

## Differences in antioxidant properties of ginkgo leaves collected from male and female trees

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Total phenolic content and antioxidant capacity (FRAP method) of *Ginkgo biloba* L. leaves collected from male and female trees were determined and compared. Different water and aqueous ethanolic (water/ethanol 80/20, V/V) extracts were prepared by varying the time of infusing, boiling and steeping in order to determine the effect of the extraction method on the above parameters. Antioxidant activity and phenolic content of ginkgo leaf extracts correlated well with significant correlation coefficients. Slopes of linear regression lines were not statistically different for either sex.

*Keywords:* *Ginkgo biloba* L., male ginkgo, female ginkgo, ginkgo extracts phenolics, FRAP

Products prepared from *Ginkgo biloba* are among the most widely sold phytopharmaceuticals and dietary supplements in Europe and in the United States. The various compounds found in ginkgo improve blood circulation by opening up blood vessels, mainly in the brain. That is why they can be used for treating dementia and other vasoregulating problems in older age. Ginkgo preparations have been shown to help slow down Alzheimer's disease (1–3). Ginkgo leaves have also antioxidant properties, which are mainly connected to their polyphenolic constituents, particularly phenolic acids, flavonoids, proanthocyanidins and catechins (4–7).

Ginkgo is a dioecious species; female trees produce malodorous seeds. That is why only male trees are cultivated in ginkgo plantations in western countries, while both male and female trees are used for leaf production in Asia (8). There are not many literature data about the differences between antioxidant properties of the sexes. In our former study (9) we found significant differences in metal ion composition, H-donating activity, reducing power property and total scavenger capacity between leaf extracts of male and female ginkgo trees.

Besides standardized ginkgo extracts, also ginkgo teas are recently being recommended for consumers with vasoregulating problems. These products are consumed on a

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daily basis for their stimulant properties. The recommended preparation method for commercial ginkgo teas is to make an infusion with a 5–10 min steeping time. Based on our preliminary experiments, it seemed to be important to investigate the effect of different extraction methods.

The main objective of this study was to compare the phenolic content and antioxidant activity of extracts of *Ginkgo biloba* L. leaves collected from male and female trees. Effects of the extraction method – aqueous ethanolic extraction, water infusions and decoctions with different steeping and boiling times – on these properties were also investigated.

## EXPERIMENTAL

### *Materials*

Ginkgo (*Ginkgo biloba* L., *Ginkgoaceae*) leaves were collected in botanical gardens in Budapest, from sexually mature male and female trees aged between 80 and 120 years. Collection places were chosen where the male and female trees were of similar age and planted next to each other. Altogether, five male and five female samples were collected in early August of two consecutive years (2011 and 2012). After collection, leaf samples were dried at 30 °C and pulverized.

### *Sample preparation*

Water extracts were prepared by addition of 100 mL water to 1 g of dried leaves. Infusions were steeped for 3, 5 and 10 min and for 24 h, while decoctions were boiled for 3, 5 and 10 min and steeped for 10 min. A 10-min boiling followed by 24-h steeping decoction method was also applied. Aqueous ethanolic extracts were made from 1 g of dried leaves by addition of 100 mL aqueous ethanol (20 °C, water/ethanol 80/20, V/V) and were stored at room temperature for 72 h (10).

All extraction methods were repeated three times. After steeping for the given time, water and aqueous ethanolic extracts were filtered and centrifuged (12281  $\times$  g, 10 min). The supernatants were analyzed. All analytical determinations were performed in two parallel measurements.

### *Total phenolic content*

Total phenolic content was measured using the method of Singleton and Rossi (11) and was expressed as milligram of gallic acid equivalent per gram of dry mass.

### *Antioxidant capacity*

To determine the antioxidant capacity, the FRAP assay (ferric reducing ability of plasma) was used (12). This method depends upon reduction of the ferric tripyridyltriazine [Fe(III)-TPTZ] complex to the ferrous complex at low pH. Fe(II)-TPTZ has an intensive blue colour and can be monitored at 593 nm. Standard curve was prepared using different concentrations of ascorbic acid. Data are given in millimole of ascorbic acid equivalent per gram of dry mass.

### Statistical analysis

Results are presented as the means of five samples  $\pm$  standard deviation, separately for male and for female trees. Unpaired two tailed Student's *t*-test (in case of homoscedasticity) or Welch's *t*-test (in case of heteroscedasticity) was performed, separately for each extraction method, to determine the level of significance between male and female trees. Effects of the extraction method were analyzed by one-way analysis of variance, separately for male and female trees; the means were separated by Fisher's protected least significant difference test at  $p \leq 0.05$ . Correlations between the phenolic content and antioxidant capacity, and between maceration time and the afore-mentioned properties, were investigated by performing correlation and regression analyses. All statistical analyses were realized using Microsoft Excel 2007 software.

## RESULTS AND DISCUSSION

### Total phenolic content

As shown in Table I, total phenolic contents of ginkgo leaf extracts exhibited significant differences, depending on the sex of the tree. More phenolics were measured for leaves collected from male trees than from female trees, in almost every extraction method. The extent of these differences was up to 10 % for decoctions and 6 to 30 % for infusions. The largest difference was recorded for aqueous ethanolic extracts; the average data for male trees was by 45 % higher than that for the female trees.

Table I. Effect of the sex of the tree and extraction method on total phenolic content (gallic acid equivalents,  $\text{mg g}^{-1}$  dry mass) and on antioxidant capacity (ascorbic acid equivalents,  $\text{mmol g}^{-1}$  dry mass) of ginkgo leaf extracts

Extraction method <sup>a</sup>	Total phenolic content			Antioxidant capacity		
	Male trees <sup>b</sup>	Female trees <sup>b</sup>	<i>p</i> <sup>c</sup>	Male trees <sup>b</sup>	Female trees <sup>b</sup>	<i>p</i> <sup>d</sup>
I3min	21.40 $\pm$ 0.39	16.46 $\pm$ 0.17	5.23 $\times 10^{-10}$	16.20 $\pm$ 0.41	13.05 $\pm$ 0.17	8.09 $\times 10^{-7}$
I5min	22.87 $\pm$ 0.58	17.70 $\pm$ 0.19	1.66 $\times 10^{-7}$	17.57 $\pm$ 0.42	15.50 $\pm$ 0.24	3.00 $\times 10^{-4}$
I10min	25.77 $\pm$ 0.27	20.39 $\pm$ 0.41	1.42 $\times 10^{-11}$	20.32 $\pm$ 0.30	18.17 $\pm$ 0.14	2.57 $\times 10^{-6}$
I24h	32.23 $\pm$ 0.22	30.29 $\pm$ 0.42	5.25 $\times 10^{-4}$	24.49 $\pm$ 0.64	20.85 $\pm$ 0.12	4.91 $\times 10^{-5}$
D3min+S10min	24.08 $\pm$ 0.80	21.88 $\pm$ 0.20	1.66 $\times 10^{-2}$	22.11 $\pm$ 0.30	17.30 $\pm$ 0.15	2.27 $\times 10^{-12}$
D5min+S10min	23.92 $\pm$ 0.30	22.57 $\pm$ 0.30	3.40 $\times 10^{-3}$	24.38 $\pm$ 0.23	21.17 $\pm$ 0.32	7.94 $\times 10^{-9}$
D10min+S10min	28.29 $\pm$ 0.35	28.59 $\pm$ 0.17	4.51 $\times 10^{-1}$	27.59 $\pm$ 0.35	26.75 $\pm$ 0.17	4.01 $\times 10^{-2}$
D10min+S24h	32.69 $\pm$ 0.30	31.06 $\pm$ 0.12	5.87 $\times 10^{-5}$	29.61 $\pm$ 0.55	27.23 $\pm$ 0.14	7.00 $\times 10^{-4}$
E	79.77 $\pm$ 0.83	55.22 $\pm$ 0.45	5.49 $\times 10^{-18}$	62.60 $\pm$ 1.00	50.37 $\pm$ 0.38	1.02 $\times 10^{-9}$
LSD 5% <sup>d</sup>	1.40	0.82		1.44	0.62	

<sup>a</sup> I – infusion, steeping for the indicated time; D – decoction, boiling followed by steeping for the indicated times, E – aqueous ethanolic.

<sup>b</sup> Mean  $\pm$  standard deviation ( $n = 15$ ).

<sup>c</sup> Calculated by unpaired two-tailed *t*-test.

<sup>d</sup> Calculated by Fisher's protected least significant difference (LSD) test at  $p \leq 0.05$ .

A significant difference was also found in the efficacy of extraction solvents. For both sexes, aqueous ethanolic extraction resulted in almost three times higher phenolic contents than in water extractions (Table I). Similar data were reported by other authors for water extracts (6, 13, 14) and alcoholic extracts (15). Although extraction with aqueous ethanol was much more effective than water extraction, ginkgo teas are used as herbal teas, that is, as water extracts.

Significant differences were also found between different water extraction methods. As expected, increasing steeping time for infusions resulted in a significantly higher phenolic content. However, except for the 24-h steeping time, decoction extracted significantly more phenolic compounds than infusion (Table I). Excluding the results of water extractions employing a 24-h steeping time, we have found a strong positive correlation between the maceration time (time of steeping for infusions, or time of boiling plus time of steeping for decoctions) and the phenolic content both for male ( $R^2 = 0.421$ ,  $N = 90$ ,  $p = 4.57 \times 10^{-12}$ ) and female ( $R^2 = 0.897$ ,  $N = 90$ ,  $p = 3.19 \times 10^{-45}$ ) samples. Thus, it seems that the maceration time had a greater effect on the phenolic level of water extracts than the employed method (infusion or decoction).

### *Antioxidant capacity*

Measured antioxidant capacity values (using FRAP assay) of the investigated samples of extracts are presented in Table I. FRAP values for water extracts are in accord with the results of Goh (16). Similarly to the trend of phenolic contents, extracts of leaves collected from male trees had higher antioxidant capacity than that for female trees, regardless of the extraction method. Similarly, the highest antioxidant capacity resulted from aqueous ethanolic extractions, it was on average about 2.5 times higher than that from water extractions.

Statistically significant differences were found between the antioxidant capacity of the different water extracts (Table I). Similarly to the phenolic content, longer steeping and boiling times resulted in higher FRAP values. Strong positive correlations were also found between the maceration time and the antioxidant capacity of extracts both for male ( $R^2 = 0.896$ ,  $N = 90$ ,  $p = 5.46 \times 10^{-45}$ ) and female ( $R^2 = 0.869$ ,  $N = 90$ ,  $p = 1.49 \times 10^{-40}$ ) samples.

### *Correlation between total phenolic content and antioxidant capacity*

Based on all water extract data, a very strong positive correlation was found between total phenolic content and antioxidant capacity of ginkgo leaf extracts (Fig. 1). The correlation was stronger for female than for the male trees. The highly significant correlations with relatively high  $R^2$  values demonstrate that the phenolic content had a determinant role in the antioxidant capacity of ginkgo leaf extracts. Slopes of linear regression lines were not statistically different for either sex ( $0.803 \pm 0.066$  for male and  $0.769 \pm 0.040$  for female trees), thus the sex of the tree did not influence the trend of this correlation.

Our results are in agreement with some other studies of antioxidant properties of ginkgo leaf extracts. Aoshima *et al.* (14) as well as Pietta *et al.* (17) reported close correlations between the phenolic content and antioxidant capacity of ginkgo leaf extracts. In case of collected ginkgo leaves, Sati *et al.* (18) found a strong positive correlation between the phenolic content and antioxidant capacity measured by the FRAP assay. However, Ronowicz

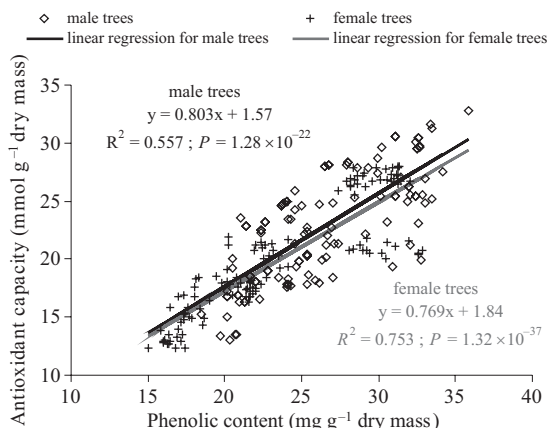


Fig. 1. Correlation between gallic acid equivalent to phenolic content and ascorbic acid equivalent to antioxidant capacity of ginkgo leaf water extracts.

*et al.* (19) documented a significant negative correlation ( $R = -0.7668$ ,  $p \leq 0.01$ ) between the total phenolic content and antioxidant activity (measured by DPPH method) for commercial ginkgo preparations.

## CONCLUSIONS

Leaves collected from male trees had higher phenolic content and higher antioxidant capacity, regardless of the extraction method, than leaves from female trees. Aqueous ethanolic extraction proved to be a much more effective method for extraction of phenolic materials and other antioxidative compounds from ginkgo leaves than aqueous extraction. Thus, the use of tinctures prepared from ginkgo leaves should be emphasized for pharmaceutical use. In case of water extracts, steeping and boiling times had a pronounced effect on the phenolic content and antioxidative capacity. Based on our results, a reasonable, but relatively long, maceration time at least 20 minutes should be recommended for preparing ginkgo teas in order to utilize the potential antioxidant capacity of ginkgo leaves as much as possible.

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