra of methanol solutions of ABTS at \( \lambda = 744 \text{ nm} \) the absorbance of control is 0.99425. Spectra of ABTS methanol solutions at \( \lambda = 744 \text{ nm} \) after 10 min. interaction with Creatine monohydrate solutions (10 mM ÷ 100 mM) are illustrated on Figure 1.
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.

<table>
<thead>
<tr>
<th>C [mM]</th>
<th>A [AU]</th>
<th>RSA [%]</th>
<th>R [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.001</td>
<td>0.89273</td>
<td>10.21</td>
<td>89.79</td>
</tr>
<tr>
<td>0.01</td>
<td>0.81888</td>
<td>17.64</td>
<td>82.36</td>
</tr>
<tr>
<td>0.05</td>
<td>0.75548</td>
<td>24.02</td>
<td>75.98</td>
</tr>
<tr>
<td>0.075</td>
<td>0.70116</td>
<td>29.48</td>
<td>70.52</td>
</tr>
<tr>
<td>0.1</td>
<td>0.64565</td>
<td>35.06</td>
<td>64.94</td>
</tr>
<tr>
<td>0.125</td>
<td>0.59406</td>
<td>40.25</td>
<td>59.75</td>
</tr>
<tr>
<td>0.150</td>
<td>0.54890</td>
<td>44.79</td>
<td>55.21</td>
</tr>
<tr>
<td>0.175</td>
<td>0.50122</td>
<td>49.59</td>
<td>50.41</td>
</tr>
<tr>
<td>0.200</td>
<td>0.46295</td>
<td>51.44</td>
<td>46.56</td>
</tr>
<tr>
<td>0.225</td>
<td>0.42628</td>
<td>57.13</td>
<td>42.87</td>
</tr>
<tr>
<td>0.250</td>
<td>0.39632</td>
<td>60.14</td>
<td>39.86</td>
</tr>
<tr>
<td>0.275</td>
<td>0.36690</td>
<td>63.10</td>
<td>36.90</td>
</tr>
<tr>
<td>0.300</td>
<td>0.33975</td>
<td>65.83</td>
<td>34.17</td>
</tr>
<tr>
<td>0.325</td>
<td>0.31114</td>
<td>68.71</td>
<td>31.29</td>
</tr>
<tr>
<td>0.350</td>
<td>0.28517</td>
<td>71.32</td>
<td>28.68</td>
</tr>
<tr>
<td>0.375</td>
<td>0.26250</td>
<td>73.60</td>
<td>26.40</td>
</tr>
</tbody>
</table>

The results for the absorbance values of ABTS methanol solution at λ = 744 nm after 10 min. interaction 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 R² = 0.974 for Trolox and R² = 0.991 for Creatine monohydrate.

### 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 monohydrate (10 mM ÷ 100 mM) with ABTS-radical solution.

<table>
<thead>
<tr>
<th>N:</th>
<th>C [mM]</th>
<th>A [AU]</th>
<th>RSA [%]</th>
<th>R ABTS [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10</td>
<td>0.91673</td>
<td>7.80</td>
<td>92.20</td>
</tr>
<tr>
<td>2.</td>
<td>20</td>
<td>0.82971</td>
<td>16.15</td>
<td>83.45</td>
</tr>
<tr>
<td>3.</td>
<td>30</td>
<td>0.80888</td>
<td>16.64</td>
<td>81.36</td>
</tr>
<tr>
<td>4.</td>
<td>40</td>
<td>0.77180</td>
<td>22.37</td>
<td>77.63</td>
</tr>
</tbody>
</table>

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.
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.

**Conclusion**

From the experimental results it was observed that the ABTS-radical scavenging effect of Creatine monohydrate (IC₅₀ = 100.98 mM) is lower compared to the standard Trolox (IC₅₀ = 0.2 mM), which antioxidant power (1/IC₅₀ = 5) is higher in comparison with Creatine monohydrate (1/IC₅₀ = 0.01).

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**Conflict of interest:** None
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Ethics statement: None

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