EXPERIMENTAL PAPER

Study of total antioxidant activity of green tea leaves
(Camellia sinensis L.)

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Summary

Introduction: There is a high interest in creating medicines, dietary supplements, cosmetics including plant extract with antioxidant activity. For understanding whether plant extract has a maximum level of antioxidant activity it is important to know the total antioxidant activity of raw material.

Objective: The main goal of study was to find out the green tea leaves total antioxidant activity.

Methods: The antioxidant activity was measured by potentiometric method. Total phenolic, flavonoids, catechins and hydrocinnamic acids derivatives were quantified using Folin-Ciocalteu, aluminium chloride, vanillin and sodium molibdate methods, respectively.

Results: The green tea leaves total antioxidant activity was 660.75 mmol-eqv./m res. dry weight. A significant correlation was observed between the amount of phytochemicals and antioxidant activity, which indicated its main role in antioxidant activity.

Conclusion: The research showed that the green tea leaves possess a high value of antioxidant activity and it is a good source of phenolic constituents.

Key words: Camellia sinensis L., total antioxidant activity, potentiometric method, phytochemistry, analysis

Słowa kluczowe: Camellia sinensis L., całkowita aktywność antyoksydacyjna, metoda potencjometryczna, fitochemia, analiza

INTRODUCTION

Tea (Camellia sinensis L.) is a green shrub which grows mainly in China, India and Japan. The global tea market was estimated at 55.144 million dollars in 2019 and there is tendency to reach 68.950 million dollars by 2027. The main reason of popularity and high consumption of tea over the world is a variety of health benefits [1].

Literature data shows that the chemical composition of green tea leaves (Camellia sinensis) has been well documented. The main constituents are
catechins (15.0–35.0%), flavonols (5.0%), caffeine (1.5–3.5%), organic acids (1.5%), phenolic acids (0.5–5%), free amino acids (1–5.5%), carbohydrates (10–20%) and proteins (5.0–10.0%) [2, 3]. The 80% of phenolic compounds are flavan-3-ols or catechins [4]. The laboratory studies shown that the beneficial health effect of tea are attributed to the flavan-3-ols in tea. According to available clinical researches, the flavan-3-ols possess antioxidant [5, 6], antimutagenic [7], anticancear [8], anti-allergic [9] activities.

Recent publications indexed in PubMed and Scopus show that there is a high interest among researchers in the determination of antioxidant activity of plant extracts [10], tinctures [11], and dietary supplements [12]. It can be explained by the important role of antioxidants to scavenge oxygen-free radicals. Literature survey presents that antioxidant activity (AOA) of green tea leaves (Camellia sinensis) was investigated by electrochemical [13], titrimetric [14], chromatographic [15] and spectral methods [16]. Hu et al. [17] assessed the antioxidant activity (AOA) of a 75% ethanol green tea leaves extract by DPPH method. In our opinion, the results of their study on the determination of the AOA of the extract may not be reliable as they did not take into account the AOA of ethyl alcohol. In our previous studies [18], it was found that ethyl alcohol contributes to overall level of antioxidant activity.

In researches of Zeng et al. [19] and Shpigun et al. [20], the antioxidant activity of infusions and extracts of green tea leaves (Camellia sinensis) obtained by water was estimated. There is an assumption that the antioxidant activity (AOA) of these infusions and extracts cannot estimate the entire AOA level of the green tea leaves (Camellia sinensis) due to the fact, that complete extraction of all biologically active substances is not achieved by water extraction, because only hydrophilic compounds are extracted. According to literary sources, green tea leaves contain flavonoids aglycones and C-glycosides, which are hydrophobic components and cannot be obtained by aqueous extraction. Available scientific studies show that the aglycones of flavonoids have antioxidant activity [21], so in our view, the neglect of these compounds in the determination of the AOA of green tea leaves (Camellia sinensis) is inappropriate. However, there is no reviews about determination the green tea leaves (Camellia sinensis) total AOA. In our view, it is worth estimating antioxidant activity of raw material in order to understand antioxidant capacity of it and further apply this information in developing drug, dietary supplements and cosmetological products.

The aim of the study was find out the total AOA of green tea leaves (Camellia sinensis).

**MATERIALS AND METHODS**

The object of the study was green tea leaves (Camellia sinensis L.), collected in Anhui province, China. Potentiometric measurements were conducted by pH meter Hanna 2550 (Germany) with a combined platinum electrode EZDO 50PO. All reagents and solvents were analytical grade. A ultraviolet-visible spectrophotometer (UV-1000, China) with matched 1 cm quartz cell was used to carry out measurements of optical densities of analysed solutions.

The procedure of determining the level of AOA

A 2 mmol/l solution of K₃[Fe(CN)₆] was prepared by weighing 0.8232 g into a 25.0 ml volumetric flask, dissolving a compound in a distilled water and filling the flask to volume with the same solvent. A 0.02 mmol/l of K₃[Fe(CN)₆] was prepared by weighing 0.0921 g into a 250.0 ml volumetric flask, dissolving a compound in a distilled water and filling the flask to volume with the same solvent. Then, 5.00 ml aliquot of both prepared solutions was taken and transferred into a 250.0 ml measuring flask and made up to the mark by 0.067 mol/l phosphate buffer solution. A 50.0 ml of prepared mediator solution was transferred in an electrochemical cell. The initial potential of mediator solution was measured after initial one was established, a 1.00 ml of aliquot of the prepared solutions was added and a final potential was measured. The difference (∆E) between the initial (E₀) and final (Eₜ) potentials was found [22]. The shift of potential is explained by the change of ratio of oxidized and reduced forms of the mediator system. The following equation was used to calculate AOA and expressed as mmol-equiv./m res dry weight [18, 23]:

\[
\text{AOA} = \frac{C_{\text{oxy}} - \alpha C_{\text{red}}}{1 + \alpha} \cdot K_{\text{dl}} \cdot 10^{\frac{\Delta E - \text{E} - \text{ethanol}}{2.3RT}},
\]

where \(\alpha = C_{\text{oxy}} / C_{\text{red}}\); \(\Delta E\) – change of potential; \(F=96485.33\) C/mol – Faraday constant; \(n=1\) – number of electrons; \(R=8.314\) J/mol·K – universal gas constant; \(T=298\) K; \(\alpha\) – coefficient of dilution; \(m_{r}\) – mass of dry

\(C_{\text{oxy}}\) – concentration of potassium ferricyanide, mol/l; \(C_{\text{red}}\) – concentration of potassium ferrocyanide, mol/l; \(E_{\text{ethanol}}\) – 0.0546 C₆H₅NO₆ – 0.0091; \(C_{\text{oxy}}\) – concentration of ethanol; \(\alpha\) – change of potential; \(F=96485.33\) C/mol – Faraday constant; \(n=1\) – number of electrons; \(R=8.314\) J/mol·K – universal gas constant; \(T=298\) K; \(\alpha\) – coefficient of dilution; \(m_{r}\) – mass of dry
residue; \( m_2 \) – mass of dry residue in 1.0 ml of extract.

The antioxidant potential of extracts was carried out five times, the results of determination AOA was presented as the mean ± confident error of five determinations.

**Preparation of extracts**

A 10.0 g (exact mass) of dried leaves of green tea were grinded in the size 1–2 mm. The extraction was carried out one by one using 96% ethanol, 60% ethanol, 40% ethanol, 20% ethanol, distilled water on water bath at 80°C, material/solvent ratio 1/20, extraction time was 60 min. The solvent was refluxed by the condenser system. After cooling, the solution was filtered and concentrated by vacuum evaporator at a temperature of 50–60°C to a ratio of extract to raw material 1:2.

**Quantification of sum of phenolic compounds**

The Folin-Ciocalteu method was applied to evaluate the total amount of phenolic compounds [24]. A 1.0 ml of extract solution was mixed with 1.0 ml of 1 M Folin-Ciocalteu reagent. The mixture was mixed and made up to the mark by the addition of 20% Na\(_2\)CO\(_3\). The optical density was recorded at 760 nm after 30 min. The calibration curve was plotted with interval concentrations 1.0–5.0 \( \times 10^{-6} \) g/ml, the calibration equation \( Y=0.1055X+0.1745 \) (\( R^2=0.9951 \)). The amount of phenolic compounds in extract was found out according to the equation [25]:

\[
X(mg/ml) = \frac{C_x \cdot m \cdot K_{dil} \cdot 1000}{V_{ext}}
\]

where \( C_x \) – concentration of gallic acid according to calibration curve, C-10\(^{-4}\), g/ml; \( V_{ext} \) – volume of extract, ml; \( m \) – mass of sample; \( K_{dil} \) – coefficient of dilution.

**Quantification of the sum of catechin**

The sum of catechin was estimated using vanillin reagent assay [26]. A 1.0 ml of prepared extract, 7.5 ml of 1% vanillin solution in 96% ethanol were added in a 25 ml volumetric flask. Then, the solution was made up by the addition 0.5 mol/l HCl in 96% ethanol solution. The mixture was analysed at 505 nm after standing for 30 min. The total content of catechins was determined using the epigallocatechin-3-O-gallate. The calibration curve was plotted with interval concentrations 100–400 \( \cdot 10^{-6} \) g/ml, the calibration equation \( Y=0.0025X-0.0851 \) (\( R^2=0.9951 \)). The amount of catechins in extract, expressed as epigallocatechin-3-O-gallate was found out according to equation [25]:

\[
X(mg/ml) = \frac{C_x \cdot m \cdot K_{dil} \cdot 1000}{V_{ext}}
\]

where \( C_x \) – concentration of epigallocatechin-3-O-gallate according to calibration curve, C-10\(^{-6}\), g/ml; \( V_{ext} \) – volume of extract, ml; \( m \) – mass of sample; \( K_{dil} \) – coefficient of dilution.

**Quantification of sum of flavonoids**

The sum of flavonoid content was determined by AlCl\(_3\) assay [27]. A 1.0 ml of prepared extract, 1.0 ml of 2% AlCl\(_3\) solution in 5% glacial acid in methanol were added into 25.0 ml measuring flask and made up to the mark with a 5% solution of glacial acetic acid in methanol. The absorbance was measured at 417 nm after 30 min as a compensation liquid was 1.0 ml of extract solution, which was diluted to 25.0 ml by a 5% solution of glacial acetic in methanol. The amount of flavonoid in extract, expressed as rutin was calculated according to following equation [25]:

\[
X(mg/ml) = \frac{A \cdot m \cdot K_{dil} \cdot 1000}{V_{ext}}
\]

where \( A \) – absorbance of analysed solution, \( A_x \) – absorbance of standard solution of rutin; \( V_{ext} \) – volume of extract, ml; \( m \) – mass of sample; \( K_{dil} \) – coefficient of dilution.

**Quantification of sum of hydroxycinnamic acids derivatives**

The sum of hydroxycinnamic acids derivatives was assessed by NaNO\(_2\)-Na\(_2\)MoO\(_4\) assay [28]. A 1.0 ml of extract solution, 2.0 ml of 0.5 M HCl, 2.0 ml of 10% NaNO\(_2\), 2.0 ml of 10% Na\(_2\)MoO\(_4\), 2.0 ml of 8.5% NaOH were added into 25.0 ml measuring flask and made up to the mark by distilled water. The optical density was recorded immediately at 525 nm as a compensation liquid was consisted of 1.0 ml of extract solution, 2.0 ml of 0.5 M HCl, 2.0 ml of 8.5% NaOH.
NaOH, which was mixed and diluted to the mark by distilled water to 25.0 ml. The total content of hydroxycinnamic in extract, expressed as chlorogenic acid was calculated according to following equation [25]:

$$X(mg/ml) = \frac{4 \cdot m \cdot K_{dil} \cdot 1000}{188 \cdot V_{ext}}$$

where A – absorbance of analysed solution, 188 – specific adsorption coefficient of chlorogenic acid; $V_{ext}$ – volume of extract, ml; m – mass of sample; $K_{dil}$ – coefficient of dilution.

**Data analysis**

Data analysis was performed in Microsoft Excel 2010 and STATISTICA 6.0, the results were presented as mean ± confident interval from five measurements with the accepted significance level (p<0.05).

**Ethical approval:** The conducted research is not related to either human or animal use.

**RESULTS AND DISCUSSIONS**

Exhaustive extraction was performed to determine the total AOA of the green tea leaves. This extraction method allows the complete extraction of both glycoside and aglycone forms of flavonoids, which have different polarities. The method is based on the alternate extraction of 96, 60, 40, 20% ethanol and water. In the case of the exhaustive extraction, the raw material was not dried after extraction, therefore, the volume of the extractant that was absorbed by the raw material was taken into account. Hence, the concentration of the extractant is 60+14%, 40+4%, 20+5%. In addition, it should be noted that 96% ethanol extracted compounds that would be recovered with 60% ethanol. In the case of extraction by 60% ethanol, some part of the compounds would take from 40% extraction as well as 20% ethanol would extracted a part of the compounds recovered from aqueous extraction.

Phenolic compounds have been revealed different pharmacological effects such as antioxidant, antimicrobial, anti-inflammatory [29]. The results of determination the total phenolic constituents, expressed as gallic acid. Table 1 shows that 96% ethanol extract had the greatest content of phenolic compounds (56.64±0.94 mg/ml), other green tea extracts showed much lower content of phenolic compounds: 22.84±0.40, 5.57±0.10, 1.52±0.05, 1.01±0.05 mg/ml for 60, 40, 20% ethanol and water extracts, respectively. The sum of total phenolic content was 87.58 mg/ml. In scientific research of Qiong et al. [30], total phenolic content was 120 mg/ml and 76 mg/ml in 96% ethanol and aqueous extracts, respectively. If compare the results with our study it can be seen that a value of total phenolic content is higher the value that obtained in the case of aqueous extract.

Total amount of catechins was expressed in epigallocatechin-3-O-gallate equivalent. Catechins are the main phenolic constituents of green tea leaves. EGCG makes up about 40% of the total catechins content and is widely accepted as major antioxidant ingredient in green tea [31]. The highest content of catechins was observed in 96% ethanol extract (61.20±1.02 mg/ml), followed by 60% ethanol extract (24.68±0.46 mg/ml), 40% ethanol extract (6.17±0.11 mg/ml), 20% ethanol extract (1.76±0.11 mg/ml) and aqueous extract (1.17±0.06 mg/ml). The sum of total catechin content was 94.98 mg/ml. Friedman et al. [32] presented data to proof the highest content of catechins in 60% ethanol extract.

**Table 1.**

Quantitative content of the sum of phenolic compounds, catechins, flavonoids, hydroxycinnamic acids derivatives, antioxidant activity with exhaustive sequential extraction of green tea leaves

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Total phenolic content [mg/ml]</th>
<th>Total catechin content [mg/ml]</th>
<th>Total flavonoid content [mg/ml]</th>
<th>Total hydroxycinnamic acids derivatives content [mg/ml]</th>
<th>AOA, mmol-eq./m&lt;sub&gt;ext dry weight&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>96% ethanol</td>
<td>56.64±0.94</td>
<td>61.20±1.02</td>
<td>4.66±0.12</td>
<td>6.14±0.14</td>
<td>357.50±10.75</td>
</tr>
<tr>
<td>60% ethanol</td>
<td>22.84±0.40</td>
<td>24.68±0.46</td>
<td>1.85±0.05</td>
<td>2.44±0.09</td>
<td>204.50±4.09</td>
</tr>
<tr>
<td>40% ethanol</td>
<td>5.57±0.10</td>
<td>6.17±0.11</td>
<td>0.46±0.02</td>
<td>0.61±0.02</td>
<td>65.72±1.31</td>
</tr>
<tr>
<td>20% ethanol</td>
<td>1.52±0.05</td>
<td>1.76±0.11</td>
<td>0.29±0.01</td>
<td>0.38±0.01</td>
<td>25.03±0.50</td>
</tr>
<tr>
<td>Water</td>
<td>1.01±0.05</td>
<td>1.17±0.06</td>
<td>0.20±0.01</td>
<td>0.26±0.01</td>
<td>8.00±0.16</td>
</tr>
<tr>
<td>The total content</td>
<td>87.58</td>
<td>94.98</td>
<td>7.46</td>
<td>9.83</td>
<td>660.75</td>
</tr>
</tbody>
</table>
established total catechin content in 80% ethanol and aqueous extracts of 24 green teas. In the case of 80% ethanol extract, the sum of catechins was in the range from 12.3 to 69.51 mg/ml, whereas in the case of water extracts was from 2.24 to 51 mg/ml. Compared to our study the total catechin content is much higher.

Flavonoids is one of the biggest class of biological active substances in plant, it plays an essential role in scavenging free radicals [33]. The total flavonoid content was expressed in rutin equivalent. The high amount of flavonoids was found in 96% ethanol extract (4.66±0.12 mg/ml), followed by 60% (1.85±0.05 mg/ml), 40% ethanol extract (0.46±0.02 mg/ml), 20% ethanol extract (0.29±0.01 mg/ml) and aqueous extract (0.20±0.01). The sum of total flavonoids content was 7.46 mg/ml. In the study of Bansode [34], the total flavonoid content of aqueous extracts of 7 different green teas was determined in the range from 11.56 to 21.90 mg/ml. In our study the sum of total amount of flavonoids was lower, which can be related with different green tea species.

Not only flavonoids and polyphenolic compounds are potent antioxidants, but also hydroxycinnamic acids derivatives, which are antioxidants widely analysed by scientists. However, the hydroxycinnamic acids derivatives inferior in AOA to catechins. The mechanism of AOA is the same as in other phenolic compounds and depends on electro donating ability [35]. As presented in table 1, the amount of hydroxycinnamic acids derivatives ranged from 6.14 to 0.26 mg/ml. In addition, in all extracts the amount of hydroxycinnamic acids derivatives is higher than amount of flavonoids. The greater content of hydroxycinnamic acids derivatives was observed in 96% ethanol extract (6.14±0.14 mg/ml). Total content of hydroxycinnamic acids derivatives was 9.83 mg/ml (tab. 1). In recent study of Jeszka-Skowron et al. [36], total hydroxycinnamic acids derivatives were 8.15, 11.41 and 34.25 mg/ml in three aqueous extracts of three green teas. Compared to our results it can be seen that in our study the value of total hydroxycinnamic acids derivatives content is similar.

The total phenolic, catechin, flavonoid, hydroxycinnamic acids derivatives content increases as follows: aqueous extract <20% extract <40% extract <60% extract <96% extract.

The differences in results can be related with sample preparation method, different brewing times, leaves/extractant ratio used, tea species, climate and geographical position.

As shown in table 1, total catechin content dominates in all extracts. Even amount of phenolic compounds is lower, this fact can be related with a high sensitivity of reaction between vanillin reagent and catechins.

The AOA values of investigated extracts were estimated with potentiometric method. This method was chosen due to its high sensitivity, rapid analysis procedure, relatively low cost of equipment and reagents, and hence the analysis as a whole [37]. In order to evaluate the total AOA of green tea leaves, extraction one by one using 96%, 60%, 40%, 20% ethanol and water was provided. Such a type of extraction was used to extract all biological active substances from green tea leaves completely.

According to obtained results, the total AOA of green tea leaves equals 660.75 mmol-equiv./mg res. dry weight. The antioxidant activity increases in following order: aqueous extract (8.00±0.16) >20% extract (25.03±0.50) >40% extract (65.72±1.31) >60% extract (204.50±4.09) >96% extract (357.50±10.75).

It can be seen that 96% ethanol extract had the biggest part in total AOA of green tea leaves. It is explained by a high level of biologically active substances among other extracts. The lowest antioxidant activity had aqueous extract as the amount of phenolic constituents quite low.

In the study of Qiong et al. [30], AOA of 96% ethanol and aqueous green tea extracts was found by FRAP, DPPH and ABTS assays. As a result, 96% of ethanol extract possessed higher AOA than aqueous extract due to better solubility of catechins in 96% ethanol than in water. In our research, exactly 96% ethanol extract contributed to greater value of AOA than other extracts.

The Pearson’s (r) and rank Spearman’s (r) correlation coefficients were used to analyse the correlation between the AOA and the amount of phenolic, catechin, flavonoid, hydroxycinnamic acids derivatives. Correlation coefficient is able to take a value in the range from −1 to +1. There is classification of correlation according to it the range from 0.90 to 1.00 is very high; from 0.70 to 0.90 is high; from 0.50 to 0.70 is moderate; from 0.30 to 0.50 is low; from 0.00 to 0.30 negligible [38].

As shown in table 2 and figure 2, there is a significant, very high positive correlation of AOA and total phenolic, catechin, flavonoid, hydroxycinnamic acids derivatives content in green tea leaves extracts. However, total catechins content had the strongest correlation with antioxidant activity, followed by total hydroxycinnamic acids derivatives, respectively. It indicated that catechin constituents play an important role in antioxidant activity.
CONCLUSIONS

1. Total green tea leaves antioxidant activity has been determined, owing to our results. Research can be used to elaborate drugs, dietary supplements, cosmetological products with green tea extract characterized by antioxidant properties.

2. The research showed that 96% ethanol extract possessed a significant high antioxidant activity due to the greatest amount of biologically active substances.

3. A significant correlation of antioxidant activity and amount of biological active substances has been observed.

Conflict of interest: Authors declare no conflict of interest.

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