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PCL ASSAY APPLICATION IN SUPEROXIDE ANION-RADICAL SCAVENGING CAPACITY OF TEA *CAMELLIA SINENSIS* EXTRACTS

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ABSTRACT

Background. Plant polyphenols are known for their limiting of adverse effects on reactive oxygen species (ROS) in biological systems. The photochemiluminescence (PCL) assay allows to evaluate the antiradical activity of a compound in the presence of a superoxide anion-radical ($O_2^{\bullet-}$), which is one of the ROS directly associated with the human body. In this work, determination of the superoxide anion radical scavenging activity of different tea extracts using the PCL assay was performed.
Material and methods. Investigations were conducted on different tea leaves extracts. The study included five kinds of tea leaves subjected to aqueous and ethanol extraction procedure. Catechins content was evaluated using HPLC. Antiradical activity of the samples was conducted with use of Photochem assay.
Results. Analysis of total catechins content in tea aqueous extracts enabled them to be arranged as follows: yellow > green > white > red > black, while for ethanol extracts it was: yellow = green > white > red > black. The examined tea extracts were ranked from highest to lowest water-soluble antioxidative capacity (ACW) values as follows: yellow > green > white > red > black. The results of lipid-soluble antioxidative capacity (ACL) values for aqueous extracts were similar; however, were approximately 50% lower than those presented as ACW. The second examined group were ethanol extracts, which ranked for ACW values: yellow > green = red = white > black, while ACL values ranked as follows: yellow > white = black = red > green. PCL assay results were correlated with total catechin content in aqueous extracts.
Conclusions. Antiradical activity of different tea leaves extracts in PCL assay, showed that the highest activity was found in extracts of yellow tea; the lowest, however, was identified in black tea extracts.

Key words: tea, *Camellia sinensis*, photochemiluminescence, PCL, superoxide radical, antioxidant activity

INTRODUCTION

Research has shown that the ability to scavenge free radicals is one of the characteristics of antioxidant compounds (Choe and Min, 2009; Forman et al., 2014; Kmiecik et al., 2015). Many studies confirm that polyphenols, i.e. compounds of plant origin, constitute the most active of such compounds and possess a significant ability to scavenge radicals (Huang et al., 1994; Heim et al., 2002; Gramza, 2007; Seifried et al., 2007). Plant polyphenols are known for their limiting of adverse effects on reactive oxygen species (ROS) in biological systems. Among the ROS inducing oxidation reactions one can distinguish peroxide radicals (ROO•), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$). The ability to scavenge free radicals may be

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the major cause of the positive effect of plant origin compounds on the human body (Hooper and Cassidy, 2006; Khan et al., 2014).

Reactive oxygen species can be generated in the human body via many biochemical pathways. The formation of oxidative stress occurs while the protective system in a tissue is unable to effectively neutralize the reactive oxygen species (Aruoma, 1994), to which the most exposed are polyunsaturated fatty acids present in the phospholipids of cell membranes. Free radicals are the most active components affecting the human organism, and the high level of impact of these radicals results in ageing and many diseases, for example cancer, cardiovascular diseases and diabetes.

Although different methods are used for the determination of antioxidant activity, it is important to apply a consistent and rapid method (Krishnaiah et al., 2011; Amorati and Valgimigli, 2015). Considering the mechanism of action, the methods for assessing antioxidant activity can be divided into SET (single electron transfer) and HAT (hydrogen atom transfer) mechanisms, the result of which can be similar, while the accompanying reaction kinetics and force varies. The HAT mechanism is a measure of the ability to scavenge a radical via hydrogen donation, which is a rapid reaction, with short finalization. That mechanism is characteristic for the ORAC assay (Oxygen Radical Absorbance Capacity), inhibition of linoleic acid oxidation and photochemiluminescence assay (PCL) (Prior et al., 2005; Gramza-Michałowska and Korczak, 2013). The PCL assay is performed in the presence of a superoxide anion-radical ($O_2^{\bullet-}$), which is one of the most reactive oxygen species present in the human body (Schlester et al., 2002). Photochemiluminescence assay principle is based on generating superoxide anion-radicals ($O_2^{\bullet-}$) under the influence of UV light (hv_1), and a photosensitizer, followed by their detection during the reaction with a chemiluminogenous compound (luminol). The measure of radical quantity in the system is the intensity of the emitted light (hv_2), and the radical scavenging compounds attenuate the photochemiluminescence intensity in proportion to the amount and activity of the tested antioxidant (Popov and Lewin, 1999; 2005; Upadhay and Mishra, 2014; Vertuani et al., 2004b).

$$LH^- + hv_1 \rightarrow L^{\bullet} + O_2^{\bullet-} \rightarrow N_2 + AP^{2-} + hv_2$$

where:

L – luminol,

hv_1 – UV radiation required for excitation of luminol,

$L^{\bullet}$ – luminol radical,

$O_2^{\bullet-}$ – superoxide anion radical,

AP^{2-} – excited aminophthalate radical,

hv_2 – chemiluminescence.

Plant products are a very important source of polyphenols in the human diet (Gramza and Reguła, 2007; Kobus-Cisowska et al., 2014; Kulczyński and Gramza-Michałowska, 2016; Sidor and Gramza-Michałowska, 2015). One of the most widely consumed beverages is brewed from tea leaves. Beverages such as tea or herbal infusions have no significant nutritional value; however, they contain compounds essential for our body – i.e. antioxidants (Warren, 1999; Gadkari and Balaraman, 2015). The leaves of the tea plant *Camellia sinensis* contain phenolic compounds with anticancerogenic and anti-inflammatory, bactericidal and bacteriostatic properties. These compounds strengthen the immune system, protect against atherosclerosis and have antitumor properties (Friedman et al., 2007; Khan and Mukhtar, 2007; Sirk et al., 2008). Tea leaves contain many biologically active compounds; however, catechins are effective scavengers of reactive oxygen species (ROS) *in vitro* and *in vivo*, which can also act as an antioxidant by affecting the transcription and activity of enzymes (Vertuani et al., 2004a; Jankun et al., 2012; Bettaieb et al., 2014). Results suggest that, due to their numerous and proven properties in biological systems, tea polyphenols may be a new nutraceutical, introducing a wide range of health benefits (Sajilata et al., 2008). Sumpio et al. (2006) analysed the results of many studies and presented the so-called "Asian paradox", characterized by a low incidence of atherosclerosis and lung cancer among Asian smokers who consumed at least 1.2 liters of green tea infusion daily. Despite the differences in climate and cultivation methods, the greatest impact on tea plant composition diversity derives from the fermentation process of fresh tea leaves. Many authors have noted the differences between the composition of green and black tea, but a comprehensive evaluation of different teas in terms of their superoxide anion radicals ($O_2^{\bullet-}$) scavenging activity has not yet been conducted.

Results from the last decade show the high antioxidant activity of components derived from the leaves of the tea plant *Camellia sinensis*, of which the dominant group are catechins (Gramza and Reguła, 2007; Almajano et al., 2008; Horzic et al., 2009; Unachukwu et al., 2010). Reports from around the world are primarily focused on selected compounds belonging to the polyphenol group (e.g., catechins) which are principal components responsible for the antiradical capacity of tea. Major catechins include (−)-epicatechin (EC), (+)-catechin (C), (+)-gallocatechin (GC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), and (−)-epigallocatechin gallate (EGCG) (Harbowy et al., 1997). The basic steps in the production of tea are based on several processes, including different stages of processing: (a) white (a set of undeveloped buds and leaves, blanching, rolling and drying), (b) green (a collection of young leaves, wilting, blanching, rolling and drying), (c) yellow (a set of leaves, wilting, subjecting to short fermentation: 10–20%, rolling and drying), (d) red (a collection of leaves, wilting, partial fermentation: 20–50%, burning, rolling and drying), (e) and black (a set of leaves, wilting, curling, subjecting to a total fermentation of 100%, burning, rolling and drying) (Hara, 2001; Hilal and Engelhardt, 2007).

The present research was undertaken to determine the superoxide anion radical scavenging activity of aqueous and ethanol tea extracts differed with catechins content as a result of various fermentation degrees of tea leaves.

MATERIAL AND METHODS

Preparation of tea extracts

The plant materials were Chinese tea leaves from *Camellia sinensis* (L.). From among the available types, five varieties of tea leaves with different degrees of fermentation were selected. The study was performed on non-fermented tea leaves: White (Pai Mu Tan-Fujian, China) and green (Lung Ching China-Zhejiang, China), partially fermented leaves: yellow (China Kekecha-Guangzhou, China) and red (Formosa Oolong-Taiwan) and fully fermented leaves: black (Yunan Golden Leaf-Yunnan, China). Tea leaves were subjected to the optimized extraction procedure published by Gramza et al. (2006). Tea leaves (100 g) were ground and extracted with 1000 ml H_2O for 15 min at 80°C. The extraction process was run in triplicate, then the extract was filtered, centrifuged, and the supernatants were combined and lyophilized to produce the powdered extract. Ethanol tea extract was produced after the maceration of ground leaves in 250 ml of 80% ethanol for 24 h, at 21°C. The extraction process was performed in triplicate; then, the mixture was filtered and centrifuged; the supernatants were combined and the solvent evaporated on a rotary vacuum evaporator; and the water residue was lyophilized to give the powdered extract. Final extracts were kept frozen and further analysed. The catechin contents of the examined tea extracts were analysed using the HPLC method described by Khokhar and Magnusdottir (2002). Extracts were processed through dispersion by sonication in water (ACW assay) and ethanol (ACL assay), followed by filtration with syringe filters (Membrane Solutions PTFE 0.45 μm), and the supernatant was stored in a dark, cool place prior to further analysis of antiradical activity.

Determination of antiradical activity with the PCL assay

The procedure was based on the methodology published by Popov and Lewin (1999). The principle of the PCL assay is based on the fact that the superoxide anion radicals ($O_2^{\bullet-}$) generated upon exposure to light and the presence of a photosensitizer are detected by their reaction with a photosensitizer, chemiluminogenous compound-luminol (5-amino-2,3-dihydro-1,4--phthalazinedione). Analysis are conducted using a Photochem® apparatus (Analytik Jena, Germany). PCL evaluations allow for precise, reproducible and rapid analysis of antioxidant activity in both lipid-soluble (ACL) and water-soluble (ACW) fractions due to kits provided by the manufacturer (Analytik Jena, Germany). For the studies, all working solutions were prepared daily according to the protocol. The presence of an antioxidant in the reaction solution results in a retardation of the luminescence. PCL assays were carried out in triplicate for each sample, as 20 μL of sample solution in HPLC grade water for ACW and HPLC grade methanol for ACL measurements were sufficient to correspond with the standard curve. Results were expressed as μmol of Trolox equivalents (TE) per 1 g of tested sample. The lag time duration

of each sample was calculated with the PCL software, and was plotted against the Trolox concentration added to the reaction medium. The results were also presented as integral antioxidant capacity (IAC), enabling a determination of the ratios of various factions in the total activity, and to state the potential synergistic properties in a mixture of compounds, for example the examined plant extracts (Besco et al., 2007).

Statistical analysis

All experiments represent the means of three independent experiments (means ±SD, $n \geq 3$) performed in three repetitions. Data were analysed using ANOVA one-way analysis of variance and differences were considered statistically significant at $P < 0.05$. Statistica 10.0 software (StatSoft) was used for the analysis.

RESULTS

The present research included evaluations of selected catechin contents in tea extracts. The results of this study concerning the phenolic compound composition in tea extracts are shown in Figures 1 and 2. Among the catechins the predominant compounds were EGCG and EGC, while either no or low levels of C in aqueous tea extracts were evaluated (Fig. 1). The aqueous extract of yellow tea was characterized by the highest C and GCG content in comparison to other extracts. EGCG content in yellow tea extract was similar to that in red tea and 30% lower than that in white tea aqueous extract.

Ethanol tea extracts were characterised by a great variation in catechin content (Fig. 2). Analysis of the ethanol extract composition showed that the greatest differences resulted from the EGCG content. This changed with the increasing fermentation degree of the leaves, and was higher for green and white tea, and the lowest for black tea ethanol extract. Among the catechins in the ethanolic extracts of tea, EGCG and ECG were the predominant compounds; however, GC and EGC were not found. Similar to the aqueous extract, higher levels of C and GCG were also evaluated in the ethanol extract of yellow tea leaves compared to other tea extracts. Analysis of the total content of catechins in tea aqueous extracts allowed them to be arranged in the following order: yellow > green > white > red > black, while for ethanol extracts this was: yellow = green > white > red > black.

Photochemiluminescence assay results of aqueous and ethanol tea extracts were found to be affected by the tea leaf variety used for the extraction procedure. Evaluation with the PCL method can be performed according to two protocols – ACW and ACL, allowing the measurement of the antioxidant

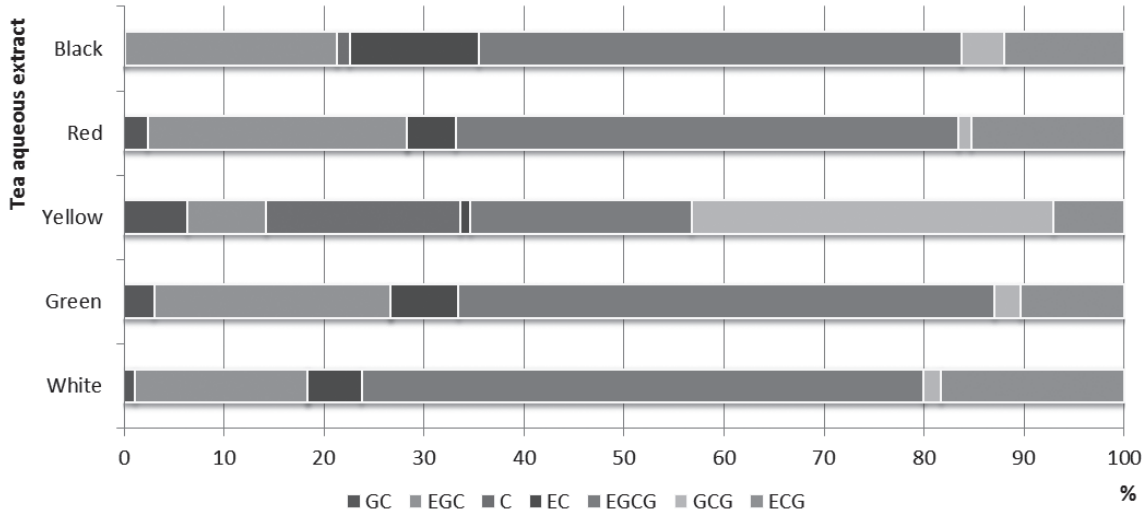

Fig. 1. Proportion of individual catechins in tea aqueous extracts (%), means ±SD, $n = 3$

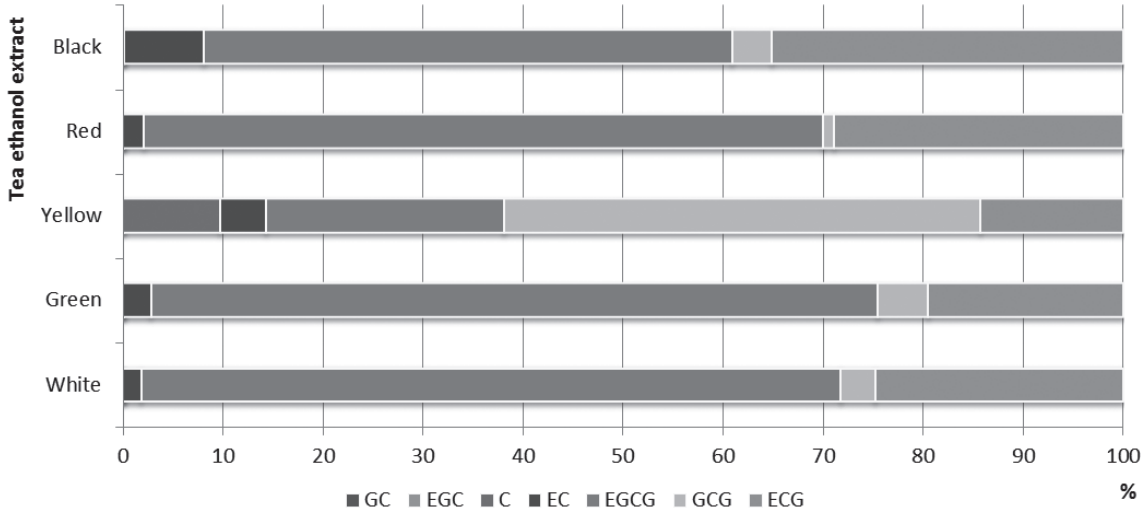

Fig. 2. Proportion of individual catechins in tea ethanol extracts (%), means ±SD, $n = 3$

capacity of water-soluble and lipid-soluble components (Popov and Lewin, 1999). Gallic acid, sinapic acid, quercetin and catechin were selected as standard compounds to compare with tea extract antiradical activity (Fig. 3). It was demonstrated that among all standard compounds catechin was characterised with the highest ACW (14 152.56 µmol Trolox/g), while quercetin had the lowest ACW value (3095.77 µmol Trolox/g). The results of ACL evaluation showed that gallic acid and sinapic acid were more active than catechin or quercetin, showing a 30% lower activity. The results for tea leaf aqueous extract activity in photochemiluminescence assays varied. It was demonstrated that of all the extracts yellow tea leaf extract possessed the highest ACW (3241.17 µmol Trolox/g) and ACL (1586.03 µmol Trolox/g) values. Other extracts were less active, while red and black tea extract showed 50–60% lower values than yellow tea (Fig. 4). The extracts were ranked from highest to lowest ACW values as follows: yellow > green > white > red > black. The results for ACL values for aqueous extracts were similar; however, the results were approximately 50% lower than those presented as ACW.

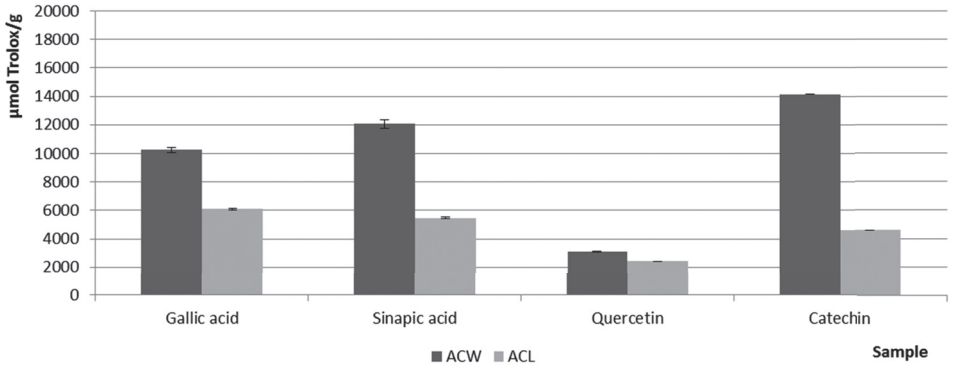

Fig. 3. Antioxidant activity of gallic acid, sinapic acid, quercetin and catechin as evaluated by photochemiluminescence in ACW and ACL assay (µmol Trolox/g), means ±SD, $n = 3$

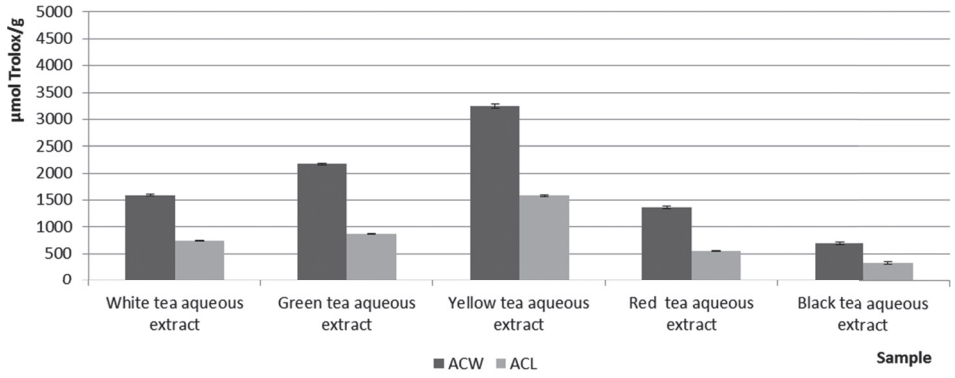

Fig. 4. Antioxidant activity of aqueous tea extracts as evaluated by photochemiluminescence in ACW and ACL assay (µmol Trolox/g), means ±SD, $n = 3$

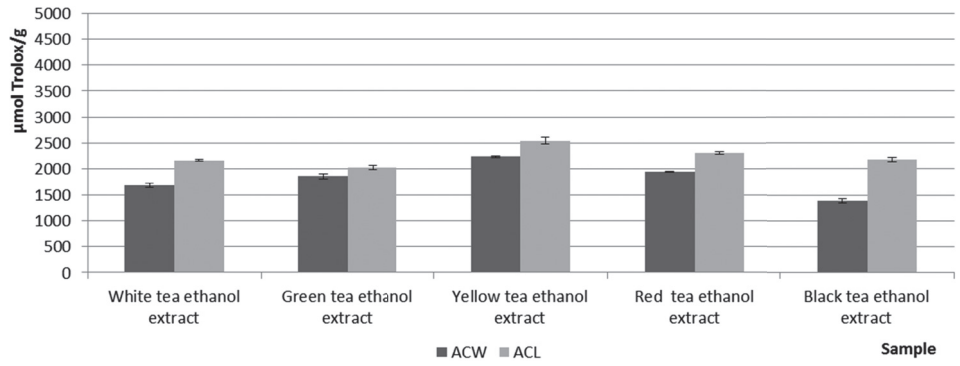

Fig. 5. Antioxidant activity of ethanol tea extracts as evaluated by photochemiluminescence in ACW and ACL assay (µmol Trolox/g), means ±SD, $n = 3$

The second group of examined extracts were ethanol extracts, which were ranked from highest to lowest ACW values as follows: yellow > green = red = white > black (Fig. 5). The results for the ACL values ranked as follows: yellow > white = black = red > green.

In the analysis of the results, the IAC index (Integral Antioxidant Capacity) was evaluated as the sum of the lipophilic and hydrophilic compound activity of each tested sample (Fig. 6). According to the IAC values, pure compounds were ranked as follows: catechin > sinapic acid > gallic acid > quercetin. From the above results, it could be concluded that catechin-rich tea extracts could exert high antiradical activity in the presence of superoxide anion radicals. Total activity in PCL assays was higher in samples of yellow tea (4849.24 µmol Trolox/g) than in red tea (4311.51 µmol Trolox/g), the remaining samples showed 25% lower activity. No differences were found between green and white tea ethanol extracts IAC (3951.50 and 3901.51 µmol Trolox/g, respectively). The total activity of both fractions in PCL assays allowed the antiradical activity of aqueous tea extracts to be arranged in the following order: yellow > green > white > red > black, and ethanolic extracts: yellow > red > green = white > black. Comparing the activity of tea extracts in scavenging the superoxide anion radical, it was found that the aqueous extract hydrophilic fraction was approx. 70%, while the lipophilic activity accounted for 30% of the total activity. The percentage of the activity of both fractions in the ethanolic extracts was similar and amounted to 43% (ACW) and 57% (ACL).

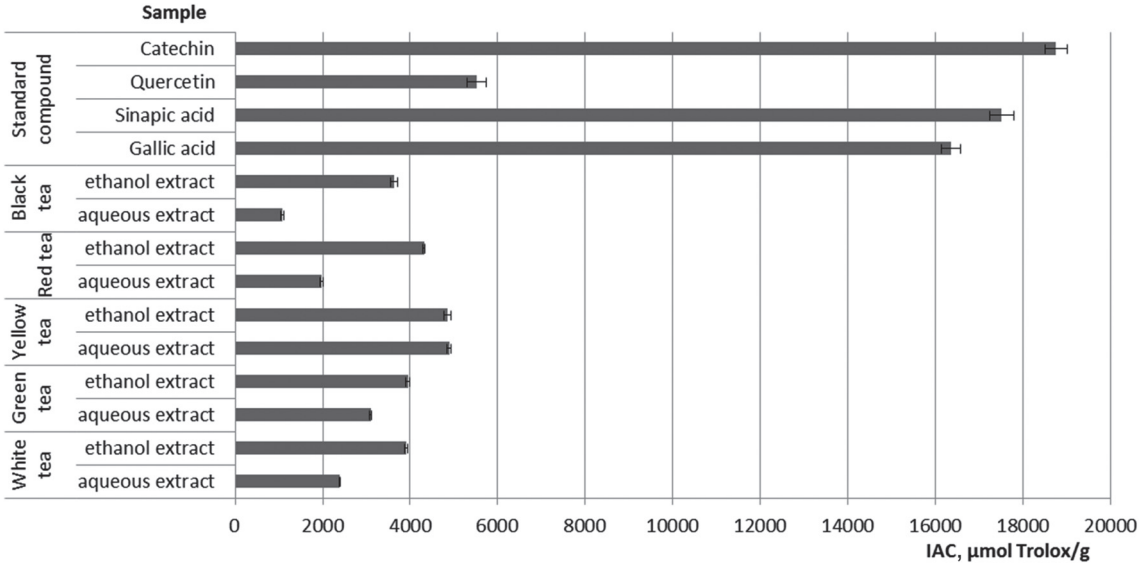

Fig. 6. Integral antioxidant capacity (IAC) of catechin, quercetin, gallic acid, sinapic acid and tea extracts as evaluated by photochemiluminescence assay (µmol Trolox/g), means ±SD, $n = 3$

These results confirmed the highest superoxide radical scavenging activity of the yellow tea extracts for both aqueous and ethanol extracts, with the lowest being for black tea. Summarizing the results of tea extract antiradical activity assays in both ACW and ACL assays, the highest activity was found in both water and ethanol extracts of yellow tea. Results of statistical analysis showed significant correlations between PCL results and catechin content in aqueous extracts (0.83, $P < 0.05$); however, no correlation was found in the ethanol extracts (0.34, $P < 0.05$).

DISCUSSION

The antiradical activity of tea extracts in the PCL assay is the result of the qualitative composition of individual extracts. It has been found that a higher ACW activity may be due to a higher content of phenolic acids (e.g., gallic acid, caffeic, or chlorogenic acid), while for ACL: quercetin and gallates (Vertuani et al., 2004b). Besco et al. (2007) reported that the activity in ACW assays corresponds with the presence of flavonoids and ascorbic acid, while the ACL fraction corresponds with the presence of carotenoids and tocopherols. Antiradical activity is attributed mainly to the presence of catechins, which tea leaves contain in the largest quantities. There are, however, few reports from around the world indicating the superoxide anion scavenging potential of tea leaf extracts measured using the PCL method. Vertuani et al. (2004b) determined the superoxide radical scavenging activity of the aqueous extracts of tea, expressed as the activity of hydrophilic compounds (ACW), and ranked them as follows: green > black > white > red tea. The results of the present evaluations allowed tea extract ACW to be arranged in a different sequence: yellow > green > white > red > black for aqueous, and yellow > red = green = white > black for ethanol extracts. Statistical analysis showed that tea extract antiradical activity in PCL assay was found to be correlated with catechin content in aqueous extracts, which was not found in the ethanol extracts. Similar trends have been observed in other methods of radical scavenging activity. The highest DPPH radical scavenging ability has also been found in both extracts yellow tea, what has also been confirmed in the presence of ABTS radical-cation (Gramza-Michałowska, 2007).

One of the factors affecting the activity of different tea leaf extracts could be the changes occurring during the fermentation process, which is mainly a flavanol polymerization reaction. During oxidation, part of the catechins are converted into theaflavins

and thearubigins (Muthuman and Kumar, 2007). Literature reports have confirmed not only the higher antioxidant activity of non-fermented teas, but there are also reports of high levels of activity in fermented teas. It has been found that fermentation of tea leaves does not reduce antioxidant activity; indeed, this may even lead to its growth (Leung et al., 2001). Research by Rice-Evans et al. (1997) indicated that the properties of the physical, chemical, biological activity and the metabolism of polyphenolic compounds depend on the number, type and location of substituents of each molecule. Polyphenolic compounds are weak acids, and easily relinquish the hydrogen of the hydroxyl groups directly attached to the ring. Hydroxyl groups (OH) are strong electron donors, and therefore the amount of its increase in the compound increases the nucleophilic nature of the ring *A* (Murkies et al., 1998). The activity of scavenging free radicals usually depends on the number of hydroxyl groups in the chemical structure of the compound; however, the reaction and the type of radical scavengers are also important factors (1). The studies by Sang et al. (2002) showed that in the case of scavenging DPPH radicals both rings *A* and *B* of flavonoid structures play an equally important role in this reaction. According to Salah et al. (1995), the antioxidant capacity of tea catechins is due to the structure of the molecule, and especially the presence of at least five hydroxyl groups. It has been found that antioxidant activity is strongly correlated with the amount of hydroxyl groups in ring *B* (Husain et al., 1987), especially in the *ortho* position with carbon 3' and 4'. In ring B, catechin structures include *o*-dihydroxyl groups, and epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) contain 5, 6, 7 and 8 groups, respectively. It has also been found that the antioxidant activity of the hydroxyl group in position 5' does not affect the activity of catechins in the lipid phase; in the aqueous phase, however, it may increase their antioxidant activity (Seifried et al., 2007). In other research, Yaping et al. (2003) found comparable antiradical activity in scavenging the superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) radicals of tea catechins and its colorants, which have been identified as polymers of catechins. Aruoma et al. (1993) found potential prooxidant properties of gallic acid in combination with superoxide radicals ($O_2^{\bullet-}$).

CONCLUSION

The application of a photochemiluminescence method is regarded as a very appropriate but expensive approach for fast and reliable measurement of the antioxidant activity of plant samples. PCL method allows the determination of hydrophilic as well as lipophilic activity in terms of their contribution to the overall activity of pure components and their mixtures. The examined tea extracts water-soluble antioxidative capacity (ACW) values were ranked as follows: yellow > green > white > red > black. The results of lipid-soluble antioxidative capacity (ACL) values for aqueous extracts were similar; however, these were approximately 50% lower than those presented as ACW. The second group of examined extracts were ethanol extracts, which ACW values ranked as follows: yellow > green = red = white > black, while ACL values: yellow > white = black = red > green. Results of tea leaf extract antiradical activity in both ACW and ACL assays showed that the highest activity was found in both water and ethanol extracts of yellow tea; the lowest, however, was identified in black tea extracts.

Plant extracts and preparations are increasingly used as an active components of functional foods, where antiradical activity can be measured using different assays, among them photochemiluminescence. Results of the above experiments could be an important step in the evaluation of the activity of pro-health components in scavenging free radicals, such as superoxide anion radicals ($O_2^{\bullet-}$).

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OZNACZANIE ZDOLNOŚCI ZMIATANIA ANIONORODNIKÓW PONADTLENKOWYCH PRZEZ EKSTRAKTY HERBATY *CAMELLIA SINENSIS* Z WYKORZYSTANIEM PCL

STRESZCZENIE

Wstęp. Polifenole są związkami ograniczającymi niekorzystny wpływ reaktywnych form tlenu (RFT) na funkcjonowanie organizmów żywych. Metoda z wykorzystaniem fotochemiluminescencji (PCL) pozwala na oznaczenie aktywności przeciwrodnikowej składników, np. polifenoli, wobec anionorodnika ponadtlenkowego ($O_2^{\bullet-}$), bezpośrednio związanego z funkcjonowaniem organizmu człowieka. W pracy przedstawiono wyniki analiz zdolności ekstraktów różnych herbat do zmiatania anionorodników ponadtlenkowych z wykorzystaniem metody PCL.

Materiał i metody. Analizą objęto pięć rodzajów liści herbat, które poddano procesowi ekstrakcji wodnej i etanolowej. Zawartość wybranych katechin w ekstraktach herbat oznaczono z zastosowaniem HPLC, natomiast aktywność przeciwrodnikową oznaczono w aparacie Photochem®, wykorzystującym reakcje fotochemiluminescencji (PCL).

Wyniki i dyskusja. Uzyskane wyniki zawartości katechin pozwoliły na ich odpowiednie uszeregowanie w ekstraktach wodnych: żółta > zielona > biała > czerwona > czarna, natomiast w ekstraktach etanolowych: żółta = zielona > biała > czerwona > czarna. Aktywność przeciwrodnikowa składników rozpuszczalnych w wodzie (ACW) dla ekstraktów herbat kształtowała się następująco: żółta > zielona > biała > czerwona > czarna, natomiast aktywność przeciwrodnikowa składników rozpuszczalnych w tłuszczach (ACL) była niższa o 50%. W grupie ekstraktów etanolowych aktywność mierzona jako ACW kształtowała się następująco: żółta > zielona = czerwona = biała > czarna, natomiast aktywność określona jako ACL: żółta > biała = czarna = czerwona > zielona. Stwierdzono zależność aktywności przeciwrodnikowej oznaczonej metodą PCL od zawartości katechin w ekstraktach wodnych herbat.

Wnioski. Ocena aktywności przeciwrodnikowej ekstraktów różnych herbat oznaczanej za pomocą testu PCL wykazała, że najwyższe wartości uzyskano w próbach herbaty żółtej, natomiast najniższe – w czarnej.

Słowa kluczowe: herbata, *Camellia sinensis*, fotochemiluminescencja, PCL, anionorodnik ponadtlenkowy, aktywność przeciwutleniająca

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