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Variations in the free radical scavenging activity of *Ginkgo biloba* L. leaves in the period of complete development of green leaves to fall of yellow ones

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Abstract

The scavenging effect against the DPPH radical has been determined for *Ginkgo* leaves in the growing season from 28 June to 28 October using an EPR method. The influence of the storage time on the leaves scavenging activity has also been assayed. The antioxidant properties decreased as the storage time increased. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Ginkgo biloba L. is a sole living member of the family *Ginkgoaceae* that has survived unchanged for some 200 million years (Bauer & Zschocke, 1996; Foster, 1998; Korszun et al., 1993). The extract from *Ginkgo* leaves, one of the oldest phytomedicines, is currently used extensively in therapy (Lugasi, Horvahovich, & Dworschak, 1999; Newall, Anderson, & Philipson, 1996; *PDR for Herbal Medicines*, 2000; Rigney, Kimber, & Hindmarch, 1999). Studies of *Ginkgo* cultivation have resulted in development of over 20 *Ginkgo* variations that differ in the tree habit, and also shape, colour and size of the leaves which are the material for the pharmaceutical industry (Korszun et al., 1993; Wichtl, 1997). Investigations on the influence of fertilization have shown the possibility of an increase of both leaf weight and content of active constituents, such as flavonoids (Korszun et al., 1993). The standardization of the dry extract EGb-761, an essential component of many phytomedicines, is based on the content of flavonoids (24%) and terpenoids (6%). These compounds are responsible for the wide biological activity of *Ginkgo* preparations (Foster, 1998; Laves, 2000; Lugasi et al., 1999; Newall et al., 1996; *PDR for Herbal Medicines*, 2000; Wichtl, 1997). The main range of application of these preparations is primarily related to diseases appearing with advanced age, such as cerebrovascular and peripheral circulatory insufficiencies and memory disturbance (Newall et al., 1996). The *Ginkgo* extract is an effective free radical scavenger showing antioxidant activity and protecting against the damage caused by free radicals. Therefore the extract is useful in diseases, in which free radicals are involved, e.g. anoxia and ischaemia of brain, heart and eye as well as arteriosclerosis, rheumatism and cancer (Dina, 1997/1998; Joyeux, Lobstein, Anton, & Mortier, 1995; Lugasi et al., 1999; O'Brien, 1999). The activity of the *Ginkgo* extract is determined mainly by flavonoids which scavenge and destroy free radicals and reactive forms of oxygen, such as superoxide radical anions ($^{\bullet}O_2^-$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Małolepsza & Urbanek, 2000; Zając & Pawełczyk, 2000). Flavonoids, acting as antioxidants, scavenge free radicals and, undergoing one-electron oxidation, form fewer reactive flavonoid radicals capable of transferring unpaired electrons (Małolepsza & Urbanek, 2000).

It has been shown that there is a link between the diet rich in flavonoids and the decreased incidence of cardiovascular diseases and cancer (O'Brien, 1999; Vinson, Dabbagh, Serry, & Jang, 1995; Vinson & Hontz, 1995). Flavonoids are polyphenolics, widely distributed in plants. Glycosylated flavonoid monomers are soluble in the cell sap and are gathered intracellularly, whereas

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their aglycones and dimers, i.e. weakly soluble biflavones, occur extracellularly in the epidermis (Wollenweber et al., 1998).

Plants may use flavonoids for protection against ultraviolet radiation which, beside other external factors, such as drought or temperature fluctuations, generate a large amount of free radicals (Małolepsza & Urbanek, 2000; Vinson, Jang, Dabbagh, Serry, & Cai, 1995). Free radicals are also produced internally in metabolic processes controlled by enzymatic systems such as photosynthesis and respiration (Przybytniak & Ambroz, 1999). Antioxidant properties of flavonoids depend on their structure, and mainly the number and position of hydroxyl groups within the molecule. The presence of a 3′,4′-dihydroxy function at the ring B is particularly significant for this activity as well as hydroxyl groups at C_3 in the ring C and at C_5, C_7 in the ring A. Moreover, a keto group at C_4 and a double bond between C_2 and C_3 in the pyrone ring seem to be of some importance (Gadow, Joubert, & Hansmann, 1997; Cos et al., 1998; Małolepsza & Urbanek, 2000).

The antioxidant properties of flavonoids can also result from their ability to complex metal ions such as Cu^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}. These ions are bonded to the followwng functionalities: 4-keto and 3-hydroxy, 4-keto and 5-hydroxy groups or 3′, 4′-dihydroxy function at the ring B. The C_2–C_3 double bond increases the chelating potency of the molecule (Małolepsza & Urbanek, 2000; Thompson & Williams, 1976). Complexing with a metal does not decrease the flavonoid ability to scavenge free radicals. However, some metal complexes, e.g. these including iron ions, inhibit the Fenton reaction, thus preventing the formation of reactive oxygen molecules, especially very toxic hydroxyl radicals (Małolepsza & Urbanek, 2000; Zając & Pawełczyk, 2000).

In a previous paper we determined the content of flavonoids by the Christ-Müller method, phenolic acids by HPLC, and also free radicals and Cu^{2+} ions by EPR in *Ginkgo* leaves collected from June to October (Ellnain-Wojtaszek, Kruczyński, & Kasprzak, 2001). The aim of the present study was to determine the free radical scavenging activity in the same samples of *Ginkgo* leaves as well as comparison of this activity in the period of complete development of green leaves to fall of yellow ones. Moreover, we have investigated the influence of storage time of the August leaves (a material for the pharmaceutical industry) on their scavenging effect.

2. Materials and methods

2.1. Material

The plant material consisted of *Ginkgo biloba* L. leaves collected every month from 28 June to 28 October 1997, as well as 25 August and 25 September 1999, from a male tree growing in the botanical garden of A. Mickiewicz University at Poznan. The leaves were dried at room temperature, pulverized and sifted through a 0.5 mm sieve. The leaves samples were numbered consecutively from 1 to 5 and also 3′ and 4′.

2.2. Extracts preparation

The extracts were prepared by immersing 10 mg samples of the leaves in 10 ml of methanol, leaving for 24 h and filtering through a paper filter.

2.3. DPPH solution

A 10 mg sample of DPPH (1,1-Diphenyl-2-picrylhydrazyl, Aldrich) was dissolved in 20 ml of methanol 24 h before the determination.

2.4. Registration of EPR

The EPR spectra were registered on a Brüker spectrometer (9.780 GHz) using quartz cuvettes. Before the determination, a 0.25 ml aliquot of each extract was mixed with 4 ml of the DPPH solution. The determination was carried out for 60 min and consisted in registration (up to 10 min—every minute, later—every 5

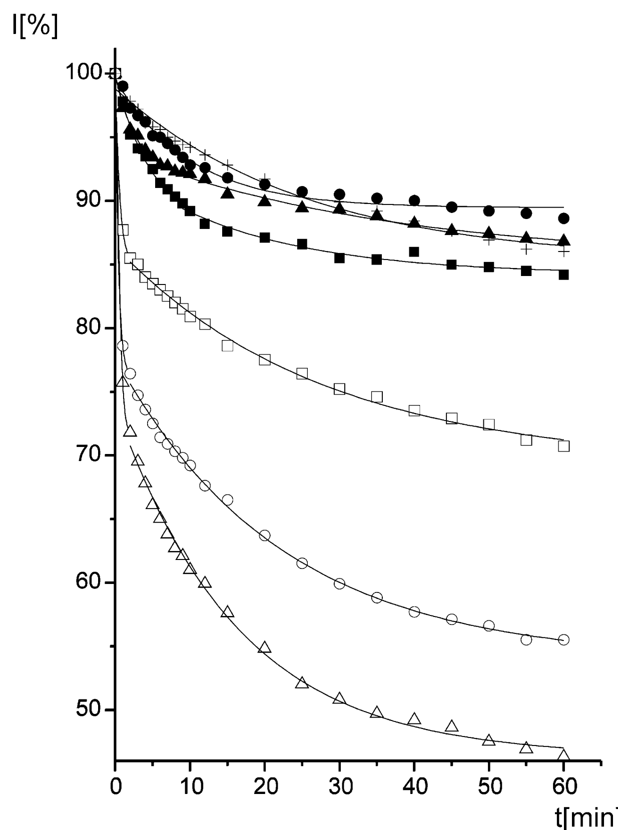

Fig. 1. Time changes of the EPR signal of DPPH radicals in solution from the moment of mixing with extract of *Ginkgo biloba* L. leaves (samples: 1—△, 2—□, 3—▲, 4—■, 5—●, 3′—○, 4′—+).

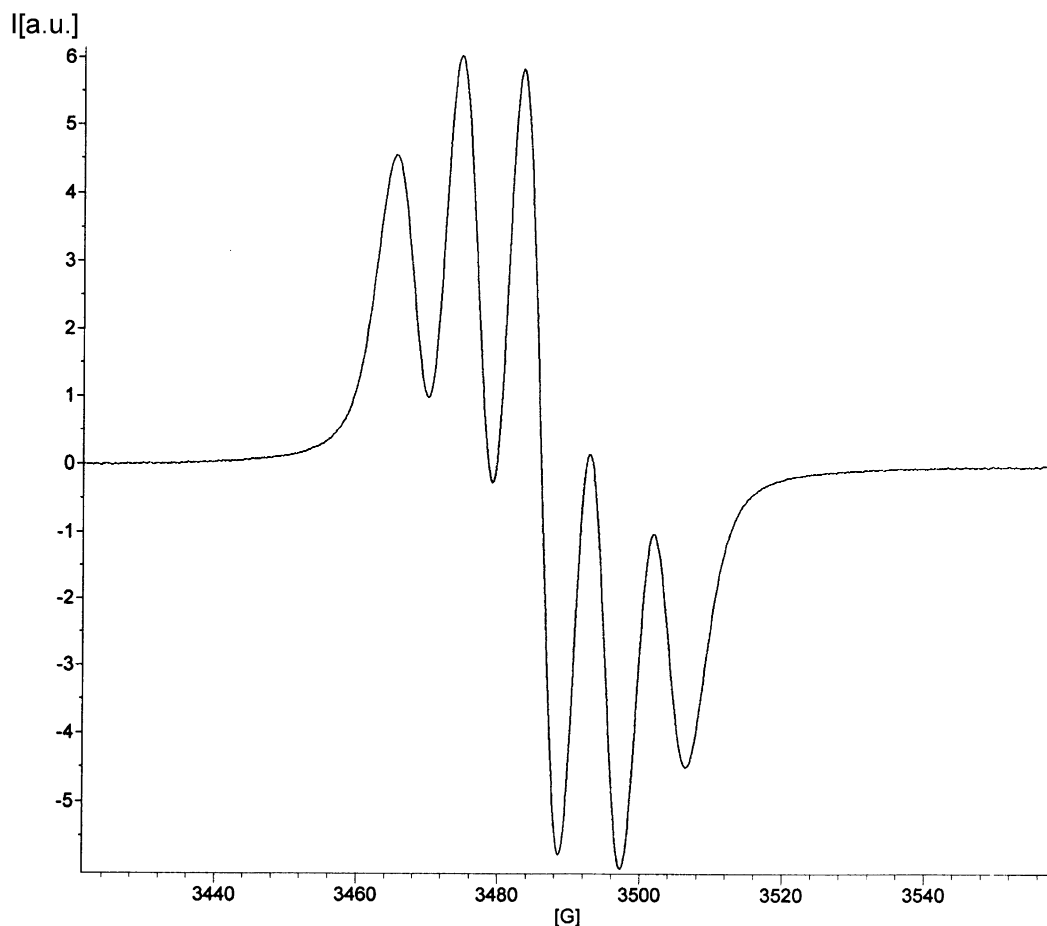

Fig. 2. The EPR signal of DPPH radicals taken in the first minute after mixing with extract from *Ginkgo biloba* L. leaves (sample 1).

min) of the concentration of free radicals in relation to the time elapsed from the moment of mixing with the DPPH solution. The radical scavenging activity was defined as change in the intensity of the DPPH signals after the addition of the antioxidant, i.e. the investigated sample (I), in relation to the DPPH standard intensity (I_0), using the following formula:

$$\frac{I_0 - I}{I_0} \times 100$$

3. Results and discussion

The ability of *Ginkgo* leaves collected in the period of complete development of green leaves to fall of yellow ones to scavenge free radicals has been determined by electron paramagnetic resonance, using the DPPH radical. The determination is based on a difference between the DPPH signals before and after the addition of the methanolic extracts from *Ginkgo* leaves.

Fig. 1 shows the relationship between the concentration of DPPH radicals and the time elapsed from the moment of mixing of the leaves' extract with the DPPH solution, registered from the first to 60th min of the determination. The increasing values of the DPPH radical concentration, registered for samples 1–5, show a decrease of the ability, to scavenge free radicals by the *Ginkgo* leaves collected from June (sample 1) to October (sample 5). Values of the scavenging activity, calculated for the samples and depicted in Table 1, are within a 24.3–1% range in the first minute and 57.7–11.4% in the 60th minute of the determination.

Fig. 2 gives an example of the EPR signal due to the DPPH radical after mixing with the *Ginkgo* extract (sample 1) in the first minute, whereas Fig. 3 shows the spectrum of the same sample in the 60th minute of the determination.

Fig. 4 shows correlation between the intensity of the EPR line of DPPH free radicals in the solution and the period after the edition of *Ginkgo* extract, registered for sample 1 during 1–60 min.

The ability of plant material to scavenge free radicals is due to an overall reaction of its active constituents and depends both on their structure and concentration (Cos et al., 1998; Gadow et al., 1997; Małolepsza & Urbanek, 2000; Zając & Pawełczyk, 2000). The amount of active constituents in plant material is connected with the extent of their accumulation during the vegetative cycle (Ellnain-Wojtaszek et al. 2001; Ellnain-Wojtaszek

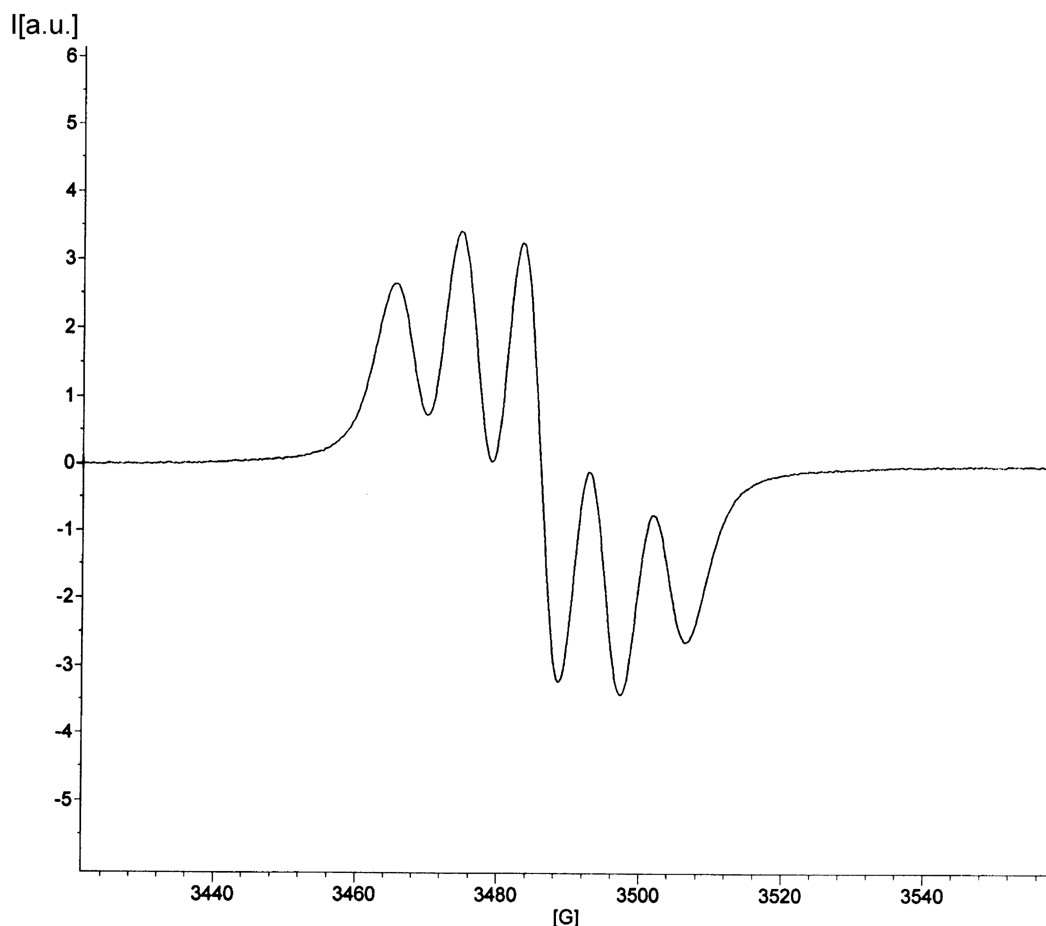

Fig. 3. The EPR signal of DPPH radicals taken in the 60th minute after mixing with *Ginkgo biloba* L, leaves (sample 1).

Table 1
The ability of *Ginkgo biloba* L. leaves—collected in the vegetative cycle—to scavenge free radicals, determined by EPR method

Sample of the leaves	The date of collection	Characteristic of the leaves	Scavenging ability S.A. (%)[a] in time		Content of Cu^{2+} ions (a.u.)
			1′	60′	
1997					
1	28.06	Green	24.3	53.7	55×10^3
2	28.07	Green	12.7	29.3	85×10^3
3	28.08	Green	2.7	13.2	103×10^3
4	28.09	Green-yellow	1.2	15.8	123×10^3
5	28.10	Yellow	1.0	11.4	206×10^3
1999					
3′	25.08	Green	21.4	44.5	60×10^3
4′	25.09	Green-yellow	1.1	14.0	110×10^3

[a] S.A. (%)—scavenging ability $\frac{I_0-I}{I_0}\times 100$ where I_0—intensity of DPPH pattern I—intensity of DPPH pattern after adding extract of the investigated sample.

& Zgórka, 1999; Lobstein, Rietsch-Jako, Haag-Berrurier, & Anton, 1991; Wang & Lin, 2000) The decreasing scavenging activity, determined for the *Ginkgo* leaves during the growing season, did not correlate with the content of flavonoids and phenolic acids (Ellnain-Wojtaszek, 1997a, b; Ellnain-Wojtaszek & Zgórka, 1999) that had been essayed in the same samples and described in a previous paper (Ellnain-Wojtaszek et al., 2001).

A similar lack of correlation was observed in investigations concerning herbal teas (Wasek, Nartowska, Haensel, Wawer, & Jeziorek, 2000). It seems that interpretation of the variations in free radical scavenging activity will be possible only if all changes, both qualitative and quantitative, that occurred in the vegetative cycle and corresponded to antioxidant constituents are taken into consideration. Seasonal alterations in the content of

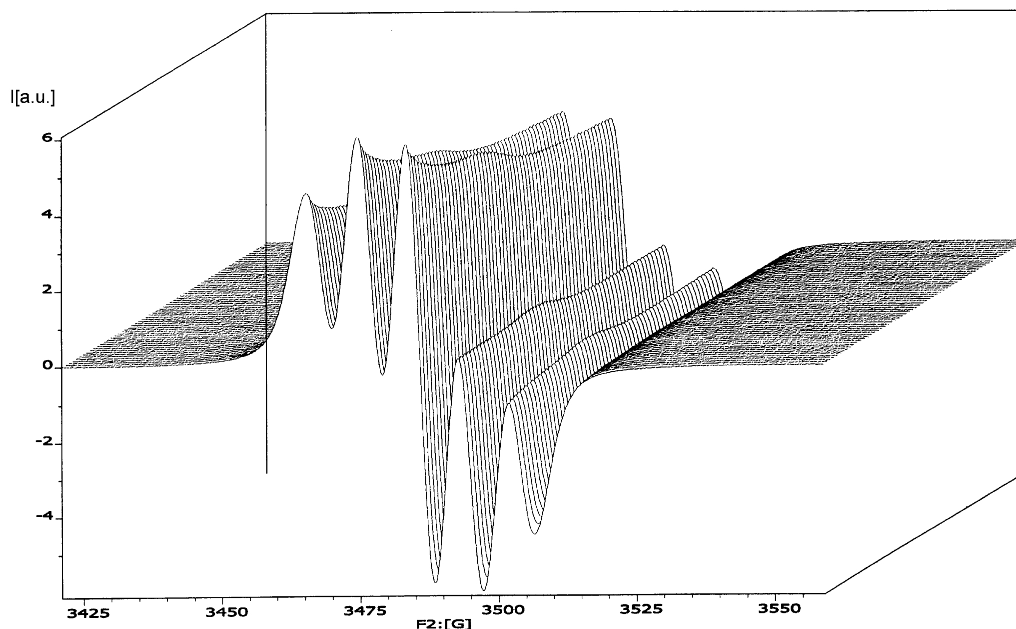

Fig. 4. 2-D illustration of time changes of the EPR signal of DPPH radicals in solution from the moment of mixing with extract from *Ginkgo biloba* L. leaves (sample 1, time range 1–60 min).

phenolic acids, revealed previously for the *Ginkgo* leaves (Ellnain-Wojtaszek & Zgórka, 1999), suggest that further studies on the activity should include not only total constituents but also individual compounds. Moreover, other researchers have shown that the free radical scavenging effect of phenolic acids is determined by their molecular structure (Gadow et al., 1997; Vinson, Dabbagh et al., 1995). The concentration of particular compounds away the total phenolic acids affect the leaves' biological activity.

Similar dependence has been observed for flavonoids, i.e. quercetin, with the ortho dihydroxyl function is a more potent free radical scavenger than kaempferol, lacking this functionality (Joyeux et al., 1995; Małolepsza & Urbanek, 2000). UV radiation shifts the quercetin–kaempferol ratio toward quercetin (Małolepsza & Urbanek, 2000). It seems that better insulation during the vegetative cycle influences the increase of concentration of constituents with the ortho dihydroxyl function. This increase will manifest itself in a higher free radical scavenging effect showed by the leaves. Furthermore, Table 1 shows a relationship between the drop in the radical scavenging activity as the growing season went by and the increase of the Cu^{2+} ion concentration, determined by EPR in a previous paper (Ellnain-Wojtaszek et al., 2001). Such determination of Cu^{2+} ions relative to the stage of the plant development has not been up to now reported. Present knowledge neither allows us to conclude whether Cu^{2+} ions participate in the complexation of flavonoids nor whether such copper complexes have higher free radical scavenging activity than uncomplexed flavonoids.

Finally, the influence of storage time on the scavenging activity was analysed. Samples 3 and 3′ of the leaves collected in August 1997 and August 1999 showed scavenging activities equal to 2.7 and 21.4%, respectively, in the first minute of the determination, and 13.2 and 44.5% in the 60th minute. These results demonstrate that storing time plays a significant role in the decrease of the free radical scavenging activity of *Ginkgo* leaves. Samples 4 and 4′, that varied in storage time identically, like samples 3 and 3′, showed a smaller difference in the scavenging activity. However, this drop in the difference level might be connected with a lower concentration of flavonols (Ellnain-Wojtaszek et al., 2001) and higher concentration of biflavones (Lobstein et al., 1991), which are weaker antioxidants, in the yellow-green leaves (Joyeux et al., 1995).

Ginkgo leaves are collected for the pharmaceutical industry in August. This particular time of harvest is due both to the maximum concentration of active constituents and the lowest possible damage to the tree caused by the leaves collection (Ellnain-Wojtaszek et al., 2001). The present study shows that the leaves should be stored for as short a time as possible to avoid the decrease in their free radical scavenging activity.

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