J. Pharm. Pharmacol. 2000, 52: 451–459
Received October 25, 1999
Accepted December 6, 1999

Identification of Kaempferol as a Monoamine Oxidase Inhibitor and Potential Neuroprotectant in Extracts of *Ginkgo Biloba* Leaves

B. D. SLOLEY, L. J. URICHUK, P. MORLEY*, J. DURKIN*, J. J. SHAN, P. K. T. PANG AND R. T. COUTTS†

*CV Technologies, Suite 308, Campus Towers, 8625-112 Street, Edmonton, Alberta, T6G 1K8, *National Research Council, Cellular Neurobiology Group, Building M-45, Ottawa, Ontario, K1A 0R6 and †Neurochemical Research Unit, Department of Psychiatry, University of Alberta, Edmonton, Alberta, T6G 2B7, Canada*

Abstract

The effects of *Ginkgo biloba* leaf extract on rat brain or livermonoamine oxidase (MAO)-A and -B activity, biogenic amine concentration in nervous tissue, *N*-methyl-D-aspartate (NMDA)- and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4)-induced neurotoxicity and antioxidant activity was investigated to determine the effects of the extract on monoamine catabolism and neuroprotection.

Ginkgo biloba leaf extract was shown to produce in-vitro inhibition of rat brain MAO-A and -B. The *Ginkgo biloba* extract was chromatographed on a reverse-phase HPLC system and two of the components isolated were shown to be MAO inhibitors (MAOIs). These MAOIs were identified by high-resolution mass spectrometry as kaempferol and isorhamnetin. Pure kaempferol and a number of related flavonoids were examined as MAOIs in-vitro. Kaempferol, apigenin and chrysin proved to be potent MAOIs, but produced more pronounced inhibition of MAO-A than MAO-B. IC50 (50% inhibition concentration) values for the ability of these three flavones to inhibit MAO-A were 7×10^{-7}, 1×10^{-6} and 2×10^{-6} M, respectively. *Ginkgo biloba* leaf extract and kaempferol were found to have no effect ex-vivo on rat or mouse brain MAO or on concentrations of dopamine, noradrenaline, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. Kaempferol was shown to protect against NMDA-induced neuronal toxicity in-vitro in rat cortical cultures, but did not prevent DSP-4-induced noradrenergic neurotoxicity in an in-vivo model. Both *Ginkgo biloba* extract and kaempferol were demonstrated to be antioxidants in a lipid-peroxidation assay.

This data indicates that the MAO-inhibiting activity of *Ginkgo biloba* extract is primarily due to the presence of kaempferol. *Ginkgo biloba* extract has properties indicative of potential neuroprotective ability.

Ginkgo biloba fruits have been used for medicinal purposes in China for centuries. More recently, extracts of the leaves have been used to protect against capillary fragility, cognitive deficits, cerebral and vascular insufficiency and other age-related problems. Recent studies have indicated that extracts of *Ginkgo biloba* leaf are effective in alleviating certain cognitive deficits in patients diagnosed with dementia of the Alzheimer type (Le Bars et al 1997; Maurer et al 1997). Extracts of *Ginkgo biloba* leaves are chemically complex and dozens of compounds have been isolated, including a variety of terpenes, catechins and flavonoids (Weinges et al 1968a, b). Standardized extracts of *Ginkgo biloba* leaf contain about 24% *Ginkgo* flavone glycosides and about 6% terpene lactones. Although some components of *Ginkgo biloba* have been evaluated and found to possess interesting pharmacological properties (Foldes-Filep & Filep 1988; Ferriola et al 1989; Medina et al 1997), the possible pharmacological relevance of many of these compounds remains to be elucidated.

Correspondence: B. D. Sloley, CV Technologies, Suite 308, Campus Towers, 8626-112 Street, Edmonton, Alberta T6G 1K8, Canada.

Several studies have determined that some natural flavonoids and related synthetic compounds are potent inhibitors of monoamine oxidase (MAO)

in-vitro (Sparenberg et al 1993; Thull & Testa 1994) and, recently, it has been reported that extracts of *Ginkgo biloba* leaves inhibit MAO type A (MAO-A) in a reversible manner (White et al 1996). Consistent with its ability to inhibit MAO-A, it has been reported that extracts of *Ginkgo biloba* elevate the levels of biogenic amines, especially 5-hydroxytryptamine (5-HT) in the brains of rodents (Petkov et al 1993). These studies suggest that *Ginkgo biloba* extract might act as an antidepressant in a manner similar to synthetic MAO inhibitors (MAOIs). Indeed, reports of *Ginkgo biloba* extracts acting as anti-anxiety or anti-stress agents in rodent models thought to be predictive of antidepressant effects in humans are now in the literature (Porsolt et al 1990; Rapin et al 1994; Satyan et al 1997).

Both *Ginkgo biloba* extracts (Ramassamy et al 1990; Marcocci et al 1994; Kose & Dogan 1995) and flavonoids (Galvez et al 1995; Chen et al 1996; Kerry & Rice-Evans 1999) have been shown to be potent antioxidants. Some flavonoids are known to scavenge radicals formed under a number of circumstances including anoxia, lipidic auto-oxidation and inflammation. Flavonoids also chelate polyvalent ions such as Fe^{3+} and Al^{3+} (reviewed by Gabor 1988). The presence of an excess amount of these ions is thought to contribute to certain aspects of neurodegenerative diseases. The scavenging of free radicals, the inhibition of MAO and the chelation of metal ions are all believed to contribute to the neuroprotective properties of certain drugs in some, but not all, neuroprotection models.

We evaluated the effects of a standardized commercial *Ginkgo biloba* extract on MAO activity and on potential neuroprotective mechanisms. Two compounds in the extract were found to be inhibitors of MAO in-vitro. The most potent MAOI, later identified as kaempferol, and the original *Ginkgo biloba* extract were examined for ex-vivo effects on MAO activity and biogenic amine concentrations in mice or rats. Kaempferol was investigated for neuroprotective effects in an in-vitro *N*-methyl-D-aspartate (NMDA)-induced neuronal toxicity assay and in an in-vivo *N*-(2-chloroethyl)- *N*-ethyl-2-bromobenzylamine (DSP-4)-induced noradrenergic toxicity model. The antioxidant capacity of *Ginkgo biloba* leaf extract and kaempferol was also assessed.

Materials and Methods

Animals

Male Sprague–Dawley rats, 200–250 g, (Charles River, Ontario) were housed two per cage and maintained on a 12-h light–dark cycle at 22°C. Male Swiss mice, 25–30 g, (Charles River, Ontario) were housed five per cage and maintained on a 12-h light–dark cycle at 22°C. All animals had free access to food and water.

Herbs and chemicals

Ginkgo biloba extract (24% *Ginkgo* flavone glycosides and 6% terpene lactones) was obtained from Acta Pharmacal (Sunnyvale, CA). Identity of the *Ginkgo* sample was confirmed using high-performance liquid chromatography (HPLC) coupled to ultraviolet absorbance and electrospray mass spectrometric detection which confirmed the presence of rutin, hyperoside, ginkgolide A, ginkgolide B and other characteristic chemical components.

Chemical reagents were obtained from either Sigma-Aldrich Chemical Co. (Oakville, ON, Canada) or Fischer Chemical Co. (Edmonton, AB, Canada), except (−)-deprenyl, daidzein, MK-801 and *N*-(2-chloroethyl)- *N*-ethyl-2-bromobenzylamine (DSP-4) which were obtained from Research Biochemicals International (Natick, MA). Radiolabelled phenylethylamine, β-[ethyl-1-^{14}C] hydrochloride and hydroxytryptamine, and 5-[2-^{14}C] binoxylate were purchased from New England Nuclear (Boston MA). Solvents were HPLC grade.

Isolation and identification of MAO-inhibiting components of Ginkgo biloba

Chromatographic isolation of MAO-inhibiting constituents of *Ginkgo biloba* extract was accomplished by dissolving the extract in 20% acetonitrile and subjecting this solution to HPLC separation. *Ginkgo biloba* extract was separated on a Gilson HPLC system equipped with model 302 pumps and a model 811 dynamic mixer using a water–acetonitrile gradient. The linear gradient ran from 0 to 70% acetonitrile over 40 min. The column was washed with 70% acetonitrile for 5 min and then re-equilibriated to 0% acetonitrile over 5 min. The column used for separation was a Zorbax C_8 (25 cm × 4·1 mm) and eluting peaks were monitored by UV absorbance at 205 and 280 nm. Eluting materials were collected as 3-min fractions, lyophilized and evaluated for effects on MAO activity. The peak corresponding to the sample which provided the most potent MAO inhibition was re-chromatographed using an isocratic 20% acetonitrile separation to obtain two chromatographically pure compounds. These samples were

examined by high-resolution mass spectrometry to determine their identity.

Determination of effects on MAO activity

The compounds were evaluated in-vitro for effects on MAO activity using a modification of the radiochemical procedure of Lyles & Callingham (1982), employing radiolabelled 5-HT and β-phenylethylamine as substrates for MAO-A and MAO-B, respectively.

To evaluate ex-vivo effects of the compounds on MAO activity, the compounds of interest were administered either intraperitoneally (using 20% DMSO as the vehicle) or orally by gavage (using water as the vehicle). Animals were killed 1 or 3 h post-administration by decapitation and the brains and livers immediately removed and stored at -80°C until the time of analysis. Tissue samples from each rat were homogenized and appropriately diluted in buffer (brain MAO-A and MAO-B 1 : 160; liver MAO-A 1 : 160; liver MAO-B 1 : 800) before incubation. The radiochemical procedure utilized was the same as the one used for in-vitro analysis with the exception that no enzyme inhibitor was added. Incubations of samples from each rat were performed in triplicate. The MAO activity was expressed as percent of control (control samples contained no drug).

Amine concentrations

Concentrations of noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC with electrochemical detection using the method of Sloley & Goldberg (1991).

Determination of effects on NMDA-induced neurotoxicity

The ability of a compound to provide protection against NMDA-induced neurotoxicity was examined in-vitro using foetal rat neuronal cultures according to the method of Black et al (1995). Cells were exposed to NMDA (100 μM) with drug (1–100 μM) or vehicle solutions for 15 min. After drug exposure the cells were incubated for a further 24 h and cell viability was estimated.

Determination of effects on DSP-4-induced neurotoxicity

Neuroprotectant activity was evaluated by examining the prevention of DSP-4-induced noradrenaline depletion in mouse hippocampus as described by Yu et al (1994). Compounds (10 mg kg^{-1} in 20% DMSO) or vehicle (20% DMSO) were administered to mice intraperitoneally. Administration of (−)-deprenyl had previously been shown to significantly protect against DSP-4-induced noradrenaline depletion and was thus used as a positive control.

Determination of effects on lipid peroxidation

The ability of plant mixtures and pure chemicals to inhibit lipid peroxidation was assessed using catecholaminergic neuroblastoma SH-SY5Y cells (a gift from Dr Peter Yu, University of Saskatoon). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% PSN (penicillin, streptomycin neomycin) at 37°C until they reached confluence. Cells were then seeded at a density of 1×10^{6} cells per well in Nunclon 40-mm diameter polystyrene tissue culture dishes. Cells were grown for 24 h (subconfluence) and then treated with various concentrations of the compounds of interest either in the presence of or absence of 0·1 mM ferrous sulphate. After 48 h the cells were harvested, and lipid peroxides were measured according to the thiobarbituric acid assay of Ohkawa et al (1979). Protein content was determined using a modification of the method of Lowry et al (1951).

Results

Ginkgo biloba extract inhibited both MAO-A and MAO-B, with IC50 (50% inhibition concentration) values of 1×10^{-4} g mL^{-1} and 2×10^{-3} g mL^{-1}, respectively. Chromatographic separation of the *Ginkgo biloba* extract (Figure 1A) yielded several fractions that inhibited MAO (defined as greater than 50% inhibition using a fraction derived from 1 mg original *Ginkgo* extract). The most potent fraction was the 3-min collection region that included peak 8. This fraction was re-chromatographed and this yielded two major compounds, both of which inhibited MAO. Compound 1 inhibited MAO-A by 55% at a concentration estimated to be about 400 ng mL^{-1} whereas compound 2 inhibited MAO-A by 90% at a concentration estimated to be 3 μg mL^{-1}. These compounds were lyophylized and subsequently analysed by high-resolution mass spectrometry. By correlating with high-resolution mass spectra of authentic samples, compound 1 was identified as isorhamnetin, (M^{-}, 316, fragments at m/z 287, 153 and 108) and compound 2 was identified as kaempferol (M^{-}, 286, fragments at m/z 229, 258, 257 and 121).

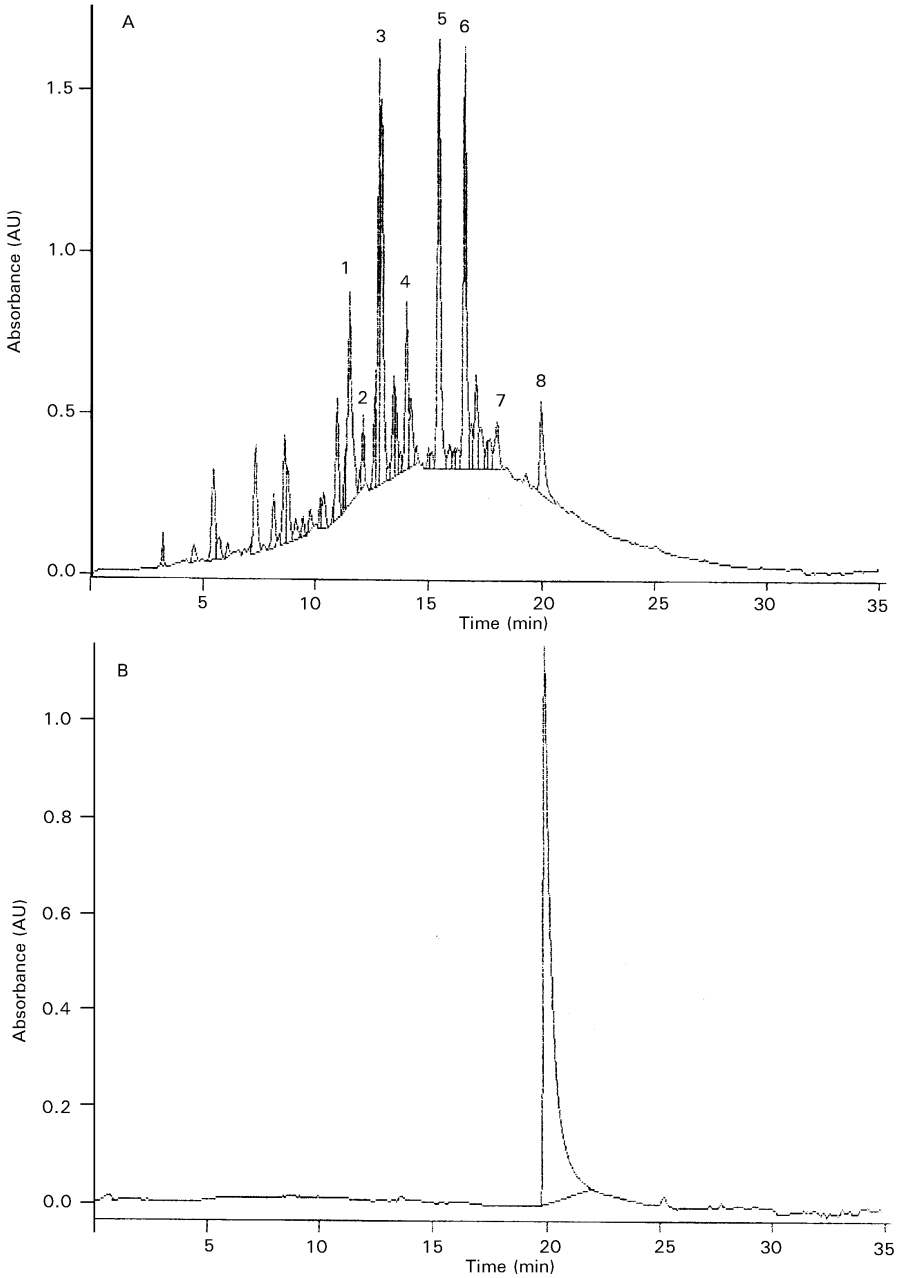

Figure 1. Typical chromatograms obtained from 1 mg *Ginkgo biloba* extract (A) and 25 μg pure kaempferol (B). The *Ginkgo biloba* sample was estimated to contain 0·60 ± 0·01% unconjugated kaempferol by weight. Other compounds were identified by electrospray mass spectrometry and comparison with compounds previously identified in similar extracts by Pietta et al (1991). Identified peaks include rutin (1), hyperoside (2), a mixture of kaempferol-3-*O*-rutinoside and astragalin (3), isorhamnetin-3-*O*-rutinoside (4), quercetin-3-*O*-rhamnosyl-(1 → 2)-rhamnosyl-(1 → 6)-glucoside (5), kaempferol-3-*O*-rhamnosyl-(1 → 2)-rhamnosyl-(1 → 6)-glucoside (6), quercetin (7) and kaempferol (8). Isorhamnetin eluted as a small peak just before kaempferol. The tailing on the pure kaempferol sample suggests the presence of a small amount of impurity in the commercially obtained reference material. The *Ginkgo biloba* sample and other reference compounds (apigenin measured at 280 nm and ginsenosides measured at 205 nm) showed no evidence of tailing when chromatographed in this system.

Kaempferol and isorhamnetin glycosides are well known constituents of *Ginkgo biloba* leaves (Pietta et al 1991) and their presence was confirmed in our extract by electrospray mass spectrometry (Figure 1). The aglycone kaempferol was found to exist in the extract in small amounts (0·60 ± 0·01% by weight) when analysed by HPLC using pure kaempferol as a reference standard (Figure 1B). The free kaempferol is believed to derive from hydrolysis of kaempferol glycosides or biflavones during extraction.

Pure kaempferol and a number of structurally related compounds inhibited MAO-A- and MAO-B-activity (Table 1). Kaempferol, apigenin and

chrysin were the most effective MAO-A inhibitors. The IC50 values for MAO-A inhibition by kaempferol, apigenin, chrysin and quercetin were 7×10^{-7}, 1×10^{-6}, 2×10^{-6} and 5×10^{-5} M, respectively. In these assays the IC50 for inhibition of MAO-A by phenelzine, which was used as a reference compound, was found to be 4×10^{-8} M.

Oral administration of kaempferol or *Ginkgo biloba* extracts did not inhibit rat brain or liver MAO activity in *ex-vivo* experiments (Table 2). Furthermore, the levels of 5-HT and 5-HIAA in rat striatum, hypothalamus and hippocampus remained unaltered following this treatment (Table 3). Intraperitoneal injection of kaempferol (50 mg kg^{-1}, 3 h) had no effect on brain MAO-A or MAO-B activity, whereas phenelzine (1·5 mg kg^{-1}, 3 h) inhibited MAO-A by 75% and MAO-B by 37% (data not shown).

Injection of a high dose (200 mg kg^{-1}, i.p., 3 h) of *Ginkgo biloba* extract into mice resulted in no change in the concentrations of noradrenaline, dopamine, 5-HT and 5-HIAA in the cortex, striatum and hypothalamus (Table 4). A slight increase in the DOPAC concentrations in the hypothalamus was determined (Table 4).

Kaempferol was evaluated for its ability to prevent NMDA-induced neuronal toxicity in rat neuronal cultures. It was able to prevent some neuronal toxicity at 100 μM in this model (Table 5). MK-801 a potent NMDA-receptor antagonist was able to completely block the NMDA-induced neuronal toxicity at a concentration of 10 μM.

Kaempferol (10 mg kg^{-1}, i.p.) was unable to prevent DSP-4-induced noradrenaline depletion in mouse hippocampus (Table 6). In contrast, (−)-deprenyl provided significant protection.

Ginkgo biloba extract and kaempferol (0·50–0·05 mg mL^{-1}) were able to significantly reduce lipid peroxidation as estimated by malondialdehyde production in cultures of SH-SY5Y cells which were incubated with 0·1 mM Fe^{2+} (Table 7). Furthermore, kaempferol alone (0·5 and 0·1 mg mL^{-1})

Table 1. In-vitro inhibition of rat-brain MAO-A and MAO-B by pure flavones, flavonols and related compounds.

Treatment	Inhibition of MAO-A (%) Pure compound				Inhibition of MAO-B (%) Pure compound			
	1×10^{-4} M	1×10^{-5} M	1×10^{-6} M	1×10^{-7} M	1×10^{-4} M	1×10^{-5} M	1×10^{-6} M	1×10^{-5} M
Kaempferol	96·7 ± 1·0	92·6 ± 0·5	58·2 ± 0·9	11·4 ± 1·7	49·3 ± 2·5	30·3 ± 2·4	8·3 ± 7·7	5·9 ± 5·5
Apigenin	93·6 ± 0·3	84·7 ± 1·2	55·9 ± 3·1	21·3 ± 2·1	75·6 ± 1·9	59·3 ± 1·9	30·3 ± 1·7	22·8 ± 6·0
Chrysin	88·1 ± 0·7	81·6 ± 1·7	33·4 ± 1·3	10·9 ± 6·9	26·0 ± 2·8	11·0 ± 4·9	4·8 ± 0·0	6·1 ± 2·7
Quercetin	62·3 ± 4·5	21·0 ± 4·5	10·2 ± 0·0	ND	15·1 ± 11·6	0	0	ND
Daidzein	60·4 ± 0·7	31·6 ± 1·6	11·2 ± 3·6	−2·6 ± 6·1	35·3 ± 3·9	21·2 ± 2·5	15·5 ± 1·6	−0·8 ± 1·8
Myricetin	49·6 ± 4·2	5·2 ± 0·0	ND	ND	24·2 ± 1·8	0	ND	ND
Naringenin	9·7 ± 4·0	1·9 ± 7·1	2·5 ± 5·3	−7·9 ± 3·5	11·1 ± 2·4	14·6 ± 5·5	4·7 ± 3·5	3·0 ± 5·4
Pelarogonidin chloride	64·7 ± 1·1	24·8 ± 2·5	18·0 ± 4·8	11·1 ± 1·8	39·6 ± 1·2	16·4 ± 1·6	14·6 ± 3·2	8·6 ± 2·0
Chromone	74·4 ± 1·7	25·3 ± 2·9	10·7 ± 3·0	−1·0 ± 1·0	63·5 ± 0·4	21·7 ± 1·2	18·9 ± 1·4	10·1 ± 0·7
Chromone-2-carboxylic acid	1·3 ± 3·9	8·5 ± 3·6	−4·3 ± 1·1	1·8 ± 0·5	82·4 ± 1·2	32·1 ± 1·0	16·9 ± 0·6	5·0 ± 3·0

Values are mean ± s.e.m.. ND, not determined, n = 3.

Table 2. Ex-vivo effects of oral administration of kaempferol or *Ginkgo biloba* leaf extract on rat brain and liver MAO-A and MAO-B activity.

Treatment	Tissue	Inhibition (%)	
		MAO-A	MAO-B
Control	Brain	0·0 ± 2·8	0·0 ± 5·3
Kaempferol (10 mg kg^{-1}, 1 h)	Brain	−11·1 ± 5·4	−6·7 ± 7·7
Kaempferol (10 mg kg^{-1}, 3 h)	Brain	9·6 ± 9·8	8·8 ± 6·7
Ginkgo extract (15 mg kg^{-1}, 3 h)	Brain	−13·7 ± 6·9	−14·1 ± 9·9
Control	Liver	0·0 ± 4·1	0·0 ± 3·4
Kaempferol (10 mg kg^{-1}, 1 h)	Liver	−10·2 ± 3·4	−0·2 ± 4·6
Kaempferol (10 mg kg^{-1}, 3 h)	Liver	2·0 ± 6·9	11·6 ± 6·5
Ginkgo extract (15 mg kg^{-1}, 3 h)	Liver	−16·2 ± 5·4	−17·2 ± 4·2

Values are mean ± s.e.m., n = 4. No significant inhibition of MAO was determined in any treatment group. MAO-A activity in control brains and liver was 3·1 and 47·4 nmol h^{-1} (mg tissue)$^{-1}$, respectively. MAO-B activity in control brains and liver was 10·0 and 60·3 nmol h^{-1} (mg tissue)$^{-1}$, respectively.

Table 3. Effects of oral administration of kaempferol or *Ginkgo biloba* leaf extract on concentrations of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the rat brain.

Treatment	Tissue	5-HT ($ng\,g^{-1}$)	5-HIAA ($ng\,g^{-1}$)
Control	Striatum	590 ± 64	636 ± 25
Kaempferol ($10\,mg\,kg^{-1}$, 1 h)	Striatum	558 ± 76	667 ± 71
Kaempferol ($10\,mg\,kg^{-1}$, 3 h)	Striatum	553 ± 31	618 ± 29
Ginkgo extract ($15\,mg\,kg^{-1}$, 3 h)	Striatum	629 ± 77	738 ± 63
Control	Hypothalamus	668 ± 82	545 ± 66
Kaempferol ($10\,mg\,kg^{-1}$, 1 h)	Hypothalamus	620 ± 41	570 ± 70
Kaempferol ($10\,mg\,kg^{-1}$, 3 h)	Hypothalamus	678 ± 32	556 ± 50
Ginkgo extract ($15\,mg\,kg^{-1}$, 3 h)	Hypothalamus	578 ± 13	556 ± 64
Control	Hippocampus	376 ± 36	372 ± 33
Kaempferol ($10\,mg\,kg^{-1}$, 1 h)	Hippocampus	360 ± 41	399 ± 31
Kaempferol ($10\,mg\,kg^{-1}$, 3 h)	Hippocampus	382 ± 41	364 ± 19
Ginkgo extract ($15\,mg\,kg^{-1}$, 3 h)	Hippocampus	405 ± 35	437 ± 19

Values are means ± s.e.m., n = 4.

Table 4. Effect of single administrations of *Ginkgo biloba* leaf extract ($200\,mg\,kg^{-1}$, i.p., 3 h) on concentrations of noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the mouse brain.

Treatment	Tissue	NA ($ng\,g^{-1}$)	DA ($ng\,g^{-1}$)	DOPAC ($ng\,g^{-1}$)	5-HT ($ng\,g^{-1}$)	5-HIAA ($ng\,g^{-1}$)
Control	Hypothalamus	1422 ± 43	857 ± 74	111 ± 7	992 ± 39	374 ± 14
Ginkgo extract	Hypothalamus	1326 ± 58	870 ± 93	141 ± 13*	1066 ± 30	417 ± 19
Control	Striatum	128 ± 21	8801 ± 494	703 ± 56	485 ± 18	377 ± 30
Ginkgo extract	Striatum	119 ± 19	8480 ± 426	666 ± 33	527 ± 29	462 ± 21
Control	Cortex	257 ± 11	1031 ± 195	88 ± 22	469 ± 49	193 ± 4
Ginkgo extract	Cortex	293 ± 15	888 ± 162	122 ± 19	516 ± 35	215 ± 13

Values are means ± s.e.m., n = 8. *$P < 0.05$ compared with control.

Table 5. Neuroprotective effect of kaempferol on NMDA-induced neuronal toxicity in rat neuronal cultures.

Treatment	Viability (% of control)	n
NMDA (100 μM)	17·7 ± 2·9	3
MK-801 (10 μM)	102·3 ± 3·5***	4
Kaempferol (1 μM)	27·1 ± 12·4	5
Kaempferol (10 μM)	26·6 ± 3·5	5
Kaempferol (100 μM)	62·8 ± 15·3**	5

Values are means ± s.e.m. **$P < 0.01$, ***$P < 0.001$ compared with NMDA group.

Table 6. Effect of (−)-deprenyl ($10\,mg\,kg^{-1}$, i.p.) and kaempferol ($10\,mg\,kg^{-1}$, i.p.) on DSP-4-induced noradrenaline depletion in mouse hippocampus.

Treatment	Noradrenaline ($ng\,g^{-1}$)
Control (20% DMSO/saline)	355 ± 18
Deprenyl/DSP-4	294 ± 15**
Kaempferol/DSP-4	136 ± 11*
20% DMSO/DSP-4	164 ± 23*

Values are means ± s.e.m., n = 5. *$P < 0.05$ compared with control. **$P < 0.05$ compared with 20% DMSO/DSP-4. Mice received vehicle, (−)-deprenyl or kaempferol intraperitoneally. After 1 h they received intraperitoneal injections of DSP-4. One week later the mice were killed, the hippocampus was removed and the noradrenaline concentration was measured.

was able to reduce lipid peroxidation in cell cultures which were not exposed to Fe^{2+} (Table 7).

Discussion

This study has shown that *Ginkgo biloba* contains compounds that possess in-vitro MAO-inhibiting properties. Among these compounds are kaempferol and isorhamnetin. Unconjugated kaempferol and isorhamnetin make up only a small amount of the *Ginkgo biloba* extract (0·6% by weight for kaempferol, much less for isorhamnetin). These aglycones are thought to arise during processing by hydrolysis of the flavonol glycosides known to exist in extracts of *Ginkgo biloba* leaves (Pietta et al 1991).

Inhibition of MAO by these related flavone compounds appears to be dependent on the presence of a phenyl or hydroxyphenyl ring in the 2 position and a double bond in the 2,3 position of the flavonoid nucleus. Moving the hydroxyphenyl ring from the 2 to the 3 position (as in daidzein) greatly

Table 7. Effect of *Ginkgo biloba* extract and kaempferol on lipid peroxidation of SH-SY5Y cells.

Treatment	Malondialdehyde (nmol (mg protein)$^{-1}$)	
	With Fe^{2+}	Without Fe^{2+}
Control	2·00 ± 0·21	0·72 ± 0·01
Ginkgo biloba extract (0·5 mg mL^{-1})	0·54 ± 0·13*	0·50 ± 0·06
Ginkgo biloba extract (0·1 mg mL^{-1})	0·70 ± 0·10*	0·40 ± 0·07
Ginkgo biloba extract (0·05 mg mL^{-1})	0·58 ± 0·04*	0·33 ± 0·01
Kaempferol (0·5 mg mL^{-1})	0·17 ± 0·01*	0·11 ± 0·02†
Kaempferol (0·1 mg mL^{-1})	0·40 ± 0·02*	0·15 ± 0·03†
Kaempferol (0·05 mg mL^{-1})	0·79 ± 0·02*	0·32 ± 0·02

Values are means ± s.e.m., n = 3. *$P < 0·05$ compared control with Fe^{2+}. $^{\dagger}P < 0·05$ compared with control without Fe^{2+}.

reduces activity. The absence of a double bond in the 2,3 position (as in naringenin) also reduces activity. The presence of a single hydroxyl group in the para position of the 2-phenyl group also appears to be important for MAO-inhibiting activity. Its absence in chrysin or the presence of more than one hydroxyl group in the 2-phenyl ring, as in quercetin and myricetin, results in decreased MAO-inhibiting activity.

The presence of MAOIs in plant extracts suggests that such preparations could be useful in the treatment of depression. Indeed, *Ginkgo biloba* extract has been reported to affect certain aspects of rat behaviour in a manner indicative of antidepressant activity (Porsolt et al 1990; Chermat et al 1997; Satyan et al 1998). Despite the fact that *Ginkgo biloba* extract contains small amounts of free flavones as well as substantially greater amounts of their sugar conjugates, our ex-vivo studies provide no evidence that these materials can inhibit MAO in the brain or liver. They also fail to alter levels of monoamine neurotransmitters or their metabolites in rat brain in a manner consistent with MAO inhibition. This observation is inconsistent with the findings of Petkov et al (1993) who reported elevated concentrations of 5-HT and Satyan et al (1997) who reported decreased levels of 5-HT in rodent brains following administration of *Ginkgo biloba* extracts. Such discrepancies may be due to different treatment regimes or extract qualities. The observed elevation in DOPAC in the mouse hypothalamus resulting from a high dose of *Ginkgo biloba* extract may reflect the fact that DOPAC is a major metabolite derived from the degradation of certain flavones found in *Ginkgo biloba* (Pietta et al 1995).

Previous reports of MAO inhibition by *Ginkgo biloba* extracts (White et al 1996) suggest that the MAO inhibition is reversible. The ex-vivo assay employed in the present study to determine MAO activity, while excellent for determining irreversible inhibition of MAO, may not reliably detect reversible MAO inhibition. This is due to dissociation of the enzyme-inhibitor complex when the tissue homogenate is diluted (in ex-vivo studies tissue homogenates are diluted from 160 to 800 fold). However, the absence of any effect on 5-HIAA or 5-HT concentrations in the brains of animals receiving *Ginkgo biloba* extract or kaempferol would appear to support the fact that, at the dose and time used here, these materials have little effect on brain MAO in-vivo.

Ginkgo biloba extract has been shown to protect against certain types of experimentally-induced neurotoxicity (Ramassamy et al 1990; Smith et al 1996). In this study it was determined that kaempferol had neuroprotective effects in an in-vitro NMDA-mediated neurotoxicity model, but not in the DSP-4-induced noradrenergic neurotoxicity model. These two models are believed to act through different mechanisms and it is not surprising that a particular compound might act as a neuroprotective agent in one model and not in the other.

Although the neuroprotective effects of kaempferol appear modest, it must be remembered that, in man, it is estimated that the daily consumption of flavonoids in a balanced diet containing vegetables, fruits, fruit juices and teas is of the order of 1 g. Even modest neuroprotectants might prove effective at this dose.

This work also demonstrates that *Ginkgo biloba* extract and kaempferol are potent antioxidants. This property may contribute to the demonstrated neuroprotective effects by reducing oxidative stress in damaged cells through either free-radical scavenging or iron chelation, but the evidence seems to suggest that aspects of both mechanisms are involved.

In summary, *Ginkgo biloba* extract possesses a number of characteristics which could conceivably contribute to previously reported antidepressant

and neuroprotective properties. It can inhibit both MAO-A and MAO-B in-vitro due to the presence of flavonoids such as kaempferol. Furthermore, kaempferol can protect against NMDA-induced neurotoxicity in-vitro and, as this and other work indicates, flavonoid components found in *Ginkgo biloba* extract protect against lipid peroxidation. While each individual protective aspect of *Ginkgo biloba* extract may not be sufficient to provide substantial protection against oxidative processes, the combination of a number of independent mechanisms may be beneficial.

Acknowledgements

The authors would like to thank Richard Strel and Dongjaing Song for technical assistance.

References

Black, M. A, Tremblay, R., Mealing, G., Ray, R., Durkin, J. P., Whitfield, J. F., Blosser, J., Morley, P. (1995) N-methyl-D-aspartate- or glutamate-mediated toxicity in cultured rat cortical neurons is antagonized by FPL 15896AR. J. Neurochem. 65: 2170–2177

Chen, Z. Y., Chan, P. T., Ho, K. Y., Fung, P., Wang, J. (1996) Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. Chem. Phys. Lipids 79: 157–163

Chermat, R., Brochet, D., DeFeudis, F. V., Drieu, K. (1997) Interactions of *Ginkgo biloba* extract (EGB 761), diazepam and ethyl beta-carboline-3-carboxylate on social behaviour of the rat. Pharmacol. Biochem. Behav. 56: 333–339

Ferriola, P. C., Cody, V., Middleton, E. (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem. Pharmacol. 38: 1617–1624

Foldes-Filep, E., Filep, J. (1988) BN 52021 (Ginkgolide B) inhibits the binding of tritiated platelet-activating factor to human neutrophil granulocytes. In: Braquet, P. (ed.) New Trends in Lipid Mediators Research. Vol. 1. Platelet-activating Factor and Cell Immunology. S. Karger, Basel, Switzerland, pp 113–120

Gabor, M. (1988) Szent-Gyorgyi and the bioflavonoids: new results and perspectives of pharmacological research into benzo-pyrone derivatives. In: Cody, V., Middleton, E., Harborne, J. B., Beretz, A. (eds) Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties. Alan R. Liss Inc., New York, pp 1–15

Galvez, J., de la Cruz, J. P., Zarzuelo, A., Sancha de la Cuesta, F. (1995) Flavonoid inhibition of enzymic and nonenzymic lipid peroxidation in rat liver differs from its influence on the glutathione-related enzymes. Pharmacology 51: 127–133

Kerry, N., Rice-Evans, C. (1999) Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationships. J. Neurochem. 73: 247–253

Kose, K., Dogan, P. (1995) Lipoperoxidation induced by hydrogen peroxide in human erythrocyte membranes. 2. Comparison of the antioxidant effect of *Ginkgo biloba* extract (EGb 761) with those of water-soluble and lipid-soluble antioxidants. J. Int. Med. Res. 23: 9–18

Le Bars, P. L., Katz, M. M., Berman, N., Itil, T. M., Freedman, A. M. Schatzberg, A. F. (1997) A placebo-controlled, double-blind, randomized trial of an extract of *Ginkgo biloba* for dementia. J. Am. Med. Assoc. 278: 1327–1332

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Lyles, G. A., Callingham, B. A. (1982) In-vitro and in-vivo inhibition by benserazide of clorgyline-resistant amine oxidases in rat cardiovascular tissues. Biochem. Pharmacol. 31: 1417–1424

Marcocci, L., Packer, L., Droy-Lefaix, M. T., Sekaki, A., Gardes-Albert, M. (1994) Antioxidant action of *Ginkgo biloba* extract EGb 761. Meth. Enzymol. 234: 462–475

Maurer, K., Ihl, R., Dierks, T., Frolich, L. (1997) Clinical efficacy of *Ginkgo biloba* extract Egb 761 in dementia of the Alzheimer type. J. Psychiat. Res. 31: 645–655

Medina, J. H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A. C. (1997) Overview – flavonoids: a new family of benzodiazepine receptor ligands. Neurochem. Res. 22: 419–425

Ohkawa, H., Ohishi, N., Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351–358

Petkov, V. D., Hadjiivanova, C., Petkov, V. V., Milanov, S., Visheva, N., Boyadjieva, N. (1993) Effects of standardized extracts GK501, from *Ginkgo biloba* L., G115 from *Panax ginseng* C. A. Meyer, and their combination, Gincosan(TM) (PHL-00701), on the levels of biogenic monoamines and on the serum content of prolactin, growth hormone and ACTH. Phytother. Res. 7: 139–145

Pietta, P., Mauri, P., Bruno, A., Rava, A., Manera, E., Ceva, P. (1991) Identification of flavonoids from *Ginkgo biloba* L., *Anthemis nobilis* L. and *Equisetum arvense* L. by high-performance liquid chromatography with diode-array UV detection. J. Chromatogr. 553: 223–231

Pietta, P. G., Gardana, C., Mauri, P. L., Maffei-Facino, R., Carini, M. (1995) Identification of flavonoid metabolites after oral administration to rats of a *Ginkgo biloba* extract. J. Chromatogr. B 673: 75–80

Porsolt, R. D., Martin, P., Lenegre, A., Fromage, S., Drieu, K. (1990) Effects of an extract of *Ginkgo biloba* (EGb 761) on "learned helplessness" and other models of stress in rodents. Biochem. Behav. 36: 963–971

Ramassamy, C., Clostre, F., Christen, Y., Costentin, J. (1990) Prevention by a *Ginkgo biloba* extract (GBE 761) of the dopaminergic neurotoxicity of MPTP. J. Pharm. Pharmacol. 42: 785–789

Rapin, J. R., Lamproglan, I., Drien, K., Defeudir, F. U. (1994) Demonitration of the "anti-stress" activity of an extract of *Ginkgo biloba* (Egb 761) using a discrimination learning task. Gen. Pharmac. 25: 1009–1016

Satyan, K. S., Jaiswal, A. K., Ghosal, S., Battacharya, S. K. (1997) Effect of ginkgolic acid conjugates on the brain monoamines and metabolites in rodents. Biogenic Amines 13: 143–151

Satyan, K. S., Jaiswal, A. K., Ghosal, S., Battacharya, S. K. (1998) Anxiolitic activity of ginkgolic acid conjugates from Indian *Ginkgo biloba*. Psychopharmacology 136: 148–152

Sloley, B. D., Goldberg, J. I. (1991) Determination of γ-glutamyl conjugates of monoamines by means of high-performance liquid chromatography with electrochemical detection and application to gastropod tissues. J. Chromatogr. 567: 49–56

Smith, P. F., Maclennan, K., Darlington, C. L. (1996) The neuroprotective effect of the *Ginkgo biloba* leaf: a review of

the possible relationship to platelet-activating factor (PAF). J. Ethnopharmacol. 50: 131–139

Sparenberg, B., Demisch, L., Hoelzl, J. (1993) Untersuchungen uber antidepressive Wirkstoffe von Johanniskraut. Pharm. Ztg. Wiss. 6/138: 50–54

Thull, U., Testa, B. (1994) Screening of unsubstituted cyclic compounds as inhibitors of monoamine oxidases. Biochem. Pharmacol. 47: 2307–2310

Weinges, K., Bahr, W., Kloss, P. (1968a) Natural phenolic compounds XI. Review of the components of *Ginkgo biloba* leaves. Arzneim. Forsch. 18: 537–539

Weinges, K., Bahr, W., Kloss, P. (1968b) Natural phenolic compounds X. Phenolic compounds of *Ginkgo biloba* leaves. Arzneim. Forsch. 18: 539–543

White, H. L., Scates, P. W., Cooper, B. R. (1996) Extracts of *Ginkgo biloba* leaves inhibit monoamine oxidase. Life Sci. 58: 1315–1321

Yu, P. H., Davis, B. A., Fang, J., Boulton, A. A. (1994) Neuroprotective effect of some monoamine oxidase-B inhibitors against DSP-4-induced noradrenaline depletion in the mouse hippocampus. J. Neurochem. 63: 1820–1828