nations for the impairment of endothelium-dependent relaxation can be made: 1) structural damage of endothelium, which causes nonspecific impairment in the ability to produce EDRF, 2) reduced sensitivity to acetylcholine, 3) impairment in the coupling between endothelium and smooth muscle, 4) reduced sensitivity of smooth muscle to EDRF. Since maximal or supramaximal concentrations of acetylcholine, both for WKY and SHRSP preparations, were applied as described above, the difference in the sensitivity of endothelium to this drug would not be involved. Although no further experiment to investigate the mechanism of the impairment of endothelium-dependent relaxation was performed, the structural damage of the endothelium due to the exposure to hypertension[17,18] seems to be the most provable one among the possible causes of the impairment. Recovery from the morphological changes after antihypertensive treatment[18] is also coincident with the recovery of the relaxation observed in the present experiment.

The relationship between the blood pressure of various Kyoto strain rats and the impairment of endothelium-dependent relaxation indicates a causal relationship between blood pressure and the impairment of relaxation. When the blood pressure is high, impairment of the release of EDRF progresses faster than when blood pressure is low, as shown in the age-dependency experiments. It was also observed that the degree of the impairment of endothelium-dependent relaxation increased when the duration of exposure to high blood pressure increased (fig. 2). The effect of antihypertensive treatment also supports the possibility that the decreases in the endothelium-dependent relaxation in spontaneously hypertensive rats are not genetically controlled but are the secondary results of protracted high blood pressure. Thus it can be concluded that impairment of the endothelium cannot be a cause of the initiation of hypertension in spontaneously hypertensive rats of the Kyoto strain, although it may be able to accelerate the elevation of blood pressure.

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## Superoxide anion scavenging effect and superoxide dismutase activity of *Ginkgo biloba* extract

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*Summary. Ginkgo biloba* extract is known to be efficient in diseases associated with free radical generation. The purpose of this work was to study, under in vitro conditions, the action of *Ginkgo biloba* extract (Gbe) against superoxide anion ($O_2^{\cdot-}$), which is directly or indirectly implicated in cell damage.

Gbe appears to have both an $O_2^{\cdot-}$ scavenging effect and also a superoxide dismutase activity. Its antiradical effect was demonstrated by low temperature electron spin resonance and in a non-enzymatic system (phenazine methosulfate-NADH), and its enzymatic activity was shown by polarographic determination.
*Key words. Ginkgo biloba*; superoxide anion; superoxide dismutase.

In some pathological situations, $O_2$ is reduced in a univalent pathway to yield superoxide anion ($O_2^{\cdot-}$). This oxygenated free radical is implicated in cell damage either by a direct nucleophilic effect[1,2] on membrane phospholipids, or by an indirect generation of hydroxyl radical ($OH^{\cdot}$)[3] which can induce lipoperoxidation processes. The peroxidation of unsaturated fatty acids in biological membranes by oxygen free radicals is thought to be a pathogenic mechanism in degenerative or other diseases and processes like aging[4], cancer[4], and adult respiratory distress syndrome[5]. Therefore the development of substances which have free-radical scavenging activities could represent an important challenge for therapeutic research.

An extract prepared from *Ginkgo biloba* leaves could be of considerable interest in this connection. This well-defined but complex product consists of two groups of major substances, flavonoidic heteroside compounds[6-8] (24% of the whole extract) and terpenoids[9]. In the first group, 2% of the heteroside compounds consist of quercetin[10] and kaempferol[11] coumaroyl glucorhamnoside. In the second group, diterpenoids (ginkgolides) and a sesquiterpenoid (bilobalide) represent 6% of the extract. Other substances of minor interest such as organic acids are present in the extract and play a role in the solubility in water.

Recently, we demonstrated that *Ginkgo biloba* extract (Gbe) was a potent free-radical scavenger in vitro since it easily reacts with hydroxyl radical ($OH^{\cdot}$) but also with 2,2 diphenylpicryl-hydrazyl radical and adriamycyl radical. Likewise, Gbe reduces the free radical-induced lipoperoxidation generated by the NADPH-$Fe^{3+}$ system in rat microsomes[12]. In vivo studies on animal models have confirmed the antiradical properties of the extract. Gbe may reduce adriamycin-induced inflammation of the rat hindpaw[13] (100–400 mg Gbe/kg, p.o.), it prevents damage in the retina of rats made diabetic by injection of alloxan[14] (100 mg Gbe/kg taken orally during 15 days), and it has an effective protection against functional disorders observed in cerebral (50 mg Gbe/kg, p.o.) and myocardial (50 mg Gbe/kg, perfusion into jugular vein) ischemia models[15,16]. In man, Gbe has also been prescribed in diseases in which free radicals are probably implicated; in psychic and behavioral disorders of the elderly, in peripheral vascular deficiency and in retinal deficits of ischemic origin[17].

The present study was undertaken to investigate in vitro the pattern of activity of *Ginkgo biloba* extract (Gbe) against $O_2^{\cdot-}$ generated in a specific way. The reduction of either cytochrome C[18] or nitrobluetetrazolium[19,20] salt is the most usual method for detecting unreacted $O_2^{\cdot-}$. Demonstration of a superoxide anion scavenging effect of Gbe was performed using a non-enzymatic system for $O_2^{\cdot-}$ production (phenazine methosulfate-NADH) and also by the electron spin resonance technique according to Fee and Hildenbrand[21]. Moreover, we attempted to study the superoxide dismutase activity of the whole extract by polarographic determination, as previously described by Rigo et al.[22].

## *Materials and methods*

*Ginkgo biloba* extract (IPS 200) was kindly supplied by Institut Henri Beaufour (France). Bovine blood Cu-Zn superoxide dismutase was obtained from the Sigma Chemical Co. Quercetin coumaroyl glucorhamnoside and kaempferol coumaroyl glucorhamnoside were a gift of Dr C. Nasr who purified the two products from *Ginkgo biloba* extract[10,11].

Superoxide anion was generated using two non-enzymatic systems. The first consisted of 630 µM of nitrobluetetrazolium (NBT), 30 µM of phenazine methosulfate and 156 µM NADH in 0.1 M phosphate buffer pH 7.4[23]. The product of the reduction of NBT was followed spectrophotometrically at 560 nm as an index of superoxide anion production. *Ginkgo biloba* extract and its two isolated compounds were dissolved in the incubation buffer.

In the second system, superoxide anion was generated by the electrolytic procedure described by Maricle and Hodgson[24] using an N,N-dimethylformamide (DMF) dry solution containing 0.1 M tetrabutylammonium bromide (TBAB, Aldrich) as carrier electrolyte. Superoxide anion was demonstrated by low temperature (– 196 °C) electron spin resonance (esr) spectrometry on a Varian E-9-X Band spectrometer. In this experiment, *Ginkgo biloba* extract was dissolved in DMF.

Superoxide dismutase activity was assayed by polarographic measurements carried out at 25 °C with a PAR Model 174 at voltages varying between + 0.1 V and – 1.9 V versus a Hg/HgCl/saturated KCl electrode (Dropping Mercury Electrode, DME) in 10 ml of 0.025 M sodium borate, pH 9.8, equilibrated with air, and containing $9 \cdot 10^{-4}$ M triphenylphosphine oxide (TPO, Aldrich). *Ginkgo biloba* extract was dissolved in the same buffer at the required concentrations.

## *Results*

*esr Measurements.* Figure 1 shows the esr spectrum of superoxide in DMF at low temperature. The presence of Gbe at 500 µg/ml in the medium severely decreases the signal intensity versus control. A slight reduction of the signal is also observed with Gbe at 125 µg/ml.

*NBT assay.* In the non-enzymatic superoxide anion generating system (phenazine metholsulfate-NADH), Gbe is able to inhibit strongly the reduction of NBT in a concentration range of 30–500 µg/ml (fig. 2). The two flavonoidic compounds of Gbe, quercetin coumaroyl glucorhamnoside and kaempferol coumaroyl glucorhamnoside, were also tested in this system. As indicated in table 1, they exhibit superoxide anion scavenging activities with a greater effect for quercetin coumaroyl glucorhamnoside.

*Polarographic measurements.* Figure 3 shows the curve currents versus potential values for the univalent reduction of $O_2$ to $O_2^{\cdot-}$. The reduction of $O_2^{\cdot-}$ on the dropping mercury electrode surface (DME) is inhibited by addition of triphenylphosphine oxide. The value of the limiting current is controlled by $O_2$ diffusion to the electrode and is therefore proportional to $O_2$ concentration. Compared with the control, addition of Gbe at 40 µg/ml to the medium increases the limiting current, thus indicating that oxygen concentration is also increased. A similar effect is observed with bovine Cu-Zn superoxide dismutase ($1.5 \cdot 10^{-9}$ M) which is known to catalyze the dismutation of $O_2^{\cdot-}$ into $H_2O_2$ and $O_2$. To avoid the registration of the whole curve, the measurement of the limiting current is performed at a fixed potential of − 0.958 V (amperometric measurements).

Table 1. Scavenging effect of quercetin and kaempferol coumaroyl glucorhamnosides on superoxide anion generated by the phenazine methosulfate/NADH system. $IC_{50}$ values were calculated from regression lines where: x was log of tested compound concentrations and y was percent inhibition. Each experiment was made in duplicate. b = slope of the regression line; r = correlation coefficient.

| | $IC_{50}$ µM | b | r |
|---|---|---|---|
| Quercetin coumaroyl glucorhamnoside | 30 ± 2 | 52.9 | 0.96 |
| Kaempferol coumaroyl glucorhamnoside | 51.8 ± 2 | 56.8 | 0.97 |

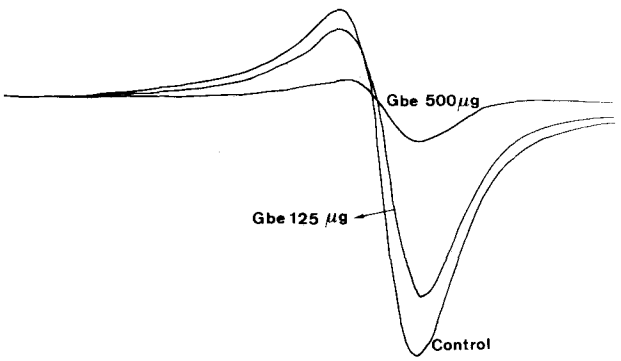


Figure 1. Scavenging effect of Gbe on superoxide anion electrochemically generated in DMF. Samples were transferred to a glass pipette and slowly frozen at − 196 °C with liquid nitrogen. Control consisted of 900 µl of DMF solution containing $O_2^{\cdot-}$(A) + 100 µl of pure DMF; assays with Gbe were made by mixing 900 µl of A with 100 µl of DMF containing 5 or 2.5 mg Gbe. The pipette was then placed in the sample cavity containing liquid nitrogen of a Varian E-9-X Band spectrometer. The spectrometer settings were as follows: modulation frequency 100 kHz; microwave frequency 9.4 GHz, field set 3395 G; modulation amplitude 10 G, time constant 0.5 s, microwave power 10 mW.

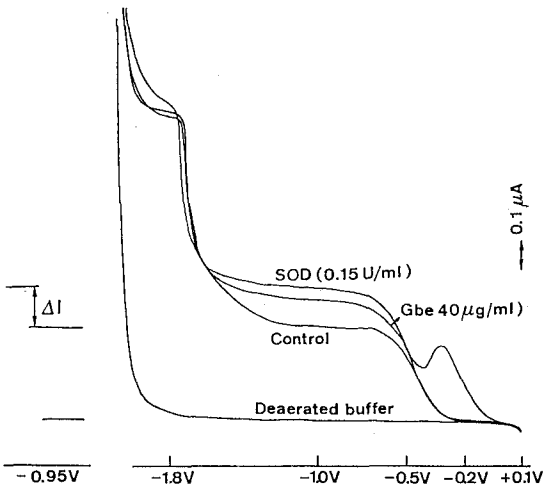


Figure 3. Effect of bovine Cu-Zn superoxide dismutase (0.15 U Fridovich/ml $1.5 \cdot 10^{-9}$M) and Gbe (40 µg/ml) on polarographic wave of $O_2$ in 0.025 M sodium borate, pH 9.8 (see Material and methods). The variation of the limiting current (µA) was registered vs the decrease of the initial potential of + 0.1 V to negative potentials (reduction potential). Control consisted of 0.025 M sodium borate pH 9.8. ΔI = current difference between SOD or Gbe solutions vs the control at a fixed potential of − 0.958 V (amperometric method). One Fridovich unit of activity is defined by the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome C by 50% under experimental conditions described in 31.

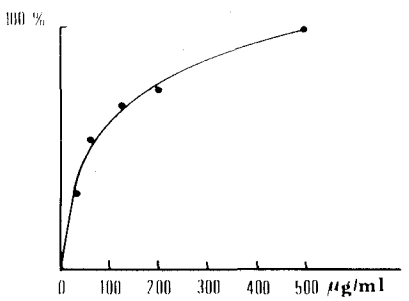


Figure 2. Inhibitory effect of Gbe on superoxide anion generation by phenazine methosulfate and NADH. Cuvettes contained 0.9 ml phosphate buffer pH 7.4 (reference) or 0.8 ml (test), 0.1 ml of water (control) or Gbe at the required concentrations, 0.1 ml NADH (156 µM final) and 0.1 ml NBT (630 µM final). Reaction was started by addition of 0.1 ml phenazine methosulfate (30 µM final) in the test cuvette. The absorbance at 560 nm was recorded during 1 min at 25 °C on a Perkin Elmer Lambda 15 UV/VIS spectrophotometer.
Results are expressed in % inhibition of the control.

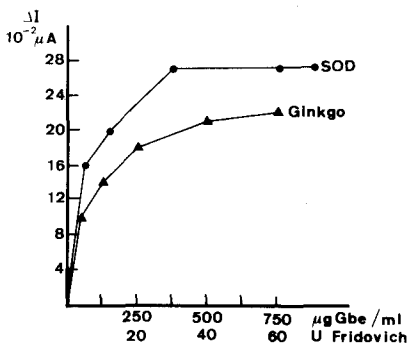


Figure 4. Superoxide dismutase activity, measured by the amperometric method, of bovine Cu-Zn superoxide dismutase and Gbe at different concentrations.
ΔI = current difference between SOD or Gbe solutions at different concentrations vs the control at a fixed potential of − 0.958 V (see fig. 3).

Table 2. Effect of cyanide (KCN $10^{-3}$ M) and of heating on superoxide dismutase activities of bovine Cu-Zn superoxide dismutase and Gbe measured under the same conditions as described in figure 4.

| | Control | + KCN | 1 h 100 °C |
|---|---|---|---|
| SOD 0.2 U/ml | 100% | 0% | 0% |
| Gbe 500 µg/ml | 100% | 100% | 0% |

The current difference (Δ I, see fig. 3) at this potential between Gbe or SOD at different concentrations and the control is shown in figure 4. There is a linear relationship between ΔI and concentrations of Gbe up to 100 µg/ml. At higher concentrations, the relationship becomes an asymptotic one. A similar profile is observed for concentrations of SOD ranging from $10^{-9}$ to $10^{-8}$ M (0 to 1.5 U Fridovich/ml; see legend to fig. 3).

Other amperometric measurements indicate that Cu-Zn superoxide dismutase activity is completely inhibited by cyanide ($10^{-3}$ M), whereas the Gbe enzyme activity is cyanide-insensitive (table 2). Lastly, for SOD as well as for Gbe, no activity can be observed after boiling for 1 h at 100 °C (table 2).

*Discussion*

Superoxide anion ($O_2^{\cdot-}$) is an oxygen-free radical which can be demonstrated by the low temperature esr technique (fig. 1) in aprotic media such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), or acetonitrile ($CH_3CN$). Superoxide anion has a lifetime of several hours in proton-free medium but it spontaneously dismutates into hydrogen peroxide and oxygen in aqueous medium.

Although Fee and Hildenbrand[21] have shown that electrolyzed $O_2$/DMF solution, in contrast with $O_2$/DMSO and $O_2/CH_3CN$ solutions, only contains small amounts of $O_2^{\cdot-}$ and is probably contaminated by another free radical, we used DMF solution because Gbe is highly soluble in this organic solvent.

The intensity of the esr spectrum of $O_2^{\cdot-}$ is strongly reduced in the presence of Gbe, which indicates that the extract has a direct $O_2^{\cdot-}$ scavenging activity (fig. 1). Experiments using the phenazine methosulfate and NADH system fully confirm that Gbe is a potent superoxide anion scavenger (fig. 2). This property is not surprising since Gbe is well known in vitro and in vivo for its free-radical scavenging activity[12–16].

It remains difficult to establish which compounds in the extract are responsible for the $O_2^{\cdot-}$ scavenging effect. However, it is well known that flavonoidic compounds exhibit potent free radical scavenging activities[12, 25, 26, 27], and recently Robak et al.[23] demonstrated that flavonoids were superoxide anion scavengers ($IC_{50}$ of quercetin is 12.5 µM and of rutin is 15.4 µM, in the phenazine methosulfate-NADH assay). Results given in table 1, with quercetin coumaroyl glucorhamnoside and kaempferol glucorhamnoside, two important flavonoidic compounds of Gbe, are in agreement with these results. Taking a mean molecular weight of 700 for flavonoidic heteroside compounds and their content in the extract, it can be calculated that they are in a concentration which is close to the $IC_{50}$ generally observed with flavonoids. From these considerations, it may be suggested that a large part of the $O_2^{\cdot-}$ scavenging effect of Gbe can be attributed to the presence of flavonoidic compounds in the extract.

Polarographic measurements indicate that Gbe also has superoxide dismutase activity (fig. 3). Like Cu-Zn bovine superoxide dismutase, which catalyzes the dismutation of $O_2^{\cdot-}$ ($k = 2.3 \cdot 10^9\ M^{-1}\ s^{-1}$) into hydrogen peroxide and triplet oxygen, Gbe present in the aqueous medium increases the $O_2$ concentration, as evidenced by the limiting current. In comparison with the enzymatic activity of Cu-Zn bovine superoxide dismutase, Gbe exhibits a similar activity (fig. 4). For example, 75 µg Gbe/ml gives an activity equal to that observed with 0.22 U Fridovich of bovine Cu-Zn SOD.

Using amperometry, we confirmed that the superoxide dismutase activity of Gbe is closely associated with the presence of protein in the extract. Indeed, neither Gbe nor Cu-Zn bovine superoxide dismutase presents any enzymatic activity after heating (table 2). In addition, in contrast to Cu-Zn bovine superoxide dismutase, the enzymatic activity of Gbe is insensitive to cyanide (table 2); this is typical of iron containing SOD. All these observations are in agreement with recent work of Duke and Salin[28], who purified and characterized an iron-containing superoxide dismutase from *Ginkgo biloba* leaves. This could be of great interest for clinical applications, since SOD is now considered to be an antiinflammatory drug[29].

*Conclusion*

In this study, we clearly show that *Ginkgo biloba* extract is efficient against superoxide anion, either because of its ability directly to scavenge the radical, or because of the presence in the extract of an iron superoxide dismutase. These observations can therefore explain the preventive effect of Gbe against functional disorders of ischemic origin in vivo. In the course of ischemia, there is a conversion of xanthine dehydrogenase into xanthine oxidase. During the reperfusion, $O_2$ is the electron acceptor of the xanthine oxidase system, which results in the generation of superoxide anion.

Moreover, the extract could also have an interesting effect as a vasoprotector since the anti-radical activity of flavonoids is associated with a vasoprotective action[30] and since Robak et al.[23] recently proved that flavonoids may prolong the half-life of the endothelial-derived relaxing factor (EDRF) by their superoxide anion scavenging effects.

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# Purification and characterization of a β-glucosidase (linamarase) from the haemolymph of *Zygaena trifolii* Esper, 1783 (Insecta, Lepidoptera)

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*Summary.* A β-glucosidase (linamarase) was purified 52-fold with a recovery of 27% from the haemolymph of the larvae of *Zygaena trifolii*, ESPER, 1783 (Lepidoptera, Zygaenidae). The final enzyme preparation was found to be nearly homogeneous on both disc polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The molecular weight of the enzyme was determined to be about 130 kDa; it consisted of two subunits of about 66 kDa. The enzyme showed an optimum between pH 4.5 and 5 with linamarin and a broad optimum between pH 3.5 and 6.5 for p-nitrophenyl-β-D-glucoside; the temperature optimum was 40 °C. The β-glucosidase showed a high specificity for its endogenous substrates linamarin and lotaustralin. Among the other natural and artificial substrates tested, only prunasin and p-nitrophenyl-β-D-glucoside were hydrolyzed by the enzyme, whereas linustatin, salicin, cellobiose and trehalose were not. The enzyme is strongly inhibited by β-glucosylpiperidine.
*Key words.* Cyanogenesis; β-glucosidase; Lepidoptera; linamarin; linamarase; lotaustralin; repellent; Zygaenidae.

Cyanogenic glycosides have long been accepted as defensive compounds for organisms because of their ability to release hydrogen cyanide (HCN) upon hydrolysis; this is the phenomenon of cyanogenesis [1, 2]. For plants as well as for arthropods, the protective function of HCN has been discussed [3, 4]. Cyanogenic glucosides are hydrolyzed by β-glucosidases to give corresponding hydroxynitriles which dissociate spontaneously above pH 6. At lower pH values, the reaction is catalyzed by hydroxynitrile lyases [5].

Several reports have shown that in plants cyanogenic glucosides and their catabolic enzymes are localized in different compartments (e.g. in the vacuole, the cytoplasmatic or apoplasmatic space) of cells belonging to either the same or different tissues. It is only after disruption of the tissue that HCN is liberated in major amounts [6–9].