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Examination of antioxidant activity of *Ginkgo biloba* leaf infusions

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Abstract

The antioxidant capacity, AOC, of *Ginkgo biloba* leaf infusions was determined and compared using FRAP and ABTS assays. Capillary electrophoresis was used for the determination of flavonoids in *G. biloba* leaves to confirm and elucidate the fingerprint compounds that contribute to the AOC. Various parameters in the preparation of the infusion, such as degree of fermentation, surface area of leaves, temperature and time of leaves infusion have been considered, in order to determine their effects on the AOC. It was found that fermentation had no significant effect on the AOC of the *G. biloba* leaves. However, increased surface area of leaves (leaves ground for 20 s), an infusion temperature of 100 °C and infusion time around 10–15 min gave the highest overall AOC.
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Keywords: $ABTS^{\bullet +}$; Antioxidant capacity; Capillary electrophoresis; *Ginkgo biloba* leaves infusion

1. Introduction

In recent years, there has been an increasing awareness of the benefit of functional foods. A major thrust of current research has been the aim of developing new food products, which have functional properties. With *Ginkgo biloba* achieving unprecedented popularity over the past decade, and the recognition of the important therapeutic effects shown by this plant, there is a growing market for phytomedicines based on its extracts.

One current example of such a phytomedicine is a yellow-green tea infusion based on *G. biloba* leaves which has a smooth, light bamboo taste. Available commercially, it is postulated to be useful in the treatment of arteriosclerosis, varicose veins and haemorrhoids (Tenney, 1996; Zuess, 1998). However, there is limited information about how the methods of preparation of the infusion may alter the postulated therapeutic effects. Using FRAP and ABTS methods, for the determination of antioxidant activity (AOC), the effects of various parameters in the preparation of the infusion, such as degree of fermentation (unfermented, 2 and 24 h fermentation), surface area of leaves (ground 5 and 20 s), temperature (60, 80 and 100 °C) and time of leaves infusion (1, 3, 5, 10, 15 and 20 min), have been examined. Of particular interest is the fermentation of the leaves as this is the normal preparation practice for many leaf infusions, including tea.

Pharmacologically, there is one significant group of compounds found in Ginkgo leaves, the flavonoids, which give Ginkgo its antioxidant activity and possible protection against the damage caused by free radicals. These compounds scavenge and destroy free radicals and reactive forms of oxygen, such as superoxide radical ($^{\bullet}O_2$) (Gardes-Albert, Ferradini, Sekaki, & Droy-Lefaix, 1993; Marcocci, Packer, Droy-Lefaix, Sekaki, & Gardes-Albert, 1994; Pincemail et al., 1985), hydroxyl radicals ($^{\bullet}OH$) (Bors, Heller, Michel, & Saran, 1990; Husain, Cillard, & Cillard, 1987), lipid peroxide radicals (Maitra, Marcocci, Packer, & Droy-Lefaix, 1995; Marcocci, Packer et al., 1994) and nitric oxide (Marcocci, Maguire, Packer, & Droy-Lefaix, 1994). ABTS assays have shown that Ginkgo leaves extract is an effective free radical scavenger (Goh & Barlow, 2002). However, there are also considerations with regard to the seasonal variations and storage conditions of the leaves (Ellnain-Wojtaszek & Zgorka, 1999; Ellnain-Wojtaszek, Kruczyński, & Kasprzak, 2002). In addition, the antioxidant activity of the flavonoids also depends greatly on their chemical structure and the relative orientation of various moieties on the molecule, particularly the number

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Nomenclature

AOC	antioxidant capacity
$ABTS^{\bullet +}$	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AEAC	ascorbic acid equivalent antioxidant capacity
FRAP	ferric reducing antioxidant power
HPLC	high performance liquid chromatography

and position of hydroxyl groups within the molecule (Cody, 1988). The presence of a hydroxyl group in position three (3-OH) of the C ring. For example, the flavonoid aglycones, such as fisetin, (+)-catechin, quercetin, myricetin and morin, are more potent inhibitors of LPO than those that lack a 3-OH substitution such as diosmetin, apigenin (flavones), hesperetin, and naringenin (flavanones) (Mora, Paya, Rios, & Alcarza, 1990; Ratty & Das, 1988).

Antioxidants are increasingly being recognised as important health promoters in conditions such as cardiovascular problems, treatments of many forms of cancer and even aging (Packer, 1999). Antioxidants are able to reduce the effects of free radicals formed in the body, either by exposure to environmental pollutants or because somatic defence mechanisms are reduced in dealing with the natural production of these compounds. Thus, it was decided that AOC would be the benchmark by which the therapeutic effects of the leaf infusion could be measured.

The AOC was based on the ability of the sample to scavenge 2,2′-azino-bis-(3-ethlybenzthiazoline-6-sulfonic acid) (free radical $ABTS^{\bullet +}$). The FRAP assay, is based on low pH (~3.6), where ferric ion is reduced to ferrous ion, forming a compound known as a ferrous–tripyridyltriazine complex (Benzie & Strain, 1996, 1999). The main advantages and disadvantages of these two methods have recently been discussed (Frankel & Meyer, 2000). In this paper both methods are used and compared.

This study will provide more information for epidemiological studies and aid in the product development of *G. biloba* leaves which can later be directed to consumers with an optimal knowledge of the content and activity of these natural antioxidants.

2. Materials and methods

2.1. Ginkgo biloba *leaves preparation*

2.1.1. Unfermented leaves

Gingko biloba leaves used for AOC determination were imported from Ventura County, California, USA. Approximately 50 g of leaves were ground using a Braun grinder for 5 s. The above steps were repeated to prepare *G. biloba* leaves ground for 20 s. The unfermented *G. biloba* leaves were kept in the oven at 25 °C and put in a desiccator for at least 24 h prior to analysis.

2.1.2. Fermented leaves

Approximately 10 g of unfermented *G. biloba* leaves (ground for 5 s) were weighed into a beaker. Distilled water (1 g of leaves: 2 ml of water) was added and they were mixed and bruised thoroughly. The bruised *Ginkgo* leaves were put into an oven at 25 °C and were allowed to ferment for 2 h, after which, the leaves were removed and dried in an oven for 24 h at a temperature of 80 °C.

The earlier steps were repeated to prepare fermented *G. biloba* leaves (ground for 5 s) fermented for 6, 10 and 24 h. The same method was used to prepare all the fermented (2, 6, 10, 24 h) *G. biloba* leaves ground for 20 s. All fermented *G. biloba* leaves were kept in a suitable desiccator after drying in the oven.

2.2. Sample preparation protocol

In all the experiments, *Ginkgo* leaf infusions were prepared according to the following standard protocol. About 0.4 g of leaves were weighed into a beaker and 40 ml of deionised water was added (100 ml of water/g of leaves). Water temperatures and infusion times were varied between 60 and 100 °C and 1–20 min, respectively, to consider the effect of infusion temperature, as well as the infusion time, on the antioxidant activity. The effect of the degree of fermentation and surface area of *Ginkgo* leaves, as well as a comparison among different tea types, on the antioxidant activity, was also determined, using similar procedures. Table 1 illustrates the various samples used for AOC determination.

2.3. Particle size determination

Particle size determination was performed on Ginkgo leaves (ground for 5 s, and 20 s), commercial green tea, and black tea, using Tyler Standard Screen Scale Sieves (Cadle, 1955).

Particle size and particle size distributions are generally measured, because of the relationships they bear

Table 1
Test samples

Samples	Fermented (F)/ unfermented (U)	Grinding time (s)
A	(U)	5
B	(U)	20
C	(F) for 2 h	5
D	(F) for 2 h	20
E	(F) for 24 h	5
F	(F) for 24 h	20

to other properties of such particles (Cadle, 1955). In this case, the particle size difference between *Ginkgo* leaves ground for 5 and 20 s, commercial green and black tea might have an effect on the release of antioxidant compounds during infusion.

2.4. Experiment 1: effect of infusion temperature

The effect of infusion temperatures was investigated using six types of *Ginkgo* leaves: samples A, B, C, D, E and F. The infusion of each was prepared by adding water at 60, 80 and 100 °C. The mixtures of each type of leaves were stirred and allowed to stand for 10 min before filtering using filter paper (Whatman No. 1). The different infusions were collected in clean test tubes.

2.5. Experiment 2: effect of infusion time

The effect of infusion time was investigated using four types of *Ginkgo* leaves: samples A, B, C, and D. The infusion of each was prepared by adding water at 100 °C. They were stirred and allowed to stand for 1 min before being filtered. All the infusions were collected in clean test tubes. The steps were repeated for infusion times of 3, 5, 10, 15 and 20 min for all the leaves.

2.6. Experiment 3: comparison studies among Ginkgo leaves, commercial green and black tea leaves

In order to benchmark the AOC of Ginkgo with similar hot beverages, green tea and Ceylon tea were used for comparison. The antioxidant activities of three different types of leaves were investigated: unfermented *Ginkgo* leaves ground for 20 s (sample B), commercial green tea and commercial black tea. The infusion of each tea was prepared by adding water at 100 °C. They were stirred and allowed to stand for 3 min before being filtered using filter paper as before. All the infusions were collected in clean test tubes. Commercial green tea infusion (1 ml) was diluted with 10 ml of distilled water and black tea with 20 ml of distilled water.

2.7. FRAP determination

The AOC of all the infusions were determined using a modification of the FRAP assay previously described by Langley-Evans (2000). The FRAP reagent was prepared from 300 mM, pH 3.6, acetate buffer, 20 mM ferric chloride and 10 mM 2, 4, 6-tripyridyl-s-triazine made up in 40 mM hydrochloric acid. All three solutions were mixed together in the ratio of 25: 2.5: 2.5 (v:v:v). The FRAP assay was performed using reagents preheated to 37 °C. Prior to analysis, the initial absorbances of 3 ml of the reagents, and a 3 ml acetate buffer used as blank, were measured at 593 nm. The infusions (40 μl) were transferred into the cuvettes containing the reagent and the mixtures were shaken thoroughly. The mixtures in the cuvettes were examined after 90 min using a UV–vis spectrophotometer and the absorbance of the mixture, after 90 min was recorded. Each measurement of sample infusions was analysed in triplicate and the FRAP equivalent, expressed as mg FRAP per 100 g of leaves, was calculated using the following equation:

$$\mathrm{FRAP} = C_{\mathrm{FS}} \times V \times (100/W) \quad (1)$$

where C_{FS} is the concentration of ferrous sulphate standard solution corresponding to the absorbance change (mg/ml), V is the volume of infusion (ml) and W is the weight of leaves used for infusion preparation (g). Experiments 1, 2 and 3, mentioned earlier, were performed using the FRAP method.

2.8. $ABTS^{\bullet +}$ determination

ABTS [2,2′-azinobis-(3-ethylbenzothiazoneline-6-sulfonic acid)] was used as the free radical provider and was generated by reacting this compound (7.4 mM) with potassium persulphate (2.45 mM) overnight (Re et al., 1999). The solution was diluted, to obtain an absorbance of between 1.5 and 2.2 at 414 nm [molar extinction coefficient, $\varepsilon = 3.6 \times 10^4$ mol^{-1} cm^{-1} (Forni et al., 1986) with 98% ethanol, before use. Reagents (3 ml) were transferred to the glass cuvettes with one of them containing 3 ml ethanol as blank. The initial absorbance of the reagents in the glass cuvettes at 414 nm was recorded. The infusions (40 μl) were transferred into the cuvettes containing the reagent and the mixtures were shaken thoroughly. The mixtures in the cuvettes were examined after 90 min using a UV–vis spectrophotometer, and the absorbance of the mixture was recorded.

Each infusion was analysed in triplicate and the ascorbic acid equivalent antioxidant capacity (AEAC) of each infusion or sample was calculated and compared. The AEAC, expressed as mg ascorbic acid (AA) per 100 g of leaves, was calculated using the following equation:

$$\mathrm{AEAC} = C_{\mathrm{AA}} \times V \times (100/W) \quad (2)$$

where C_{AA} is the concentration of the AA standard solution corresponding to the absorbance change (mg/ml), V is the volume of infusion (ml) and W is the weight of leaves used for infusion preparation (g). Experiment 2 (using only samples A and B) and 3 were repeated using the ABTS method. Ascorbic acid was used as a standard in this assay in view of the fact that earlier work (Goh & Barlow, 2002) had shown a correlation between ascorbic acid content and total antioxidant capacity.

2.9. Fingerprint of Ginkgo biloba leaves infusion compounds (unfermented vs. fermented) using capillary zone electrophoresis (CZE) separation technique

2.9.1. Materials

Sample A: unfermented *Ginkgo* leaves ground for 20 s.
Sample B: fermented 24 h *Ginkgo* leaves ground for 20 s.

2.9.2. Apparatus (CZE)

All separations were performed using a Hewlett-Packard 3D CE system supplied by HP (Waldbronn, Germany) equipped with a 48.5 cm fused silica capillary column (with effective length of 40 cm for separation), i.d. 50 μm, from Polymicro Technologies (Phoenix, AZ, USA). The analysis buffer was 50 mM sodium borate (pH 9.3) and 10% acetonitrile in water. The temperature was kept constant at 20 °C; separation voltage was 25 kV and detection was at 230 nm—the wavelength giving the best resolution. All buffers were filtered using a Sigma (St. Louis, MO, USA) 0.2-μm filter. To maintain the capillary conditions, fresh buffer was introduced into the capillary between runs. The data were collected and analysed using software for instrument control and an HP Chemstation (Edition A. 06.04) data processor.

2.10. Caffeine determination using HPLC

An HPLC system (Shimadzu HPLC, Shimadzu, Kyoto, Japan) equipped with a photo-diode array detector was used. The separation was performed on a Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) using, as mobile phase, 40:60 (v.v) methanol:water at a flow-rate of 1.0 ml/min at 40 °C. The solvent was filtered through a 0.45-μm membrane filter and degassed using an ultrasonic bath or by flushing with helium before use.

2.11. Statistical analysis

Two tailed analysis of variance was performed on the data. Student's *t*-LSD (least significant difference) ($P=0.05$) was calculated to compare means for the different teas and also for the fermented and unfermented *Ginkgo* leaves infusion.

3. Results and discussion

3.1. Particle size distribution

The particle size distribution of the leaves was determined as follows, in decreasing size order: *Ginkgo* leaves (ground 5 s) > *Ginkgo* leaves (ground 20 s) > commercial black tea leaves > commercial green tea leaves. (See Table 2).

3.2. FRAP determination

AOC was demonstrated in the *Ginkgo* leaves infusates at all temperatures studied. Generally, the FRAP values in *Ginkgo* leaves infusates increased in a linear manner across the range of temperatures studied. There was no significant difference in FRAP ($P=0.05$) between unfermented leaves and leaves fermented for 24 and 2 h (with infusion conditions of 100 °C and 10 min) as seen in Table 3. The linear increase of FRAP, illustrated in Fig. 1, was similar for leaves ground for 5 or 20 s; however, the FRAP value of those ground for 20 s was consistently higher. In addition, fermentation did not affect the FRAP results. This finding also correlated well with the chromatograms obtained from capillary electrophoresis, as shown in Fig. 2. These clearly show that the compound profiles are the same for samples A and B and there is no significant difference between the patterns of peaks obtained. It is generally accepted that unfermented tea leaves, for example, green tea, should have a higher AOC (Von Gadow, Joubert, & Hansmann, 1997) due to the flavonols present that are known potent antioxidants (Lunder, 1992; Xie, Shi, Chen, & Ho, 1993). However, this study showed otherwise. Further studies on *Ginkgo* leaves fermented for 6 and 10 h were not continued.

There was an observable increase in the FRAP value of *Ginkgo* leaves ground for 5 s and 20 s (Fig. 3) with respect to infusion times. Taking unfermented *Ginkgo* leaves infused for 10 min as an example, leaves that

Table 2
Particle size distribution[a] of the leaves passing through various sieve sizes

	Sieve opening					
	2000	850	354	177	150	<150
Ginkgo leaves (ground for 5 s)	88	26	6	2	0	0
Ginkgo leaves (ground for 20 s)	100	76	12	2	0	0
Commercial black tea leaves	100	90	10	0.8	0.7	0
Commercial green tea leaves	100	93.2	53.2	15.2	12	0

[a] Percentage passing (%).

Table 3
FRAP equivalent (mg/100 g) of fermented and unfermented *Ginkgo* leaves infusion

Type of *Ginkgo* leaves	Ground time (s)	Temperature of infusion (°C)	Time of infusion (min)	FRAP equivalent (mg/100 g leaves) (% RSD)
Fermented 2 h	20	100	10	6329 (5.48%)
Fermented 24 h	20	100	10	6409 (1.59%)
Unfermented	20	100	10	6825 (4.54%)

The AOC results are generated from three determinations.

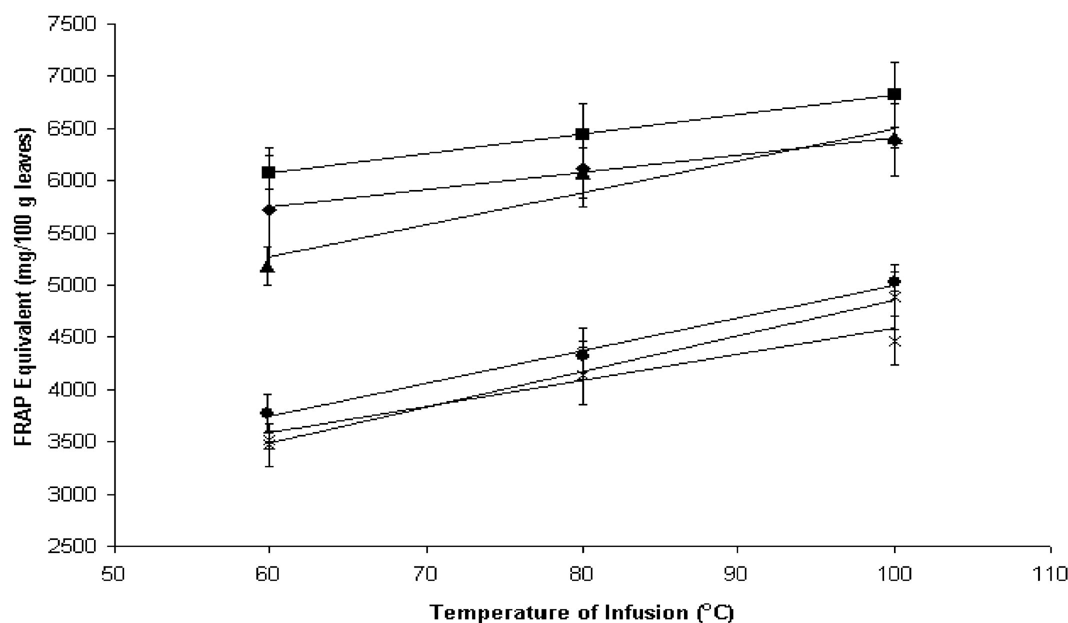

Fig. 1. FRAP equivalent (mg/100 g) of *Ginkgo* leaves infusion with respect to temperatures (°C). * Sample A: unfermented, ground for 5 s; ● sample B: unfermented, ground for 20 s. X sample C: fermented 2 h, ground for 5 s; ◆ sample D: fermented 2 h, ground for 20 s; • sample E: fermented 24 h, ground for 5 s; ▲ sample F: fermented 24 h, ground for 20 s.

were ground for 20 s (at 100 °C) gave a FRAP value of 6830 mg/100 g leaves, an increase of 2219 mg/100 g leaves from the FRAP value obtained from leaves that were ground for 5 s, at 100 °C (an increase of approximately 33%). As expected, *Ginkgo* leaves ground for 20 s had a higher FRAP value and that could be attributed to the larger surface area of the leaves that were ground for a longer time and their ability to release the antioxidant components more efficiently over the same period of time.

The FRAP values increased with infusion time for fermented leaves (samples C and D) from 1 min to a maximum FRAP value at 10 min. Similarly, for sample A, the FRAP values increased within the range of infusion time (1–20 min) under study. The trend for unfermented leaves, as seen in Fig. 3, on the other hand, was uncertain for the range of infusion time under study. For example, sample B exhibited an increase in the FRAP value obtained at $t=3$ min (6478 mg/100 g leaves) but the value decreased at $t=5$ min (6121 mg/100 g leaves) before reaching a global maximum, increasing again at $t=10$ min (6830 mg/100 g leaves). For sample A, the FRAP value was increasing within the range of infusion time (1–20 min) under study. However, most of the data (including 2-h fermented leaves) show that the release of antioxidants from *Ginkgo* leaves was maximum at $t=10$ min to $t=15$ min, as seen in Fig. 4.

3.3. $ABTS^{\bullet+}$ determination

This experiment was conducted on samples A and B. As fermentation showed no significant difference in the FRAP value, only unfermented *Ginkgo* leaves were then used in determining the AOC using the ABTS method. The AEAC value of unfermented *Ginkgo* leaves ground for 20 s was observed to be higher than that for leaves ground for 5 s, similar to the results obtained using the FRAP method. However, the optimum infusion time, for releasing the maximum antioxidants from the leaves, was different for different leaf types. The results seem contradictory because the maximum AEAC value was observed at $t=10$ min for leaves ground for 5 s whereas, for leaves ground 20 s, the maximum AEAC value was observed at $t=15$ min. However, it was anticipated that leaves of smaller particle size should be capable of releasing the maximum antioxidants after a shorter time. The results of ABTS determination do need to be viewed with care. This is because the specificity of assay involving the ABTS method for measuring the capacity of a sample to directly quench free radicals, is not always guaranteed or reproducible (Cao & Prior, 1998). However the method has been successfully used for AOC determination in alcoholic extracts of *Ginkgo biloba* nuts and leaves (Goh & Barlow, 2002).

3.4. Comparison studies among Ginkgo *leaves, commercial green and black tea leaves*

The AOC of the different beverages, as determined by the FRAP assay and ABTS decolorisation assay, decreased in the order: commercial green tea leaves > commercial fermented black tea > *Ginkgo* leaves infusion ground for 20 s as shown in Table 4. The black and green tea leaves had an approximate FRAP value 7 to

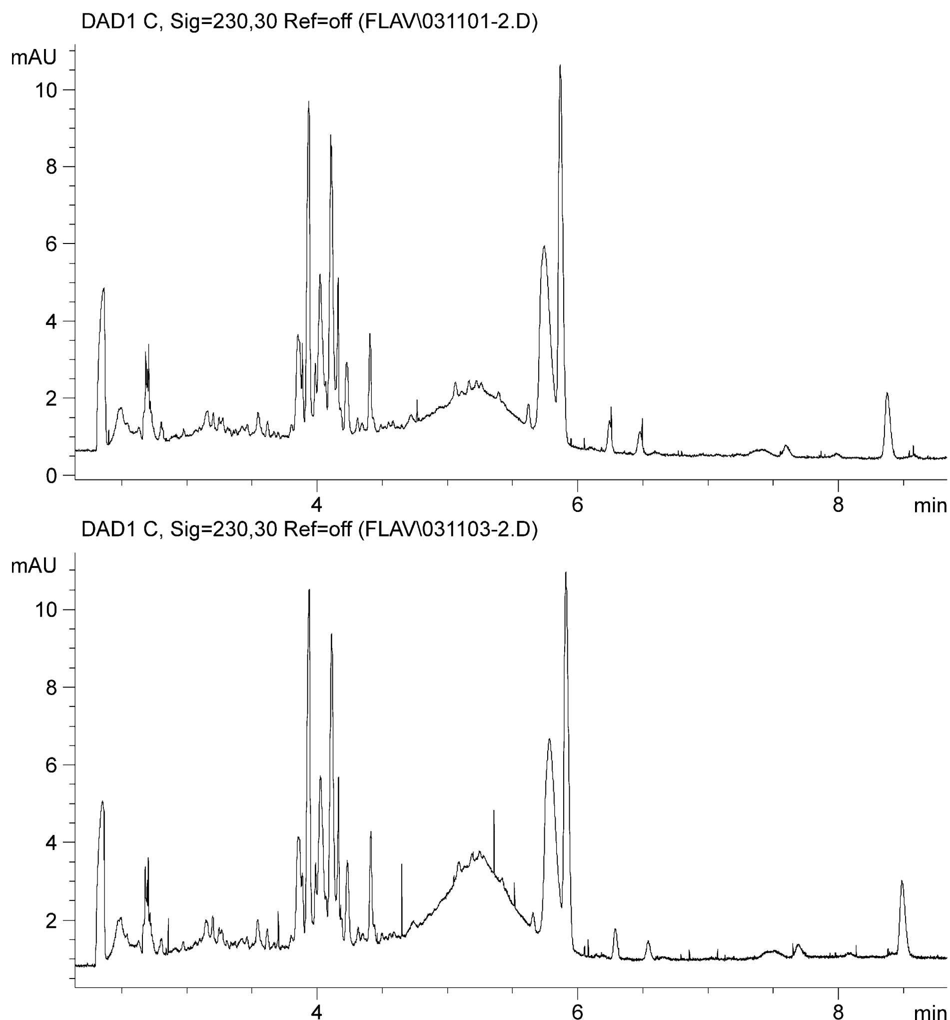

Fig. 2. A: unfermented *Ginkgo* leaves (see Section 2 for details) infusion 10 min, 0.4 g/40 ml; B: fermented 24 h *Ginkgo* leaves (see Section 2 for details) infusion 10 min 0.4 g/40 ml; capillary electrophoresis conditions: buffer, 50 mM borate and 10% ACN, injection: 50 mbar*4 s, +25 kV.

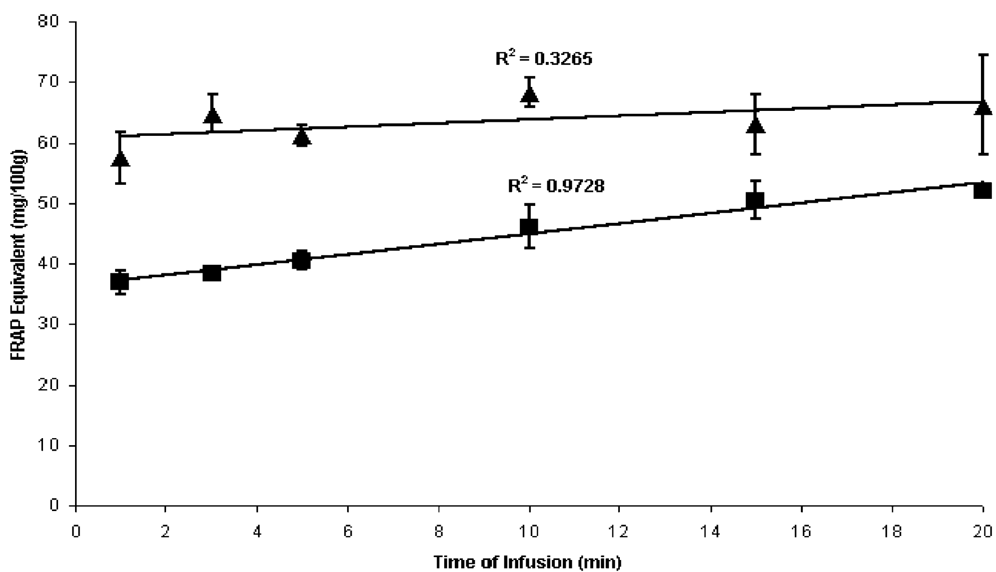

Fig. 3. FRAP equivalent (mg/100 g) with respect to time of infusion (min): ▲ unfermented *Ginkgo* leaves ground for 20 s (sample A); ■ unfermented *Ginkgo* leaves ground for 5 s (sample B).

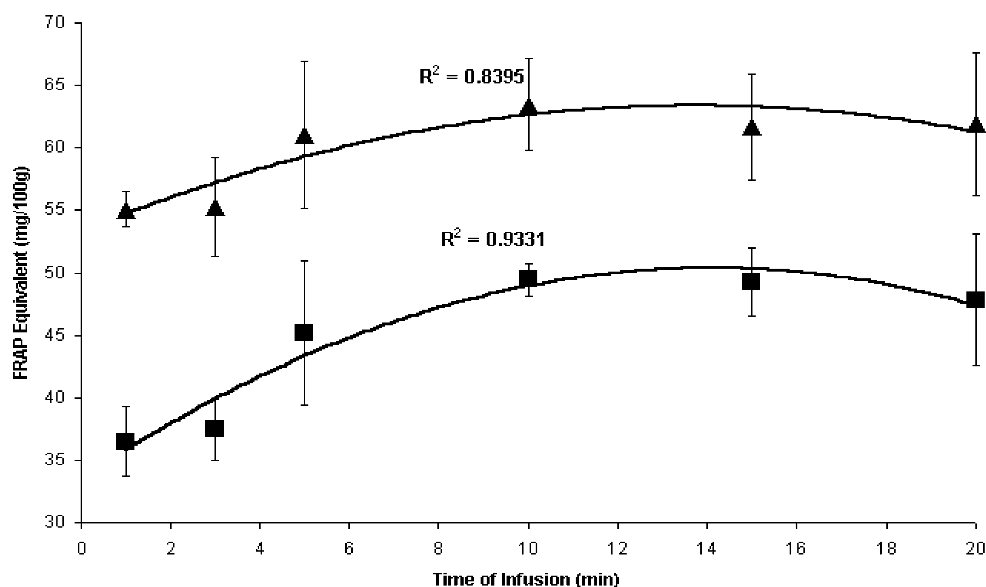

Fig. 4. FRAP equivalent (mg/100 g) with respect to time of infusion (min): ▲ fermented 2 h *Ginkgo* leaves ground for 20 s (sample D); ● fermented 2 h *Ginkgo* leaves ground for 5 s (sample C).

Table 4
AOC of the different test samples

Types of test samples	FRAP[a] (% RSD)	ABTS[b] (% RSD)
Green tea	56 810 (2.05%)	36 461 (1.67%)
English breakfast tea	44 745 (1.27%)	31 883 (1.75%)
Ginkgo leaves infusion	6211 (1.40%)	3185 (1.88%)

The AOC results are generated from three determinations.
[a] mg/100 g leaves.
[b] mg/100 g leaves.

Table 5
Caffeine content in selected beverages

Types of test samples	Caffeine content[a]
English breakfast tea	49.4±1.98
Green tea	52.0±1.41
Ginkgo leaves infusion	0

The caffeine quantifications are generated from three determinations.
[a] mg/cup (normal serving size of 250 ml).

9-fold higher than *Ginkgo* leaves. However, the difference of these values, between the green and black tea leaves and *Ginkgo* leaves, might be misleading because, from the particle size analysis of the leaves, the surface area of the green and black tea leaves was smaller than the surface area of *Ginkgo* leaves ground for 20 s. From the results presented earlier, it is seen that leaves with a larger surface area have a significantly higher FRAP value (higher AOC).

The differences of the AOC values amongst the test samples could be due to the presence of caffeine, which is present in considerable amounts in the commercial hot beverages whilst being absent in the *Ginkgo* leaves infusion. To confirm this, HPLC analysis was carried out on the *Ginkgo* leaves infusions (and also to quantify the caffeine present in the commercial hot beverages). This showed a negative result for caffeine in *Ginkgo* leaves infusion and relatively high caffeine contents in green and black tea, as shown in Table 5. Reported values, i.e. 25–110 mg of caffeine/cup (Food Facts Asia Third Quarter, 2000) coincide with the experimental results.

The ability of caffeine to scavenge the hydroxyl radical (Shi, Dalal, & Jain, 1991) displayed a reaction rate constant of approximately 5.9×10^9 mol^{-1} s^{-1} that is comparable with those of other efficient hydroxyl radical scavengers. Hence, caffeine does make a contribution to total AOC. However the degree of contribution of caffeine to total AOC in this study was not determined. In addition, there are also negative effects of caffeine, including rapid/irregular heartbeat, elevated blood sugar and cholesterol, nervousness, agitation and insomnia (Iwaoka & Brewer, 2000), a negative effect during pregnancy (Harland, 2000) and increased calcium loss (Barrett-Connor, Chang, & Edelstein, 1994; Harris & Dawson-Hughes, 1994; Lloyd, Rollings, Eggli, Kieselhorst, & Chinchilli, 1997). Ginkgo leaves infusion is caffeine-free yet capable of providing significant AOC.

4. Conclusion

Ginkgo biloba leaves ground for 5 s had a larger average particle size distribution than leaves ground for 20 s. Fermentation had no significant effect on the AOC of the *G. biloba* leaves. However, larger surface area of leaves (leaves ground for 20 s), infusion temperature of

100 °C and an infusion time around 10–15 min gave the highest AOC of *Ginkgo* leaves.

From the study, it is clearly shown that *G. biloba* leaf infusion has a lower AOC than the studied commercial hot tea beverages. However, it has an advantage over these in that caffeine is absent and this would definitely be of benefit to people who show adverse effects to caffeine.

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