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Protective Effect of *Mangifera indica L.* Extract (Vimang®) on the Injury Associated with Hepatic Ischaemia Reperfusion

Gregorio Martínez Sánchez[1]*, María A. Rodríguez H.[1], Attilia Giuliani[2], Alberto J. Núñez Sellés[3], Niurka Pons Rodríguez[1], Olga Sonia León Fernández[1] and L. Re[4]

[1]Centre for Research and Biological Evaluation, Pharmacy Institute, Havana University, San Lazaro y L, Havana 4, Cuba
[2]Department of Chemistry and Medical Biochemistry, University of Milan, via Saldini, 50-20133 Milan, Italy
[3]Center of Pharmaceutical Chemistry, P.O. Box. 16042, Havana, Cuba
[4]Institute of Experimental and Clinical Medicine, University of Ancona, via Ranieri 2, 60131, Ancona, Italy

The effect *of Mangifera indica* L. extract (Vimang®) on treatment of injury associated with hepatic ischaemia/reperfusion was tested. Vimang® protects from the oxidative damage induced by oxygen-based free radicals as shown in several *in vitro* test systems conducted. The ability of Vimang® to reduce liver damage was investigated in rats undergoing right-lobe blood flow occlusion for 45 min followed by 45 min of reperfusion. The ischaemia/reperfusion model leads to an increase of transaminase (ALT and AST), membrane lipid peroxidation, tissue neutrophil infiltration, DNA fragmentation, loss of protein -SH groups, cytosolic Ca^{2+} overload and a decrease of catalase activity. Oral administration of Vimang® (50, 110 and 250 mg/kg, b.w.) 7 days before reperfusion, reduced transaminase levels and DNA fragmentation in a dose dependent manner ($p < 0.05$). Vimang® also restored the cytosolic Ca^{2+} levels and inhibited polymorphonuclear migration at a dose of 250 mg/kg b.w., improved the oxidation of total and non protein sulfhydryl groups and prevented modification in catalase activity, uric acid and lipid peroxidation markers ($p < 0.05$). These data suggest that Vimang® could be a useful new natural drug for preventing oxidative damage during hepatic injury associated with free radical generation. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: ischaemia reperfusion; *Mangifera indica* L.; antioxidant activity; lipid peroxidation; DNA fragmentation; sulfhydryl oxidation.

INTRODUCTION

Ischaemia is a widely occurring event, which can take place generally in an organ during surgery, under conditions of shock and in transplantation. Ischaemia and reperfusion (I/R) are known to trigger a series of complex events that involve almost all cellular components (Chávez-Cartaya *et al*., 1996). Several mechanisms have been considered to explain I/R injury in the liver. These mechanisms include the involvement of reactive oxygen species (ROS), migration and activation of leukocytes, damage to endothelial cells, disturbance of the microcirculation and activation of the coagulation system (Bremer *et al.*, 1994). Endogenous defence mechanisms have been identified that use antioxidants or free radical scavengers to neutralize ROS-generated lipid peroxidation (LP); however, the extensive generation of free radicals appears to overwhelm the natural defence mechanisms, dramatically reducing the levels of endogenous antioxidants, and leading to the uncontrolled progression of peroxidative damage to cellular membranes (Campo *et al.*, 1997).

* Correspondence to: Dr G. M. Sánchez, Centre for Research and Biological Evaluation, Pharmacy Institute, Havana University, San Lazaro y L, Havana 4, Cuba. E-mail: gregorio@cieb.sld.cu
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Several authors have suggested that antioxidants, in particular plant-derived antioxidants, might have health benefits as prophylactic agents (Aruoma, 1997; Maxwel, 1997). An extract of *Mangifera indica L.* stem bark (Vimang®) was tested *in vitro* for its antioxidant activity in our laboratory. It showed a powerful scavenger activity on hydroxyl radicals and hypochlorous acid, presented a significant inhibitory effect on the peroxidation of rat brain phospholipid and inhibited DNA damage by bleomycin or copper-phenanthroline systems (Sánchez *et al.*, 2000).

Vimang® is extracted from selected varieties of *Mangifera indica* L. It has a defined mixture of components (polyphenols, terpenoid, steroid, fatty acids and microelements) (Centre of Pharmaceutical Chemistry, 1998). The main polyphenol, a C-glucosylxanthone (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside), mangiferin, was shown to have antioxidant action in *in vitro* systems (Sato *et al*., 1992; Roillard *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).

The aim of the present study was to assess the possible ability of Vimang® to reduce damage to the rat liver after I/R. Parameters of biological molecules damaged by ROS and some enzyme and biochemical indicator mediators in antioxidant/pro-oxidant balance were also studied.

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MATERIALS AND METHODS

Plant extract. A stem bark extract of *Mangifera indica* L. was prepared by decoction with a polar solvent for 1 h. The extract was concentrated by evaporation and spray dried to obtain a fine brown powder (Vimang®) which melts at 215°–210 °C with decomposition. The chemical composition of this extract has been characterized (Centre of Pharmaceutical Chemistry, 1998). Vimang® was dissolved in distilled water for pharmacological studies.

Surgical procedures. Female Wistar rats (230–250 g body weight) were anaesthetized with urethane (10 mg/kg, i.p.) and placed on a heated operating table. To induce hepatic ischaemia, laparotomy was performed and the blood supply to the right lobe of the liver was interrupted by placing a bulldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp (Peralta *et al.*, 1997). The studies were performed in concordance with the European Union regulations for animal experiments.

Treatment schedule. The protocol consisted of six experimental groups ($n = 6$). (1) The negative control group was subjected to anaesthesia and laparotomy only; (2) control group, the same procedure as group 1, but with previous per oral treatment (250 mg/kg, b.w.) of Vimang® once a day, 7 days before laparotomy; (3) positive control group, animals subjected to 45 min of right lobe hepatic ischaemia followed by 45 min of reperfusion; (4,5,6) the same procedure as group 3, but with previous per oral treatment with Vimang® (50, 110 and 250 mg/kg, b.w., respectively) 7 days before I/R period.

Biochemical determinations. The biochemical parameters were determined by spectrophotometric methods using an Ultrospect III Plus spectrophotometer from Pharmacia LKB. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in serum using standard commercial kits produced by Boehringer Mannheim (Germany). Liver homogenates were assayed for total superoxide dismutase (Cu/Zn and Mn SODs) activity, determining the capacity of the enzyme in inhibiting the autoxidation of pyrogallol by 50% (Boehringer Mannheim, 1987). The catalase concentration was measured through the catalytic activity which promotes the reduction of hydrogen peroxide (H_2O_2) to oxygen and water (Boehringer Mannheim, 1987). The myeloperoxidase (MPO) activity was assayed kinetically as described by Bradley *et al.* (1982). The rate at which a coloured product formed during MPO-dependent reaction of o-dianisidine (0.167 mg/mL) and H_2O_2 (0.0005%) was measured at 460 nm. One unit of MPO activity is defined as that which degrades 1 μmol of peroxide/min at 25 °C. Uric acid and calcium were measured in the supernatant of a centrifuged (13 000 g for 20 min, 4 °C) liver homogenate using standard commercial kits produced by Sigma (St Louis, MO, USA). Total (TSH) and non-protein (NPSH) sulfhydryl group determinations were performed according the method of Sedlak and Lindsay (1968) with Ellman's reagent. DNA fragmentation was measured at 600 nm as described by Ray *et al.* (1993) and expressed as the percentage of the total DNA appearing in the supernatant fractions. Malondialdehyde (MDA) plus 4-hydroxyalkenal (4-HDA) were assayed as a marker of LP (Bioxytech LPO-586 kit, Cayman Chemical Co. Ann Arbor, USA) using a colorimetric reaction (586 nm) which used 1-methyl-2-phenylindol as a chromogene (Esterbaver and Cheeseman, 1990). For the determination of tissue homogenate susceptibility to lipid peroxidation, liver homogenate was incubated with copper sulphate (2 mM, final concentration) at 37 °C for 24 h. The peroxidation potential was estimated by taking the difference between lipid peroxidation values (MDA + 4-HDA) at 24 h and at 0 h (Ozdemirler *et al.*, 1995). The proteins were measured by a standard Coomassie blue method (Spector, 1978). Chemicals and reagents were purchased from Sigma Chemical Co., St Louis, MO, USA.

Statistical analysis. The statistical analysis was started by using the OUTLIERS preliminary test for the detection of error values. Then 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 ($n = 6$ per group). Different letters indicate a statistical significance of at least $p < 0.05$.

RESULTS

As shown in Fig. 1, significant increases in AST and ALT levels were observed in the group subjected to I/R with respect to the control group. Administration of Vimang® (250 mg/kg, b.w., without I/R) resulted in the same effects on ALT and AST as the negative control group. In the Vimang® + I/R groups a reduction of ALT and AST were observed.

The enzymatic determinations (SOD, catalase and MPO, Table 1) showed that I/R caused no modification in SOD activity compared with the control group. A substantial decrease of catalase activity was noted in the positive control group. The administration of Vimang® restored the catalase to the basal levels. The polymorphonuclear leukocytes (PMNs) were monitored by MPO levels. After I/R the MPO values showed a two-fold increase compared with the negative control group. Only a dose of 250 mg/kg b.w. reduced the tissue PMNs accumulation.

An increase in uric acid levels was found in the I/R group (Table 2). However, Vimang®, at all doses, reduced this increase. The cytosolic Ca^{2+} levels (Table 2) increased in all I/R groups. Treatment with Vimang® at higher doses reduced Ca^{2+} to basal values.

The levels of liver damage attributable to oxidation of biomolecules (proteins, DNA and lipids) were assayed (Table 3). The TSH group content was significantly decreased ($p < 0.05$) in all I/R groups. However, treatment with Vimang® showed a reduction in the TSH lost compared with the positive control group. A similar reduction took place in the NPSH groups. On the other hand, in the 110 and 250 mg/kg b.w. + I/R groups NPSH reached basal values. The DNA fragmentation observed in the I/R group (33%) was prevented in animals treated with

Table 1. Effect of Vimang® on superoxide dismutase catalase, and myeloperoxidase activity in liver homogenates

	SOD (U/g protein)	Catalase (U/g protein)	MPO (U/mg protein)
Control	908 ± 97	61.5 ± 20.4[a]	8.5 ± 1.3[a]
Vimang® (250 mg/kg)	1024 ± 171	57.8 ± 21.1[a]	8.3 ± 4.1[a]
I/R	959 ± 200	39.9 ± 5.6[b]	16.1 ± 3.4[b]
Vimang® (50 mg/kg + I/R)	1059 ± 158	51.4 ± 9.6[a]	17.8 ± 5.8[b]
Vimang® (110 mg/kg + I/R)	938 ± 188	49.9 ± 10.9[a]	15.2 ± 1.4[b]
Vimang® (250 mg/kg + I/R)	874 ± 188	57.8 ± 21.1[a]	8.2 ± 2.5[a]

Values represent mean ± SD ($n = 6$). Values with non-identical superscript are statistically significant ($p < 0.05$).

Table 2. Effect of Vimang® on uric acid and cytosolic calcium levels in liver homogenates Values represent mean ± SD ($n = 6$)

	Uric acid (mg/g tissue)	Cytosolic Ca^{2+} (mg/g tissue)
Control	1.50 ± 0.24[a]	2.52 ± 0.56[a]
Vimang® (250 mg/kg)	1.00 ± 0.37[a]	2.64 ± 0.68[a]
I/R	2.07 ± 0.38[b]	3.56 ± 0.68[b]
Vimang® (50 mg/kg, b.w. + I/R)	1.03 ± 0.24[a]	3.68 ± 0.48[b]
Vimang® (110 mg/kg, b.w. + I/R)	0.85 ± 0.23[a]	3.20 ± 0.52[b]
Vimang® (250 mg/kg, b.w. + I/R)	0.86 ± 0.26[a]	2.04 ± 0.48[a]

Values with non-identical superscript are statistically significant ($p < 0.05$).

Table 3. Changes in oxidative markers of biomolecules oxidation Values represent mean ± SD ($n = 6$)

	TSH (μmol/mg protein)	NPSH (μmol/g tissue)	DNA fragmentation (%)	DNA fragmentation (%) with respect to control	MDA + 4-HDA (μmol/mg protein)	Peroxidation potential
Control	35.3 ± 2.1[a]	0.96 ± 0.13[a]	3.38 ± 0.57[a]	–	11.5 ± 0.2[a]	2.33 ± 0.65[a]
Vimang® (250 mg/kg)	36.0 ± 1.2[a]	1.00 ± 0.08[a]	3.54 ± 0.38[a]	4	11.7 ± 0.9[a]	2.54 ± 0.70[a]
I/R	10.6 ± 2.3[b]	0.65 ± 0.08[b]	4.50 ± 0.26[b]	33	17.4 ± 0.3[b]	3.27 ± 0.52[b]
Vimang® (50 mg/kg, b.w. + I/R)	20.1 ± 1.8[c]	0.74 ± 0.08[b]	4.16 ± 0.49[b]	23	11.5 ± 1.0[a]	2.99 ± 0.50[a]
Vimang® (110 mg/kg, b.w. + I/R)	26.8 ± 2.0[d]	1.18 ± 0.22[a]	4.02 ± 0.25[c]	18	9.2 ± 1.0[a]	2.30 ± 0.95[a]
Vimang® (250 mg/kg, b.w. + I/R)	23.0 ± 2.1[d]	1.22 ± 0.17[a]	3.61 ± 0.53[a]	6	9.7 ± 0.5[a]	2.18 ± 0.62[a]

TSH, total sulfhydryl; NPSH, non-protein sulfhydryl; MDA + 4-HDA, malondialdehyde + 4-hydroxyalkenal. Peroxidation potential was estimated by taking the difference between lipid peroxide value (MDA + 4HDA) at 24 h and at 0 h, incubated with copper.
Values with non-identical superscript are statistically significant ($p < 0.05$).

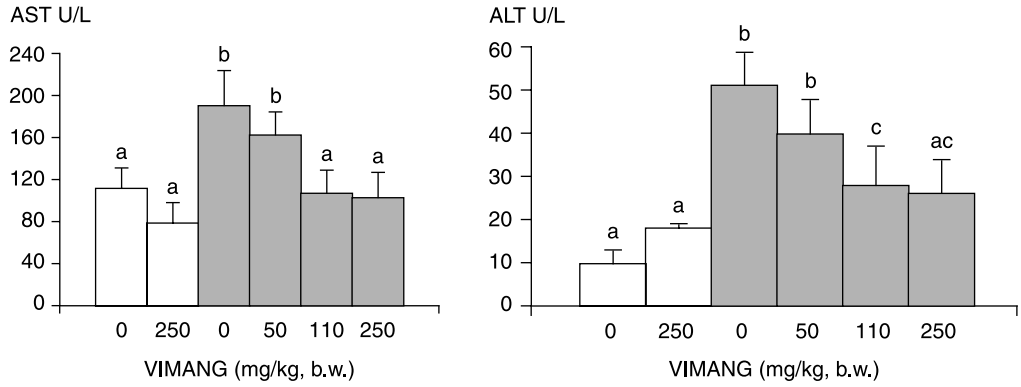

Figure 1. Serum ALT and AST levels (U/L) in the experimental groups. Bar heights represent mean ± SD ($n = 6$). The white bars represent no I/R and black bars I/R, respectively. Values with non-identical superscript are statistically significant ($p < 0.05$).

Vimang® in a dose-dependent manner. Moreover, Vimang®, at all doses, avoided the increase in LP observed in the positive control group. Therefore, the peroxidation potential indicated a better balance between prooxidant factors and antioxidant protection in Vimang® treated groups compared with the I/R control group.

DISCUSSION

The restoration of blood flow after a period of ischaemia is associated with the production of ROS and consequent tissue damage. These reactive molecules can be generated by several catalytic activities, including xanthine

oxidase (XO), cyclooxygenase and NADPH oxidase/myeloperoxidase of activated leukocytes, or cellular mechanisms such as mitochondrial or microsomal electron transport. In post-ischaemic liver, a decrease in oxygen-scavenging enzyme activity has been demonstrated, aggravating the imbalance between the generation and removal of ROS (Hamer *et al.*, 1995). In addition, during reperfusion extensive generation of ROS appears to overwhelm the natural defence mechanisms, dramatically reducing the content of endogenous antioxidants. Thus, following free radical attack, the levels of these substances have been shown to be severely depleted (Campo *et al.*, 1996). Therefore, during I/R the local control of ROS is lost, and free radicals can attack biological molecules and peroxidation begins (Panetta and McCall, 1995).

The present study was designed to evaluate the effect of treatment with Vimang® in a rat model of ischaemic and reperfused liver. In this model, Vimang® reduced the I/R injury. The increase in transaminases (ALT and AST) observed after I/R were attenuated by pretreatment with Vimang® (Fig. 1).

Catalase and SOD play an important role in the antioxidative defence system (Halliwell and Cross, 1994). Superoxide ($O_2^{\bullet-}$) by itself is unable to cause cell death, due to its short half-life and poor reactivity in aqueous solution (Fridovich, 1997). In contrast, H_2O_2, produced directly by XO or indirectly by dismutation of $O_2^{\bullet-}$, appears to be cytotoxic to cells, since exogenous catalase completely prevents cell damage (Hamer *et al.*, 1995). SOD activity after I/R (Table 1) was similar to the control group. This fact suggests that experimental conditions of I/R (45 min of I/R) lead to non-significant inhibition or activation of this enzyme. However, the catalase level was significantly decreased in the I/R positive control group together with the lowest level of TSH groups (Table 3). These results agree with the proposed hypothesis that ROS could oxidize critical protein sulfhydryl groups (Kedderis, 1996). Instead, even at a low dose of Vimang® catalase activity was preserved, thus the cell H_2O_2 -detoxification capacity did not change.

The preservation of catalase activity might be due to the ability of Vimang® to scavenge the free radicals generated during I/R and then sparing catalase.

In I/R tissue PMNs infiltration occurred and MPO is an enzyme marker for those cells (Werner and Szelenyi, 1992). PMNs, once activated and attached to endothelial cells may exacerbate tissue injury via the generation of ROS, and the secretion of several enzymes (Panetta and McCall, 1995). Vimang® (250 mg/kg, b.w.) reduced PMNs infiltration, thus confirming its protective effects. During ischaemia, xanthine dehydrogenase (XD) is converted to XO by limited proteolysis, or sulfhydryl oxidation, or both (Hamer *et al.*, 1995). This conversion leads to the production of $O_2^{\bullet-}$, H_2O_2, uric acid (UA), and subsequently to the formation of hydroxyl radicals ($OH^{\bullet}$). Our finding revealed that the UA concentration was only increased in the I/R group (Table 2). Several reports have shown that, for a short period of I/R, proteolytic conversion of XD did not occur (Parks *et al.*, 1988). In that case, conversion by sulfhydryl group oxidation seems to be the main important mechanism. However, all doses of Vimang® partially preserved TSH groups. This suggested that by means of -SH preservation Vimang® prevented the XD conversion and ROS generation.

In I/R injury intracellular Ca^{2+} overloading is perhaps the most critical factor in determining the biochemical basis of ultimate cell death (Bagchi *et al.*, 1997). Vimang® at a dose of 250 mg/kg b.w. prevented cytosolic Ca^{2+} overloading during I/R (Table 2).

Free radicals are able to produce chemical modifications of proteins, nucleotides and lipid, resulting in a variety of biological consequences (Stohs, 1995). Sulfhydryl oxidation is one of the earliest observable events during the radical-mediated oxidation of proteins. Inactivation of enzymes and conformation changes by limited -SH oxidation have been documented (Dean *et al.*, 1997). In addition, after I/R the reduced form of glutathione (GSH) is released from the hepatocyte into the sinusoidal lumen, where it can act, directly or in the presence of glutathione oxidase, as a trapping agent for ROS generated in the extracellular medium (Nakano *et al.*, 1995). By maintaining GSH levels at the time of reperfusion, it might be expected that the destructive potential of ROS is reduced. It has been assumed, according to the results shown in Table 3, that previous treatment with Vimang® may reduce reperfusion injury as a consequence of the protection against TSH and NPSH damage.

Experimental evidence suggested that during I/R the cells were able to undergo apoptosis (Gottlieb *et al.*, 1994). A distinctive feature of apoptosis at the biochemical level is DNA fragmentation (Kinloch *et al.*, 1999). An increase in Ca^{2+} can stimulate endonuclease activity and in consequence the enzymatic fragmentation of genomic DNA (Ray *et al.*, 1993). A dose dependent reduction in DNA fragmentation was found in groups treated with Vimang® (Table 3).

Lipid peroxidation is considered to be a critical mechanism of injury occurring during reperfusion. It occurs essentially in the non-parenchymal cell portion of the liver. The blockade of the GSH redox cycle and the catalase activity increase the endothelial cell susceptibility to LP (Hamer *et al.*, 1995). In the positive I/R control group the -SH and catalase activity were dramatically decreased and these conditions lead to an increase of LP (Table 3).

The levels of MDA + 4-HDA, found at the end of reperfusion in the I/R control group, strongly emphasize the damage due to reperfusion. Administration of Vimang® attenuated membrane injury, as demonstrated by MDA + 4-HDA and peroxidation potential.

Peroxidation potential shows the susceptibility of liver homogenates to copper-induced lipid peroxidation as a balance between pro-oxidant factors and antioxidant protection in the homogenates. The results indicate that this balance was better in groups treated with Vimang®. The scavenger properties of Vimang® (Sánchez *et al.*, 2000) might be justified this effect.

Summarizing, these results demonstrated that pretreatment with Vimang® (250 mg/kg b.w.) in an I/R model, *in vivo*, protected the liver from injury associated with ROS generation, which would be relevant for surgery and therapeutic applications. It also demonstrated their correlation with *in vitro* experiments, demonstrating the high antioxidant potential of Vimang®.

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