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In-vivo and In-vitro Assessment of the Free-radical-scavenger Activity of *Ginkgo* Flavone Glycosides at High Concentration

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Abstract

Free radicals are involved in numerous skin diseases, especially inflammatory reactions and photosenescence. To identify possible free-radical scavenging by an original terpene-free *Ginkgo biloba* extract containing 33% *Ginkgo* flavone glycosides, mostly quercetin and kaempferol derivatives, we studied its activity by means of in-vitro and in-vivo experiments, using superoxide dismutase (SOD) as a positive control.

By means of an in-vitro electron-spin resonance (ESR) assay we compared the activity of the *Ginkgo* extract with that of its two aglycones, quercetin and kaempferol. Quercetin and *Ginkgo* extract had significant antioxidant properties without pro-oxidant effect. In contrast, kaempferol, above an optimum antioxidant concentration, behaved as a pro-oxidant. The in-vivo experiments were conducted on an anti-inflammatory model. The cutaneous blood flux which reflects the skin inflammatory level was recorded by means of a laser Doppler perfusion imager. The data confirmed the free-radical-scavenging property of both *Ginkgo* extract and SOD. The *Ginkgo* extract significantly inhibited (37%) cutaneous blood flux to the same extent as SOD.

These data confirmed the antioxidant property of *Ginkgo* extract. A complementary spin-trapping technique would enable identification of the free radicals involved. This *Ginkgo* extract should be useful for protection of the skin against free radicals.

It is well established that free radicals and highly reactive oxygen species are involved in the mechanism leading to cutaneous damage such as photoageing, inflammatory disorders and skin cancer (Gutteridge 1993). Reactive oxygen species are generated by external or endogenous phenomena, or both, and the most deleterious environmental sources are UV radiation, heavy metals released by pollution and cigarette smoke. At a cellular level, reactive oxygen species induce oxidation of compounds such as lipids, proteins and even DNA single-strand breakage.

Flavonoids are some of the most efficient antioxidants against the superoxide anion, hydroxyl radical and peroxy radical. They inhibit the activity of key enzymes involved in the inflammatory process, e.g. xanthine oxidase (Cotelle et al 1992), cyclooxygenase, lipoxygenase (Hoult et al 1994), and phospholipase (Lindhal & Tagesson 1993). Moreover, flavonoids have free-radical-scavenging activity, because of their ability to chelate the transition metal involved in the production of reactive oxygen species via the Fenton reaction (Morel et al 1993).

These properties of flavonoids explain why the compounds are widely used as protective agents against skin alteration. Thus, much attention has been focused on *Ginkgo biloba* standard extract, a natural mixture containing flavonoids (24%) and terpenes (6%) (Drieu 1986).

Numerous investigations have reported the scavenging of hydroxyl radical and superoxide anions by *Ginkgo biloba* extract, in accordance with its SOD-like activity (Pincemail et al 1989). It has also been demonstrated that *Ginkgo biloba* stops lipoperoxidation by quenching the peroxy radical (Dumont et al 1992; Maitra et al 1995).

The aim of this study was to assess, by means of in-vitro and in-vivo experiments, the free radical activity of an original terpene-free *Ginkgo* extract characterized by a high level (33%) of *Ginkgo* flavone glycosides, mostly quercetin and kaempferol glycosides (Figure 1).

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Materials and Methods

In-vitro study

Terpene-free ginkgo extract containing 33% *Ginkgo* flavone glycosides was purchased from Nuova Linea, and used in aqueous solution. Quercetin and kaempferol, purchased from Sigma, were dissolved in ethanol. Superoxide dismutase (SOD; EC 1.15.1.1) from *Escherichia coli* was purchased from Sigma and assessed in aqueous solution. These compounds were added at 10% (v/v) to an aqueous solution of pheomelanin. The photo-irradiated pheomelanin model has been described elsewhere (Duval & Poelman 1995).

Electron-spin resonance measurements

Electron-spin resonance (ESR) spectra were recorded by means of a Bruker ER 200 D spectrometer, operating at room temperature (25°C). The ESR parameters were: field modulation 100 kHz; microwave frequency 9·7 GHz; microwave power 2 mW; modulation amplitude 4 G; time constant 1 min; and sweep width 100 G.

Apparatus

A solar simulator containing a 150-W xenon arc as light source was used for all photolysis experiments. The energy output from the source of radiation was directed on to the front of the ESR cavity from which photon fluence rates were measured by means of a radiometer (UVA/UVB Osram Centra). The values were 2·1 mW cm^{-2} for UVA and 0·125 mW cm^{-2} for UVB. The irradiance energy for 5 min irradiation was 650 mJ cm^{-2} for UVA and 37·5 mJ cm^{-2} for UVB. No significant variation was found over the time necessary to perform the ESR experiments.

Production and detection of free radicals

A solution of pheomelanin, extracted from red hair (Bolt 1967), in phosphate buffer (pH 7·4, 50 mM; 200 μL) was poured into a quartz flat cell for irradiation and ESR measurements. The output from the solar simulator was directed on to the front of the cavity. Preliminary studies enabled us to set the irradiation time at 5 min.

The ESR spectrum of pheomelanin was obtained before (intrinsic signal) and after irradiation (illuminated signal). The illuminated signal amplitude of pheomelanin was compared in both the presence and absence of the test compound. The signal amplitude of pheomelanin solution was first assessed after irradiation in the absence of the test compound. ESR measurement was then performed with increasing concentrations of the test compound. It had previously been verified that addition of ethanol (1 : 10, v/v) did not affect the pheomelanin signal.

Analysis of the pheomelanin signal

Each measurement was performed on five different samples. The mean and the standard deviation of the intrinsic (A_{In}) and illuminated (A_{Il}) signal amplitudes were calculated. The effect of the test compounds was expressed as the percentage inhibition (PI), calculated by use of equation 1:

$$PI = \{[(A_{Il} - A_{In})_{control} - (A_{Il} - A_{In})_{Test} / [(A_{Il} - A_{In})_{Control}]\} \times 100$$

where A_{In} is the amplitude of intrinsic signal, A_{Il} is the amplitude of illuminated signal, $(A_{Al}-A_{In})_{Control}$ is the difference between the amplitudes of the illuminated and intrinsic signals from pheomelanin in the absence of test compound, and $(A_{Il}-A_{In})_{Test}$ is the difference between the amplitudes of the illuminated and intrinsic signals from pheomelanin in the presence of the test compounds.

In-vivo study

Subjects and experimental design. Ten healthy women volunteers, 23–28 years, participated in the study after giving their written consent. The study was approved by the Ethics Committee. The clinical investigator assessed they were in good health and had no dermatological disease. They were asked not to ingest or apply to the skin any anti-inflammatory drug one week before the study. In addition, smoking, coffee drinking or consumption of any other stimulant were not allowed at least 3 h before and during the study. The study was performed according to a simple-blind randomized fashion.

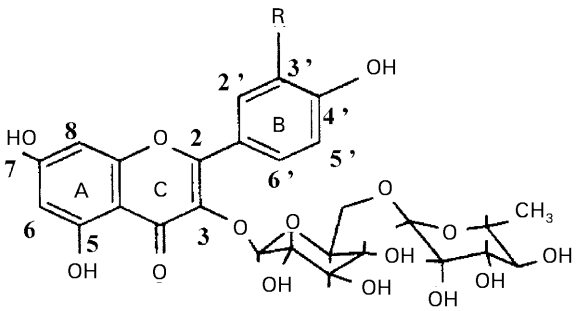

Figure 1. The structures of quercetin-3-*O*-rutinoside (rutin, R = H) and kaempferol-3-*O*-rutinoside (R = OH).

Six randomized areas (20 cm^2) were outlined on the internal side of the forearm of each volunteer. The study was performed in two stages. The first was the pretreatment phase consisting of the application of 2 mg cm^{-2} twice a day for 4 days of an oil-in-water (O/W) emulsion containing *Ginkgo* extract, its placebo, or SOD formulated in a Carbomer gel.

On the fifth day, the sites were occluded by a plastic film for 3 h. After the occlusion period each zone was gently washed and left to dry for 1 h, followed by the application of an aqueous solution of methyl nicotinate (0·5%, m/v; 100 μL; Merck).

Perfusion was measured for treated and untreated areas.

Laser perfusion imager measurements

Skin perfusion was assessed by means of the laser Doppler perfusion imager (Lisca Developpement AB, Linkoping, Sweden) based on the well-known laser Doppler principle (Nilson et al 1991) which collects data without touching the tissue and generates a colour-coded image of the spatial distribution of skin perfusion in 1 min (approx.) A computer-controlled optical scanner directed the low-power (2 mW) helium–neon laser beam on to the skin. The diameter of the laser beam at the focal point was estimated to be 100 μm. The incident monochromatic light beam ($\lambda = 633$ nm) penetrated the tissue to a depth of a 200–300 μm. The distance between scanner head and skin was 20 cm.

Statistical analysis

Results are presented as means ± s.e.m. Statistical analysis was undertaken by use of the paired Student *t*-test. Differences were accepted as statistically significant at $P < 0.05$.

Results

ESR spectroscopy

Pheomelanin has a stable ESR signal characteristic of cysteinyl dopa pheomelanin (Figure 2). Irradiation of pheomelanin at 10 mg mL^{-1} induces the generation of free radicals. Amplification of the illuminated signal means that UV induces free-radical production from pheomelanin. The recorded signal corresponds to the semiquinone stable free radical. Table 1 summarizes the percentage inhibition obtained with different concentrations of ginkgo extract (8–320 μg mL^{-1}), quercetin and kaempferol (40–320 μg mL^{-1}) and SOD (50–210 units mL^{-1}). *Ginkgo* extract clearly has dose-dependent activity, the maximum activity, 22% ($P = 0.02$) was obtained at a concentration of 160 μg mL^{-1} and was then followed by a decrease with increasing concentration until the activity was reduced by half at 320 μg mL^{-1}.

Quercetin significantly inhibited the ESR signal of pheomelanin. The inhibition was dose dependent. At 320 μg mL^{-1} inhibition was 28% ($P = 0.001$). The behaviour of kaempferol was biphasic—the activity increased with concentration up to 80 μg mL^{-1} (14%), decreased at 160 μg mL^{-1} (6·7%) and became pro-oxidant at 320 μg mL^{-1}.

The efficiency of SOD was high in the irradiated pheomelanin model. The percentage inhibition increased with increasing SOD concentration; at 210 units mL^{-1} inhibition was (approx.) 49%.

In-vivo study

There was no significant difference between control and placebo and the placebo had no effect on the methyl nicotinate model ($P = 0.4$). The percentage

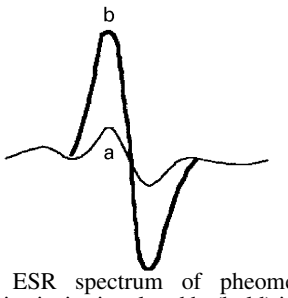

Figure 2. The ESR spectrum of pheomelanin solution (10 mg mL^{-1}): a. intrinsic signal and b. (bold) irradiated signal. Instrument settings: field modulation 100 kHz; microwave frequency 9·7; modulation amplitude 4 G; time constant 1 min; microwave power 2 mW; irradiation energy for 5 min is 650 mL cm^{-2} for UVA and 37·5 mL cm^{-2} for UVB.

Table 1. Inhibition of the pheomelanin signal by the compounds tested.

Compound	Concentration	Inhibition (%)
Ginkgo extract	8 μg mL^{-1}	4·9 ($P = 0.04$)
	40 μg mL^{-1}	9·6 ($P = 0.02$)
	80 μg mL^{-1}	16·14 ($P = 0.001$)
	160 μg mL^{-1}	21·85 ($P = 0.001$)
	320 μg mL^{-1}	11·2 ($P = 0.001$)
Quercetin	40 μg mL^{-1}	15 ($P = 0.000$)
	80 μg mL^{-1}	22·72 ($P = 0.000$)
	160 μg mL^{-1}	24·24 ($P = 0.000$)
	320 μg mL^{-1}	27·65 ($P = 0.000$)
Kaempferol	40μg mL^{-1}	8·2 ($P = 0.002$)
	80 μg mL^{-1}	14·1 ($P = 0.001$)
	160 μg mL^{-1}	6·66 ($P = 0.003$)
	320 μg mL^{-1}	4·85 ($P = 0.038$)[a]
Superoxide dismutase	50 units mL^{-1}	36·28 ($P = 0.000$)
	100 units mL^{-1}	46·20 ($P = 0.000$)
	210 units mL^{-1}	49·30 ($P = 0.000$)

[a]Pro-oxidant effect.

inhibition was calculated relative to the effect of the placebo, thus *Ginkgo* extract in-vivo significantly ($P = 0{\cdot}01$) reduces the inflammation induced by methyl nicotinate. The highest inhibition, 37%, was obtained by use of 0·1% *Ginkgo* extract emulsion. At concentrations above this the anti-inflammatory response de-creased, thus for 0·2% *Ginkgo* extract the percentage inhibition 26% was still significant ($P = 0{\cdot}02$) but for 0·3% it was not (13%; $P = 0{\cdot}3$).

SOD gel (2000 units mL^{-1}) reduced inflammation by the same amount as 0·1% *Ginkgo* extract emulsion (34%).

Discussion

In the in-vitro study the free-radical scavenging property of *Ginkgo* extract and of two of its main components were compared with that of SOD by using ESR to monitor the semiquinone radical. The results obtained with SOD as positive control are in accordance with our previous work (Duval & Poelman 1995) and confirm the generation of the superoxide anion in the course of pheomelanin photolysis. Other radical species, e.g. hydrogen peroxide and the semiquinone radical, are also produced (Lambert et al 1984; Land et al 1986; Pilas et al 1986). Our findings demonstrated the activity of *Ginkgo* extract, quercetin and kaempferol toward these free radical species generated by irradiated pheomelanin.

Comparison of the effects of ginkgo extract with quercetin and kaempferol revealed that quercetin is more efficient than *Ginkgo* extract. The maximum inhibition by quercetin (28%) was higher than that by the *Ginkgo* extract (22%). However, for identical concentrations (160 $\mu g\, mL^{-1}$) the activity of *Ginkgo* extract and quercetin is similar (22% and 24%, respectively). The activity of both *Ginkgo* extract and kaempferol is biphasic, but *Ginkgo* extract seems more efficient than kaempferol.

In addition, kaempferol at 320 $\mu g\, mL^{-1}$ has a pro-oxidant effect, with amplification of the amplitude of the ESR signal, whereas the activity of *Ginkgo* extract decreases down to 11% without pro-oxidant effect. In agreement with our results, quercetin is reported to have stronger antioxidant activity than other flavonoids against an array of radical species. This effect is attributed to its structure, in particular the *o*-dihydroxy-structure of the B ring, the 2, 3 double bond conjugated with the 4 oxo function, and the hydroxy groups at positions 5, 7 and 3′ (Figure 1). Shi & Niki (1998) reported that quercetin and kaempferol contain four and two active hydrogens, respectively, and that oxidation of quercetin and kaempferol lead, respectively, to the formation of semiquinone and phenoxy radicals.

Rice-Evans et al (1996) have reported that the phenoxy radical is involved in the pro-oxidant activity. The data obtained in our study with kaempferol are consistent with Rice-Evans's report. Metodiewa et al (1999) reported that quercetin could behave as both antioxidant and pro-oxidant depending on the concentration and the source of free radicals. With the photoirradiated pheomelanin model under our conditions no pro-oxidant behaviour is observed with quercetin.

Because irradiation of pheomelanin generates a semiquinone radical, it might be suggested that interaction between the two semiquinone radicals from pheomelanin and quercetin avoids the pro-oxidant effect. Flavonoids contained in *Ginkgo* extract are mostly flavonoid glycosides and it has been reported that the *O*-glycosylation reduces both antioxidant and pro-oxidant activity (Rice-Evans et al 1996; Cao et al 1997). This might explain the attenuated pro-oxidant effect of *Ginkgo* extract, but owing to its high flavone level, *Ginkgo* extract has stronger antioxidant activity than flavonoid glycosides.

Most experimental in-vitro studies have attempted to reproduce the biological conditions in which reactive oxygen species are generated in man. In this regard, photo-irradiated pheomelanin constitutes material from man which is closer to biological conditions and avoids the use of animals.

Our in-vivo study consists of generation of reactive oxygen species by inflammation induced by methyl nicotinate (Poelman et al 1989); this, in turn, stimulates cyclooxygenase and the prostaglandin synthesis (Wilkin et al 1985). This pathway cascade triggered the generation of endoperoxides leading to the production of reactive oxygen species. Furthermore, the stimulated inflammatory cells such as endothelial and polynuclear cells, fibroblasts and keratinocytes released the inflammatory mediators, l-1, Il-6 and reactive oxygen species, in particular the superoxide anion and hydrogen peroxide which induce lipoperoxidation by attacking polyunsaturated fatty acids. Peroxy and alkoxy radicals are then produced. Taylor et al (1983) demonstrated that reactive oxygen species are crucially important in the regulation of inflammation, and are involved in the release of arachidonic acid from the membrane phospholipids and subsequent conversion and formation of prostaglandin; he also demonstrated that free-radical scavengers inhibit all these effects.

We have also demonstrated that *Ginkgo* extract efficiently inhibits this inflammation model. Its

activity is assessed by a 37% reduction in skin perfusion obtained with 0·1% *Ginkgo* extract in the emulsion.

The blood flux, which reflects the level of inflammation, is reduced to 26% ($P = 0{\cdot}02$) and 13% ($P = 0{\cdot}3$), respectively, by 0·2 and 0·3% *Ginkgo* extract. In agreement with results from our previous in-vitro experiments, *Ginkgo* extract must be present at an optimum concentration for greatest efficiency. This phenomenon is frequently observed with antioxidant compounds. Indeed, the pro-oxidant effect of antioxidants such as Vitamin E (Kagan et al 1992) has been described when they are present at concentrations above the optimum. According to the findings of the in-vitro study, *Ginkgo* extract reduces inflammation by acting as a free-radical scavenger against the reactive oxygen species involved in the inflammation, as was emphasized by Taylor et al (1983).

The methyl nicotinate inflammation model, although indirect (but validated with SOD) reliably detects the activity of free-radical-scavenger compounds. A similar phenomenon was described after UV irradiation that stimulates phospholipase A_2 and prostaglandin synthesis (Hanson & Deleos 1989; Chen et al 1995).

Several authors (Miyachi et al 1983; Bisset et al 1990; Weber et al 1997) highlighted the protective role of antioxidants such as vitamin E and SOD against UV damage-mediated by reactive oxygen species. Indeed, UV induces skin cancer and photo-ageing and there is increasing evidence that reactive oxygen species are involved in this process. Because the SOD result is in good agreement with these authors' findings, it confirms the similarity of UV irradiation erythema and the methyl nicotinate inflammation model. In this model, *Ginkgo* extract is revealed to be as efficient as SOD ($P = 0{\cdot}34$).

The agreement between the results from in-vitro and in-vivo testing is worth noting. Although this work cannot provide precise information about its free-radical-scavenger specificity (which could be obtained by use of a complementary spin-trapping technique) the *Ginkgo* extract could actively protect the skin against exogenous free radicals induced by various oxidative stress.

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