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"*Mangifera indica* L. Extract (QF808) Reduces Ischaemia-induced Neuronal Loss and Oxidative Damage in the Gerbil Brain"

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The effect of oral administration of *Mangifera indica* L. extract (QF808) on ischemia-reperfusion-induced neuronal death in the gerbil hippocampal CA1 sector was examined. Oral administration of QF808 for 7 days dose-dependently protected against neuronal cell death following transient ischaemia and reperfusion as assessed by histopathology. In addition, locomotor activity assessment prior to ischaemia and 7 days after correlated well with the histological results. To evaluate redox alterations by reactive oxygen species, total sulfhydryl, non-protein sulfhydryl groups (NPSH), malondialdehyde+4-hydroxyalkenals and total nitrogen oxide levels were assayed in hippocampus and cortex homogenates. QF808 treatment attenuated NPSH loss, nitrogen oxide levels and lipid peroxidation in the hippocampus. These results suggest that orally administered QF808 is absorbed across the blood–brain barrier and attenuates neuronal death of the hippocampal CA1 area after ischaemia-reperfusion. These protective effects are most likely due to the antioxidant activity of QF808.

Keywords: Antioxidant activity; Gerbil; Ischaemia-reperfusion; *Mangifera indica* L.; Neuroprotection

Abbreviations: 4-HAD, 4-hydroxyalkenals; CNS, central nervous system; I/R, ischaemia/reperfusion; iNOS, immunological nitric oxide synthase; LP, lipid peroxidation; MDA, malondialdehyde; NFκB, nuclear factor κB; NMDA, N-methyl-D-aspartic acid; NO, nitric oxide; NOS, nitric oxide synthase; NPSH, non-protein sulfhydryl group; $OH^{\bullet}$, hydroxyl radical; ROS, reactive oxygen species; TBI, traumatic brain injury; TSH, total sulfhydryl group

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INTRODUCTION

During recent years the molecular mechanisms and potential treatment of acute and chronic neurological disorders have become research areas of paramount importance.[1] Worldwide, stroke remains the third most common cause of death after cardiac disease and cancer. Stroke is also the leading cause of chronic disability in the world.[2] Reactive oxygen species (ROS) are implicated as playing an important role in the pathophysiology of many central nervous system (CNS) disorders.[3] Neuronal cell death in hippocampal CA1 sector is known to be induced by transient ischaemia,[4] and the onset of this cell death is associated with the generation of ROS following reperfusion of ischaemic cerebrum. It has been demonstrated that ROS are produced by a xanthine oxidase-dependent mechanism.[5] Excessive amounts of ROS are also known to be generated in mitochondria respiratory chain by over-stimulation of N-methyl-D-aspartic acid (NMDA) subtype of glutamate receptors following cerebral ischaemia reperfusion.[6]

Elucidation of the role of oxidative injury is important because therapy with agents that scavenge ROS and augment endogenous antioxidant capacity may prove useful in the therapeutic modulation of these devastating neurological conditions.[7] It is postulated that because of the synergism between excitotoxicity and pro-oxidant events, therapeutic strategies aimed at decreasing brain injury based on combined mechanisms of action (e.g., glutamate antagonists and antioxidants) should prove more effective than monotherapy.[8] In order to attenuate the cascade of events mediated by ROS, several therapeutic approaches have been attempted, including chelating agents, low molecular weight antioxidants, spin-traps or superoxide dismutase and catalase. However, the efficacy of such strategies is limited, particularly when the protective agent does not cross the blood–brain barrier or does not arrive at intracellular compartments at effective concentrations.[9]

QF808 is an extract obtained from the stem bark of selected varieties of *Mangifera indica* L. It has a defined mixture of components (polyphenols, terpenoids, steroids, fatty acids and microelements).[10] QF808 has shown a powerful scavenger activity of hydroxyl radicals and hypochlorous acid, presented a significant inhibitory effect on the peroxidation of rat brain phospholipids and inhibited DNA damage induced by Fe/bleomycin or copper-phenanthroline systems.[11] Some of the main components of QF808 (mangiferin 20%, amentoflavone 15%, beta-sitosterol 2.5%, daucosterol 1.7%) have shown antioxidant properties in *in vitro* models and central pharmacological actions.[12–17] The presence of selenium as an organic compound in QF808 (0.05%) is also important in the antioxidant mechanism of this extract.[10] QF808 has been also tested in a broad set of toxicological tests with satisfactory results, in terms of acute and subchronic toxicity, genotoxicity and irritability demonstrating that it can be classified as a non-toxic product.[10] The objective of the present study was to evaluate the protective effects of QF808 on post-ischaemic CNS injury using global ischaemia in gerbils.

MATERIALS AND METHODS

Drug

Stem bark extract of *Mangifera indica* L. was prepared by decoction with water for 1 h. The extract was concentrated by evaporation and spray dried to obtain a fine brown powder (coded as QF808), the active ingredient of Vimang formulations, which melts at 210–215°C with decomposition. The chemical composition of this extract has been characterised.[10] The solid extract was dissolved in distilled water for pharmacological studies.

Animals

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 50–70 g, obtained from CEN-PALAB (Bejucal, La Habana, Cuba) were used. The gerbils were housed in groups of five, maintained on a 12/12 light/dark cycle, and allowed free access to food and water before and after surgical intervention. All procedures were performed as approved by the Institutional Animal Care Committees and in accordance with the European Union Guidelines for animal experimentation.

Experimental Design

The experiment consisted of 120 gerbils randomised into sham-operated animals, occluded animals that received 0.9% saline, occluded animals that received 250, 110 or 50 mg/kg b.w. of QF808 daily beginning 7 days prior to occlusion or animals treated for 7 days with QF808 (250 mg/kg b.w.) but without occlusion. Each group was of 20 animals, 10 were used for histological studies, 7 days after occlusion, and 10 for biochemical analysis 24 h after the ischaemic-reperfusion procedure.

Surgical Procedure

Surgery to induce transient global cerebral ischaemia and histological examination were performed under pentobarbital anaesthesia (50 mg/kg, i.p.).[4] Briefly, bilateral common carotid arteries were occluded with atraumatic miniature bulldog clamps for 5 min under anaesthesia. Cessation of circulation and beginning of recirculation were visually confirmed and then the skin lesion was sutured. Sham-operated animals were only anaesthetized, and their carotid arteries exposed. Body temperature during vascular occlusion was maintained close to 37.2°C with an electric heating blanket, monitored with a rectal thermometer over post-operative 2.5 h, and recorded during 8 hours after reperfusion to evaluate the degree of post-ischaemic hyperthermia. After the gerbils recovered from anaesthesia, the animals were allowed free access to water and food as in the normal condition. After 24 h or 7 days the animals were anaesthetized and perfused transcardially with 0.9% saline at 4°C for 10 min (all animals) and then for 15 min with fixative, 4% formaldehyde in 0.1 M phosphate buffer pH 7.3 at 4°C (animals for histological studies).

Tissue Processing

Brains for biochemical studies were removed promptly and dissected into different regions (cortex and hippocampus) according to the method described by Glowinsky and Iversen.[18] The homogenisation procedure was performed according to Hall *et al.*[19] Briefly, each region was placed in a 1.5 mL microcentrifuge tube containing 4 mm glass balls and 2 mL of KCl/histidine buffer (pH 7.3). Tissue was minced and vortexed for 2 min. The homogenates were centrifuged for 5 min at 12,000 rpm at 4°C. The protein content of the homogenates was determined by a standard Coomassie Blue method.[20]

Brains for histological studies were removed from the skull and post-fixed in formaldehyde 10%. Each tissue block was embedded in paraffin, and 8 μm-thick cross-sections containing the dorsal hippocampus were cut and stained with Cresyl violet (0.5%). Sections from each brain were scored by light microscopy for damage to pyramidal cells in the CA1 area of the hippocampus, with ratings being made bilaterally. The histopathological scoring system was based on the method used and illustrated by Bartus *et al.*[21] with: 0=normally stained cells, densely packed with rounded soma and a stained central nuclei; 1 = some shrinkage and irregularly shaped cells, with a pale chromatolytic region surrounded by a deeply stained peripheral rim of cytoplasm; 2 = some apparent cell loss, along with patches of pyknotic cell;

3 = more moderate cell loss and pyknosis; 4=lack of Nilssl staining indicating marked depletion of neurons, aside from an occasional neuron found among numerous microglia and macrophages.

Locomotor Activity

Locomotor activity was measured on an automated activity meter (Model 7401, Ugo Basile, Italy) for 30 min after a 10 min period of acclimation. Activity was quantified as the number of movements per animal per minute and it was done to each group before the ischaemic reperfusion procedures and 7 days after.

Biochemical Determinations

The biochemical parameters were determined by spectrophotometric methods using an Ultrospect III Plus Spectrophotometer from Pharmacia LKB (Sweden). Total (TSH) and non-protein (NPSH) sulfhydryl group determinations were performed according to the method of Sedlak and Lindsay (1968)[22] with Ellman's reagent. Malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA) were assayed as a marker of lipid peroxidation (LP) (Bioxytech LPO-586 kit, Oxis International Portland, OR, USA) using a colorimetric reaction which utilize 1-methyl-2-phenylindol as the chromogenic reagent.[23] Nitrite and nitrate content concentrations were determined by first converting cell nitrate to nitrite using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). After enzymatic reduction, samples were mixed with equal amounts of Griess reagent (sulphanilamide 1%, naphthylethyenediamide 0.1% in phosphoric acid 0.25%).[24] Samples were incubated at room temperature for 10 min and absorbance was measured at 540 nm using a microplate reader. Chemicals and reagents were purchased from Sigma Chemical Co (St Louis, MO, USA).

Statistical Analysis

One way ANOVA was used followed by homogeneity variance test (Bartlett–Box). In addition, a multiple comparison test was used (Duncan test). In the case of neuroprotective effect of different QF808 doses it was evaluated by Kruskall–Wallis test followed by the Mann–Whitney U test. Values are expressed by the mean±standard error of mean (n=10 per group). Different letters indicates a statistical significance of at least $p<0.05$.

RESULTS

Figure 1 presents photographs of sections containing the hippocampal CA1 pyramidal cells layer obtained 7 days following reperfusion of the ischaemic brain, various treatment groups and sham-operated control. Ischaemia and reperfusion in the absence of QF808 administration resulted in the disappearance of the pyramidal cells of the CA1 region. Treatment for 7 days prior to ischaemia with QF808 at 250, 110, 50 mg/kg b.w. resulted in a dose-dependent inhibition of damage score of 86, 78, 48%, respectively, compared with I/R group.

Locomotor activity was similar in all the experimental groups before I/R (Fig. 2). Reperfusion of the ischaemic brain resulted in characteristically elevated levels of motor activity with respect to pre-ischaemic basal levels. This hyperactivity was attenuated by administration of 110 or 250 mg/kg but not 50 mg/kg b.w. of QF808 (Fig. 2).

Table II shows the control and post-ischaemic alterations in different tissue metabolites. TSH were found to be unchanged during I/R. On the other hand, we observed significant decreases ($p<0.01$) in NPSH in the hippocampus and cortex of I/R group. The loss of NPSH-induced by I/R was dose-dependently inhibited by oral administration of QF808. Moreover, QF808, at all doses, attenuated the increase in MDA+4-HDA

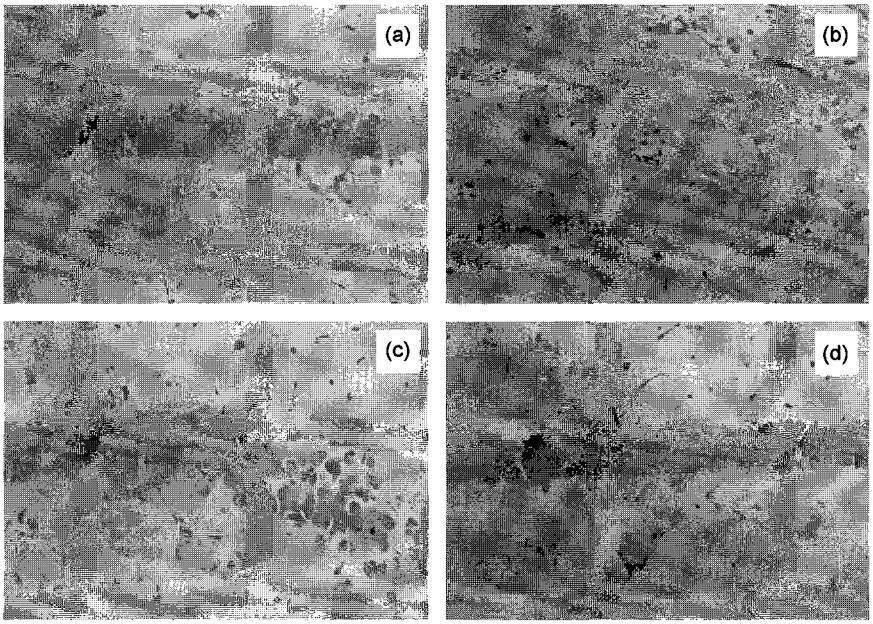


FIGURE 1 Representative photographs of pyramidal cell layer of the hippocampal CA1 region 7 days after 5 min ischaemic of the forebrain in gerbils. (A) Sham-operation (control); (B) I/R only; (C) and (D) I/R with 7 days pre-treatment of QF808 250 or 50 mg/kg b.w. respectively. Each section was stained with Cresyl violet. Magnification (A–D) ×250 (see colour plate at the rear of this issue).

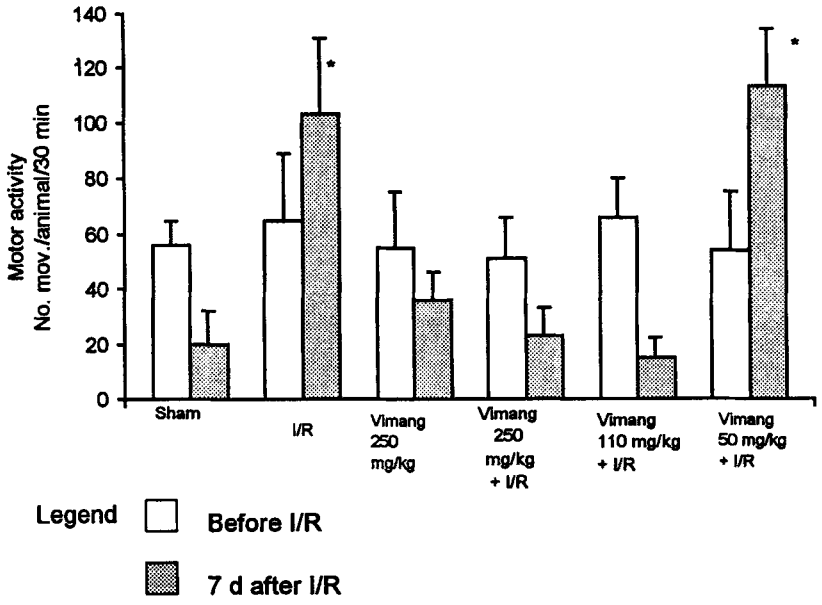
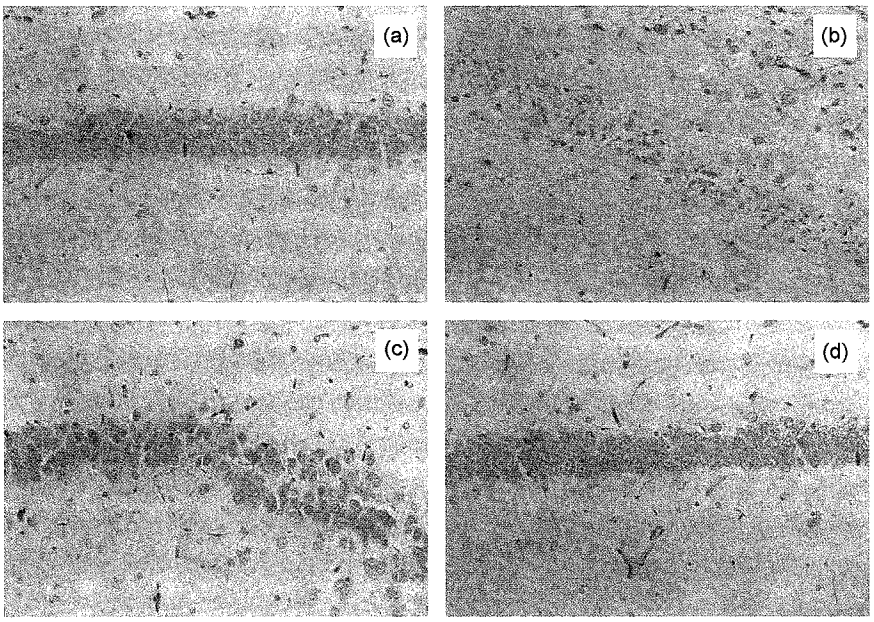


FIGURE 2 Motor activity in the experimental groups. Before (white bar) and 7 days after (black bar) ischaemia reperfusion (I/R). Animals treated with QF808 received the correspondent doses 7 days before I/R. I/R procedure consists of bilateral common carotid arteries occlusion for 5 min followed by 7 days of reperfusion. Bar heights represent mean±S.D. ($n=9$). $p<0.05$, compared with sham within the same series.



Colour Plate (See Figure 1, page 469, G. Martínez Sánchez *et al.*)
Representative photographs of pyramidal cell layer of the hippocampal CA1 region 7 days after 5 min ischaemic of the forebrain in gerbils. (A) Sham-operation (control); (B) I/R only; (C) and (D) I/R with 7 days pre-treatment of QF808 250 or 50 mg/kg b.w. respectively. Each section was stained with Cresyl violet. Magnification (A–D) ×250.

TABLE I Protective effect of QF808 on delayed neuronal death of pyramidal cells in hippocampal CA1 of gerbils

	Damage score			% of damage inhibition[a]
	Min.	Max.	Mean	
Sham operation (control)	0	1	0.20[b]	—
QF808 250 mg/kg b.w.	0	1	0.06[b]	—
I/R	1	4	2.00[c,b]	—
QF808 250 mg/kg b.w.	0	1	0.28[c,b]	86
QF808 110 mg/kg b.w.	0	2	0.44[c,b]	78
QF808 50 mg/kg b.w.	0	2	1.04[c,b]	48

The histopathological score system was based on the method of Bartus *et al.* (1998)[21] with 0=normally stained cells, densely packed with rounded soma and a stained central nuclei to 4=lack of Nilssl staining indicating marked depletion of neurons, aside from an occasional neuron found among numerous microglia and macrophages. In each group n=10.
[a] Compared with I/R group.
[b] Significant differences ($p<0.05$) from the I/R group.
[c] Significant differences ($p<0.05$) from the sham group.

TABLE II Changes in oxidative markers of biomolecules oxidation and total nitrites levels

	TSH (μmol/mg protein)		NPSH (μmol/g tissue)		MDA+4-HDA (μmol/mg protein)		Total nitrites (nmol/mg protein)
	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Sham	77.7±3.7	95.8±98	0.40±0.04[a]	0.41±0.02[a]	9.2±1.9	12.1±2.3	16.4±2.8[a]
I/R	74.3±6.2	79.7±7.6	0.21±0.02[b]	0.23±0.03[b]	11.9±1.7[c]	11.6±1.6	29.9±5.3[b]
QF808 250 mg/kg	79.5±5.2	90.5±6.2	0.39±0.04[a]	0.45±0.02[a]	10.3±0.9	12.5±2.2	12.6±3.8[a]
QF808 250 mg/kg+I/R	79.6±4.4	91.3±10.1	0.43±0.05[a]	0.53±0.08[a]	10.0±1.2	12.5±2.8	16.2±5.4[d]
QF808 110 mg/kg+I/R	80.1±6.5	90.7±8.1	0.32±0.04[c,a]	0.37±0.06[c,a]	9.5±1.4	12.5±2.3	NT
QF808 50 mg/kg+I/R	85.5±8.7	89.1±9.3	0.25±0.03[b]	0.31±0.03[c,d]	9.2±1.1	12.7±1.8	39.3±4.8[b]

TSH, Total sulfhydryl; NPSH, Non-protein sulfhydryl; MDA+4-HAD, Malondialdehyde+4-hydroxyalkenal. NT, non-tested.
[a] Significant differences ($p<0.01$) from the I/R within the same set.
[b] Significant differences ($p<0.01$) from the sham within the same set.
[c] Significant differences ($p<0.05$) from the sham within the same set.
[d] Significant differences ($p<0.05$) from the I/R within the same set.

observed in the positive control group. Nitrite and nitrate concentrations were found to be significantly increased ($p<0.01$) 24 h after ischaemia and this increase was inhibited by QF808 at a dose of 250 mg/kg b.w.

DISCUSSION

A short period of transient cerebral ischaemia produces selective pyramidal cell damage within days in the hippocampal CA1 regions in Mongolian gerbil.[25] The mechanisms for this neuronal death have not been definitely determined. However, in the early stage after ischaemia, it is believed that release of glutamate occurs in the hippocampus after transient ischaemia,[26] with the resulting stimulation of NMDA receptors in CA1 inducing Ca^{2+} influx,[27] activation of Ca^{2+}-dependent protease,[28] phospholipase C,[29] protein kinase C[27] and Ca^{2+}-dependent superoxide production by the mitochondria.[30] In the present study, we have shown that the I/R-induced cell death in the CA1 region was dose-dependently inhibited by oral administration of QF808 (Fig. 1 and Table I).

Ischaemic damage to the gerbil brain produces large increases in locomotor activity, which are correlated with the degree of neuronal degeneration in the CA1 region of the hippocampus.[31] The increase in activity

may result from a failure of the cognitive functions of the hippocampus with a reduction in the animals ability to form spatial maps, rather than being attributable to a simple form of motor hyperactivity.[32] A return to normal activity in the QF808-treated groups (110 or 250 mg/kg) correlated well with the histopathological evidence of protection against I/R.

Hypothermia has proven to be beneficial in several models of traumatic brain injury (TBI). The hypothermic neuroprotection mechanism are due to its ability to decrease the extracellular levels of glutamate and other excitatory amino acids, attenuates the generation of hydroxyl radical ($OH^{\bullet}$) and inhibit the NO synthesis after TBI.[33] According to our finding no hypothermic effect was detected in any experimental group, since the neuroprotection observed for Vimang group was not associated with an hypothermic effect.

The oxidation of proteins by ROS may be responsible for damaging enzymes critical to neuronal function.[34] Sulfhydryl oxidation is one of the earliest observable events during the ROS-mediated oxidation of proteins. Inactivation of enzymes and conformation changes by limited –SH oxidation has been documented.[35] We found NPSH levels decreased in response to cerebral I/R indicating an alteration in the redox state of the hippocampus. In addition to thiol oxidants, peroxidation of polyunsaturated fatty acids associated with the plasma membrane lipid bilayer has been suggested to account for some of the pathophysiology induced by I/R. Indeed, we found increased levels of peroxidation products (MDA + 4HDA) in the post-ischaemic brain.

Administration of QF808 dose-dependently attenuated I/R-induced increases in MDA+4-HDA levels. Although the mechanisms by which QF808 inhibit NPSH and lipid oxidation are not entirely clear, it is known that QF808 scavenges $OH^{\bullet}$.[11] Production of NO has also been reported to play an important role in models of cerebral ischaemia.[36,37] It is known for example that cerebral ischaemia promotes both the synthesis of NO and the expression of the genes encoding NO synthase (NOS). Early on in the reperfusion period, parenchymal cell and microvascular NO overproduction are enhanced via up-regulation of neuronal and endothelial NOS isoforms. At later times (>24 h), the inducible isoform of NOS is responsible for the synthesis of NO.[35] Furthermore, it has been reported that iNOS is induced by NFκB, a transcription factor that is regulated by the intracellular redox-state and antioxidants inhibit induction of iNOS redox-state.[38] Thus, the enhanced production of NO-derived nitrate and nitrite (Table II) during I/R and its inhibition by QF808 are consistent with enhanced oxidative stress within the post-ischaemic gerbil brain. It is also known that several cytokines are expressed in the injured brain and within the cerebrospinal fluid following I/R. Astrocytes and macrophages/microglia can express iNOS *in vitro* and mediate neuronal cell death after exitotoxic injury. These cells become activated after CNS trauma, and the activation is most prominent after 1 day.[39,40] Although the protective mechanism of QF808 after I/R damage remains to be elucidated, another possible mechanism for our findings might involve the inhibition of the generation of proinflammatory cytokines, that leading iNOS expression in astrocytes and macrophages injured brain.

It is generally assumed that free radical damage under ischaemia/hypoxia is induced by exhanced production of superoxide anion and $OH^{\bullet}$, the latter is the most toxic and contributes to neuronal death.[19] It has been shown by *in vitro* experiments that QF808 is an effective ROS scavenger. In a model system using desoxirribose assay, QF808 was found to decrease $OH^{\bullet}$-desoxirribose-damage with a half inhibition concentration of 0.011% w/v.[11] In addition mangiferin, the main polyphenol component of QF808 (20%) was found to react with superoxide anion radical.[41] It has also been noted that QF808 interferes with the myeloperoxidase system by neutralising the HOCI.[11]

In summary, the above described results showed that QF808 treatment enhanced the antioxidant activity in the brain and protected neurons against death. We could describe the exact protective mechanism, but considering the scavenger ability and the biological multifunction of QF808 components, it was inferred that not only reduction of ROS but also inhibition of early and late events of the cell death process contributed to protection of neuronal cells. Finally, from the point of view of the development of safer drugs, to prevent oxidative-stress-induced cell injury in the CNS, it is noteworthy that oral uptake of QF808 was effective against ischaemia-induced neuronal cell damage *in vivo*.

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