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Effect of *Mangifera indica* L. Extract (QF808) on Protein and Hepatic Microsome Peroxidation

Gregorio Martínez,[1]* Attilia Giuliani,[2] Olga Sonia León,[1] Gema Pérez[1] and Alberto J Núñez Selles[3]

[1]Center for Evaluation and Biological Research. Institute of Pharmacy, Havana University, San Lázaro y L, Havana 4, Cuba
[2]Department of Chemistry and Medical Biochemistry, via Saldini, 50-20133 Milan, Italy
[3]Center of Pharmaceutical Chemistry, PO Box 16042, Havana, Cuba

The antioxidant activities of QF808, a steam bark extract of *Mangifera indica* L., were studied on hydroxyl-mediated oxidation of bovine serum albumin (BSA) and in a hepatic microsome system. The extract was effective in reducing the oxidation of BSA, since its half- maximal inhibition concentration (IC_{50}) was 0.0049% w/v in the inhibition of carbonyl group formation and lower than 0.0025% w/v in the inhibition of sulfhydryl group loss. QF808 inhibited lipid peroxidation which was initiated enzymatically by reduced nicotinamide adenine dinucleotide phosphate (NADPH), IC_{50}= 0.00075% w/v, or non-enzymatically by ascorbic acid, IC_{50} = 0.0126% w/v. The extract tested did not inhibit NADPH-dependent cytochrome P-450 reductase activity, since it had no effect on the oxidation rate of NADPH. These results suggest that QF808 has an antioxidant activity, probably due to its ability to scavenge free radicals involved in microsome lipid peroxidation. In addition, QF808 antioxidant profile *in vitro* is probably similar to its principal polyphenolic component, mangiferin, a glycosylated xanthone.

Keywords: Mangifera indica L.; antioxidant activity; microsome lipid peroxidation; protein peroxidation.

INTRODUCTION

Free radicals are inevitable by-products of biological redox reaction. There are many pathologies in which they play an important role (Aruoma, 1998). Reaction of the reactive oxygen species (ROS) with biomolecules generally leads to an impairment or loss of biological functions. It is well known that the structure and function of proteins exposed to free radicals are altered. Depending on the free radical involved, the nature of the protein and the conditions of interaction, protein molecules can undergo scission and cross-linking, destruction of amino acids, an increase in susceptibility to proteolysis and heat denaturation, and a loss of biological function (Dean *et al.*, 1997).

Lipid peroxidation is important *in vivo*. It has potential importance in relation to the oxidative damage that occurs during cardiovascular diseases, such as pre-eclampsia and atherosclerosis, ageing and ischaemia–reperfusion injury; in addition, the end products of this process can cause damage to proteins and DNA (Aruoma, 1998; Nakagawa *et al.*, 1997).

That a role exists for free radicals and antioxidants in the pathogenesis of human diseases and in the process of ageing has led to the suggestion that antioxidants, in particular, plant-derived antioxidants, might have health benefits as prophylactic agents (Aruoma, 1997). QF808 was extracted 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) (Center of Pharmaceutical Chemistry, 1998). In Cuba, the aqueous extract of this species is used to improve the quality of life in patients with elevated physical stress (Guevara *et al.*, 1998). QF808 has shown a powerful scavenger activity of hydroxyl radicals and hypochlorous acid, presented a significant inhibitory effect on the peroxidation of rat brain phospholipid and inhibited DNA damage by bleomycin or the copper-phenantroline system (Martínez *et al.*, 1999). The main polyphenol, a C-glucosylxanthone (1,3,6,7-tetrahydroxy-xanthone-C2-β-D-glucoside), mangiferin, was shown to exhibit antioxidant action in *in vitro* systems (Sato *et al.* 1998; Rouillard *et al.*, 1998). In addition, the scavenger ability of norathyriol (aglycone of mangiferin) was demonstrated in a model of respiratory burst induction in rat neutrophils (Hsu *et al.*, 1997). In *in vivo* models QF808 (50–1000 mg/kg p.o.) exhibited potent dose dependent analgesic and antiinflammatory effects (Garrido *et al.*, 2000).

In the present work, we studied the antioxidant effect of QF808 on hydroxyl-mediated oxidation of bovine serum albumin (BSA) by measurement of carbonyl group formation and sulhydryl group loss in the protein. We also assayed the antioxidant effect of QF808 on the course of lipid peroxidation by ADP/Fe/NADPH or ascorbate–iron in rat liver microsomes.

* Correspondence to: Dr G. Martínez, Center for Evaluation and Biological Research, Institute of Pharmacy, Havana University, San Lázaro y L, Havana 4, Cuba.
E-mail: gregorio@cieb.sld.cu
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MATERIALS AND METHODS

Plant extract. A 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, QF-808 the active ingredient of QF808 formulation, which melts at 215 °–210 °C with decomposition. The chemical composition of this extract has been characterized (Center of Pharmaceutical Chemistry, 1998). The solid extract was dissolved in distilled water for pharmacological studies.

Chemicals. The following chemicals were obtained from Sigma (St Louis, MO, USA): ascorbic acid, bovine serum albumin (>98%, essentially fatty acid free), butylated hydroxytoluene (BHT), ethylenediaminetetracetic acid (EDTA), sodium salicylate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), guanidine hydrochloride, hydrogen peroxide, $FeCl_3$, 2,4-dinitrophenyl-hydrazine (DNPH). Adenosine diphosphate (ADP) was bought from BDH Chemical Co. (Poole, Dorset, UK). Trolox, 6 hydroxy-2,5,7,8-tetramethylchroman-2-carbon-saeure, was obtained from Aldrich Chemical Co. (Gillingham Dorset, UK)

Oxidation of BSA by hydroxyl radicals ($OH^{\cdot}$)

Experiment schedule. All reagents were prepared using deionized double distilled water. BSA was oxidized by a Fenton-type reaction. BSA (1 mg/mL) was oxidized at 25 °C in solution with 2.5 mM H_2O_2, 1.0 mM $FeCl_3$, 1.0 mM ascorbate and 3.0 mM EDTA in 2.0 mL polypropylene centrifuge tubes with attached lids. The BSA solution, in an equal volume of 50 mM phosphate buffer, pH 7.5, served as a control. The compounds tested in distilled water were added at the final concentration stated, sodium salicylate 0.6 mM, QF808 0.0025%–0.1% w/v.

After incubation for 45 min at 25 °C, protein was precipitated with 10% TCA (w/v, final concentration), centrifuged ($3000 \times \mathbf{g}$, 4 °C, 2 min) and the supernatants was decanted. The precipitate was washed with 1 mL of 10% TCA and the supernatant was again removed.

Determination of protein carbonyl content. This method is based on that of Oliver *et al.* (1987). The control and the oxidized protein precipitate from the preceding experiment were suspended in 1 mL of 2 M HCl and the other pellets were treated with an equal volume of 0.2% (w/v) DNPH in 2 M HCl. The pellets were evenly suspended by stirring with a small glass rod for 1–2 min. The samples were incubated at 37 °C for 1 h in a shaker-incubator, precipitated with 10% TCA (w/v, final concentration), the precipitates were extracted with 1.0 mL of ethanol–ethyl acetate (1:1. v/v) and then reprecipitated with 10% TCA. The protein pellets were dissolved in 1 mL of 6 M guanidine hydrochloride in 20 mM phosphate buffer, pH 6.5. The solutions were centrifuged ($3000 \times \mathbf{g}$, 2 min, 4 °C) to remove insoluble materials. The difference spectra of the DNPH derivatives were obtained at 370–375 nm. The concentration of carbonyl groups was calculated from a millimolar absorption of 21.0 mM/cm^{-1}.

Determination of total protein sulhydryl groups. Protein pellets from the oxidation experiment were dissolved by vortex-mixing in 1 mL of 50 mM potassium phosphate buffer, pH 7.5. Total sulhydryl group (TSH) determinations were performed according to the method of Sedlak and Lindsay (1968) with Ellman's reagent.

Assay of lipid peroxidation in rat liver microsomes

Preparation of rat liver microsomes. Male Sprague-Dawley rats weighing 250–300 g were killed by decapitation after an overnight fast, the liver was removed and homogenized. Liver microsomes were isolated essentially as described by Albro *et al.* (1987) and stored at −80 °C. The protein concentration in the microsomal suspension was determined according to Bradford (1976), using BSA as a standard.

Lipid peroxidation of microsomes. The microsome suspension (0.6–0.8 mg/mL) was preincubated with 0.4 mM ADP and 10 μM $FeSO_4$ at 37 °C for 5 min before each compound to be tested was added. After a further 5 min incubation, lipid peroxidation was initiated enzymatically by adding 200 μM NADPH or non-enzymatically by 500 μM ascorbic acid (Masuda and Murano, 1977; Ubeda *et al.*, 1993). After incubation for an appropriate time, the lipid peroxidation was assayed by the TBA method (Beuge and Aust, 1978) and the extent of peroxidation was expressed as the change in absorbance of thiobarbituric acid-reactive substances (TBA-RS) at 535 nm. The amount of TBA-RS existing in the mixture before starting the peroxidation reaction was subtracted from each value.

Determination of NADPH consumption. The microsome suspension (500 μL) containing $FeSO_4$ 100 μmol, ADP 2.5 mmol, NADPH 5 mmol was incubated at 37 °C. With the addition of the NADPH the absorption at 340 nm was registered continuously for 20 min. The oxidation rate of NADPH was calculated using an extinction coefficient of 6.2×10^3 M/cm^{-1} (Jha *et al.*, 1985).

Statistical analyses. The statistical analysis was started by using the Outliers preliminary test for detection of error values. Afterward, the ANOVA method (single way) was used followed by the homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test). Values are expressed as the mean ± SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The results in Table 1 show that QF808 was effective in reducing the oxidation of BSA under the experimental conditions. The half protective concentration was estimated as 0.0049% w/v for the inhibition of carbonyl group formation and it was lower than 0.0025% w/v for the inhibition of sulhydryl group loss. Thus, the extract showed strong antioxidant properties, but had more ability (6 fold) to protect SH group oxidation than to reduce carbonyl group formation. There is evidence that

Table 1. Loss of sulhydryl groups and carbonylation of BSA in the presence of QF808

	Amount of carbonyl[c] μmol/mg BSA	Inhibition[d] (%)	Amount of sulfhydryl[c] μmol/mg BSA	Inhibition[e] (%)
Control[a] (+)	34.2 ± 1.4		449 ± 42[g]	53
Control[b] (−)	0.0 ± 0.9	0	856 ± 56	
Salicylate 0.6 mM	5.6 ± 0.8[f]	83	505 ± 14[g]	59
QF808 (% w/v)				
0.1	0.4 ± 0.1[f]	100	870 ± 56	100
0.05	0.7 ± 0.0[f]	100	842 ± 56	98
0.01	1.3 ± 0.1[f]	96	884 ± 84	100
0.005	15.1 ± 0.2[f]	56	659 ± 42	77
0.0025	25.9 ± 0.1	24	491 ± 42[g]	57

[a] Mixture consisted of 1.0 mM $FeCl_3$, 3.0 mM EDTA, 2.5 mM H_2O_2 and 1.0 mM ascorbate.
[b] BSA solution without $OH^{\cdot}$-generating mixture.
[c] Represents the mean ± SD of five separate analyses, performed in duplicate.
[d] With respect to control (+), % inhibition of carbonyl group formation.
[e] With respect to control (−), % inhibition of sulfhydryl group loss.
[f] $p < 0.05$ significantly different compared with the control (+).
[g] $p < 0.05$ significantly different compared with the control (−).

the oxidation of proteins can occur *in vivo*, because damage typical of oxidation was found in proteins from cells and tissues exposed to ROS. In addition, recent studies suggested that some of the products of protein modification by ROS are chemically active (Gebicki *et al.*, 1994). Oxidative damage to proteins *in vivo* may affect the function of receptors, enzymes, transport proteins, etc. and perhaps generate new antigens that provoke immune responses. Products of oxidative protein damage can contribute to secondary damage to other biomolecules, e.g. inactivation of DNA repair enzymes and loss of fidelity of DNA polymerases in replicating DNA (Aruoma, 1998).

The protection of $OH^{\cdot}$-mediated oxidation of BSA, takes place essentially by reducing the H_2O_2 concentration, a fundamental component in the Fenton-type reaction, by chelating iron or by scavenging the $OH^{\cdot}$ formed on the immediate side of oxidation on the target protein (Kingu and Wei, 1997). QF808 was able to scavenge $OH^{\cdot}$ (0.011% w/v) and chelating iron (0.0117% w/v) according to our previous report (Martínez *et al.*, 1999). However, the lower half protective concentration reached to prevent $OH^{\cdot}$-mediated oxidation of BSA, suggested that additional mechanisms might mediate this effect.

The antioxidant capacity of different QF808 concentrations in the biological lipid peroxidation system was examined using rat liver microsomes. Addition of NADPH and Fe^{3+} to the microsomal suspension increased TBA-RS time-dependently up to 60 min, as

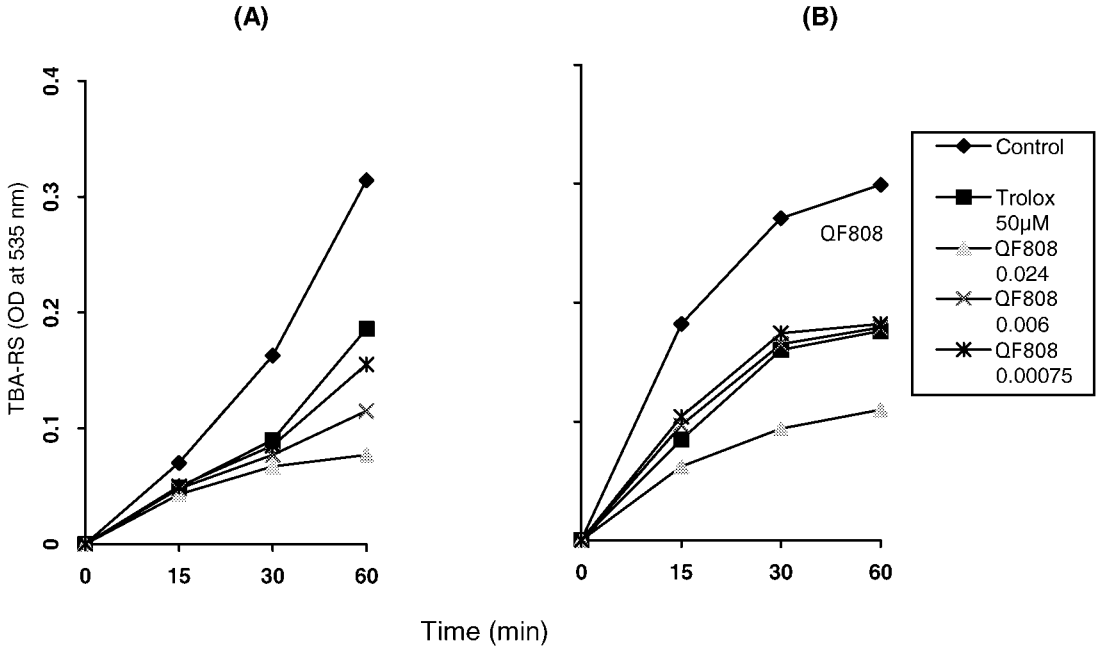

Figure 1. Inhibitory effect of the compounds tested on microsome lipid peroxidation initiated by ADP/Fe/NADPH (A) or ascorbic acid/Fe (B). For experimental details see Materials and Methods. The amount of peroxidated products is expressed as the change in absorbance of thiobarbituric acid-reactive substances (TBA-RS) at 535 nm. The QF808 concentration is in % w/v. Each point represents the mean of three separate experiments which varied by no more than 10%.

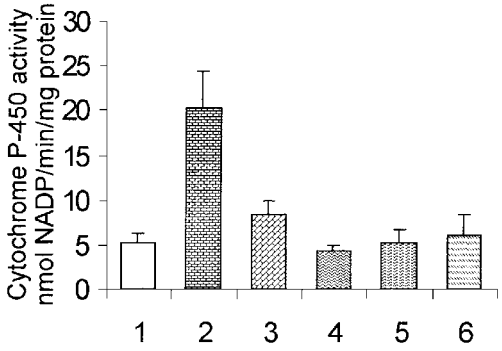

Figure 2. Effect of QF808 on cytochrome P-450 activity. For experimental details see Materials and Methods. The activity of cytochrome P-450 was based on the determination of NADPH consumption. Bar description: 1 (Control), 2 (Trolox 50 μM), 3 (BHT 5 μM), 4 (QF808 0.024% w/v), 5 (QF808 0.006% w/v), 6 (QF808 0.00075% w/v.

shown in Fig. 1A. This form of lipid peroxidation is mediated via the NADPH cytochrome P450 reductase–cytochrome P450 electron transport chain present in liver microsomes, although the precise role of the reductase and various P450s is still unclear (Sevanian *et al.*, 1990). When the microsomes had been pre-treated with each compound tested, the production of TBA-RS was inhibited markedly by QF808 and Trolox 50 μM.

The half inhibitory concentration (IC_{50}) of QF808 in this system was 0.00075% w/v. Since NADPH and Fe^{3+}-induced lipid peroxidation is triggered by the activation of NADPH-dependent cytochrome P450 reductase, the antioxidant activity of QF808 may possibly involve an inhibitory effect on the enzyme action; hence its true antioxidant capacity such as scavenging active oxygen radicals can not be evaluated in such an enzymatic system. Therefore, we also studied the effect of QF808 on the peroxidation induced non-enzymatically. As indicated in Fig. 1B, TBA-RS production induced by the addition of ascorbic acid and Fe^{3+} thus circumventing the cytochrome P450 reductase action, was inhibited markedly by QF808 (IC_{50}= 0.0126% w/v). Similar results were obtained by Sato *et al.* (1992) who evaluated mangiferin in the same model. Furthermore, the IC_{50} reached by QF808 was comparable to the IC_{50} determined for an extract of rosemary, 0.00025% w/v in NADPH/Fe-dependent lipid peroxidation and 0.0125% w/v in ascorbate/iron system (Plumb *et al.*, 1996). Rosemary extract was one of the most potent natural antioxidant extracts tested in different *in vitro* assays (Aruoma *et al.*,1996).

As demonstrated in Fig. 1, QF808 inhibited enzymatic lipid peroxidation at a lower concentration than the non-enzymatic one, suggesting its probable inhibitory action on cytochrome P-450 reductase action. Considering the fact that NADPH is oxidized during the functioning of cytochrome P-450, we studied the effect of QF808 on the oxidation rate of NADPH as an indicator of its action on the enzyme. As seen in Fig. 2, in the control sample the rate of NADPH consumption was 5.26 nmol/min/mg protein. With the concentration of QF808 used, no statistically different results were observed in the NADPH consumption rate. A similar effect was detected by Sato *et al.* (1992) in the action of mangiferine on the rat microsome system. Mangiferine (40%–60%) is the main component of the active ingredient used for Vimang® formulation.

In a comparison of the tested antioxidants, (Fig. 2), we found a slightly enhanced NADPH consumption, in accordance with the result of Jha *et al.* (1985), who evaluated similar results for butylated hydroxyanisol (BHA). In addition, an increase in NADPH consumption was found for Trolox (synthetic Vitamin E analogue). This result was in accordance with recent studies which showed that the use of synthetic antioxidants is accompanied by several toxic effects (WHO, 1995), since the induction of cytohrome P-450 may increase the carcinogen metabolism (Camus-Randon *et al.*, 1996).

QF808 had a strong antioxidative potency as indicated by the following observations: inhibition of lipid peroxidation in microsomes initiated by enzymatic (NADPH) or non-enzymatic (ascorbic acid) systems and no inhibition of NADPH-dependent cytochrome P-450 reductase activity, since QF808 had no effect on the oxidation rate of NADPH.

The potent antioxidant action of QF808 in the NADPH-dependent peroxidation system, in addition to the previously invoked mechanism, might be due to its ability to scavenge H_2O_2. In addition, the metabolic transformation of mangiferin in its aglycone norathyriol may impart additional antioxidant properties (Hsu *et al.*, 1997). Mangiferin has a catechol moiety on its structure (6,7-dihydroxylated) and studies on many flavonoid derivatives have suggested that a catechol moiety in their structure may be important for antioxidant activity (Jha *et al.*, 1985); in addition, the hydrolysis of mangiferin to its aglycone (norathyriol) adds an additional catechol moiety.

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