# PREVENTING HEPATOCYTE OXIDATIVE STRESS CYTOTOXICITY WITH *Mangifera indica* L. EXTRACT (VIMANG)

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**SUMMARY**

Vimang is an aqueous extract of *Mangifera indica* used in Cuba to improve the quality of life in patients suffering from inflammatory diseases. In the present study we evaluated the effects of Vimang at preventing reactive oxygen species (ROS) formation and lipid peroxidation in intact isolated rat hepatocytes. Vimang at 20, 50 and 100 μg/ml inhibited hepatocyte ROS formation induced by glucose-glucose oxidase. Hepatocyte cytotoxicity and lipid peroxidation induced by cumene hydroperoxide was also inhibited by Vimang in a dose and time dependent manner at the same concentration. Vimang also inhibited superoxide radical formation by xanthine oxidase and hypoxanthine. The superoxide radical scavenging and antioxidant activity of the Vimang extract was likely related to its gallates,

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catechins and mangiferin content. To our knowledge, this is the first report of cytoprotective antioxidant effects of Vimang in cellular oxidative stress models.

**KEY WORDS**

*Mangifera indica* L., Vimang, reactive oxygen species, lipid peroxidation, hepatocytes, rat

## INTRODUCTION

Cellular generation of reactive oxygen species (ROS) has been associated with or contributes to human disease states, such as inflammatory diseases, neurodegenerative diseases, ischemia-reperfusion injury, cancer and aging. Recently a fluorescent method for following cellular ROS formation has become available /1/ which was shown to increase before reoxygenation cell injury following hypoxia or anoxia /2/ and before cytotoxicity induced by metals or aflatoxin /3,4/.

The role of 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, may have health benefits as prophylactic agents /5/. Vimang is an aqueous extract of *Mangifera indica* L., used traditionally in Cuba for its anti-inflammatory, analgesic, and antioxidant properties /6,7/. It is currently being used in clinical trials in Cuba for treating various inflammatory or oxidative stress diseases at an adult dose of 300 mg/kg. Several authors have reported other pharmacological activities of extracts of *M. indica* L., including spasmolytic, anti-amoebic, antimicrobial and antipyretic effects /8-10/. These fractions contained polyphenols, triterpenes, flavonoids, phytosterols and microelements /11/. The major compound is mangiferin, a C-glucosyl-xanthone with antiviral, antitumor, antidiabetic, and antioxidant activity /12-14/.

In the present study, we evaluated the effects of Vimang on ROS formation and products derived from lipid peroxidation in intact isolated rat hepatocytes and on superoxide radical scavenging activity.

## MATERIALS AND METHODS

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 QF 808), the active ingredient of Vimang formulations, which melts at 210-215°C with decomposition. The chemical composition of this extract has been characterized /15/. The solid extract was dissolved in distilled water for the studies.

### Chemicals

2′,7′-Dichlorofluorescein (DCFH)-diacetate, Tris (hydroxymethyl-aminomethane, and nitroblue tetrazolium (NBT) were purchased from Fluka Chemie AG (Oakville, ON, Canada). All other chemicals were purchased from a local supplier and were of the highest commercial grade available.

### Animals

Male Sprague-Dawley rats (280-300 g) fed a standard chow diet and given water *ad libitum* were used in all experiments.

### Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver, as described by Moldeus *et al.* /16/. Approximately 85-90% of the hepatocytes excluded trypan blue upon isolation (i.e., 85-90% cell viability). Cells were suspended at a density of $10^6$ cells/ml in round-bottom flasks, rotating in a water bath maintained at 37°C in Krebs-Hensleit buffer (pH 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% $O_2$:85% $N_2$:5% $CO_2$.

### Cell viability

The viability of isolated hepatocytes was assessed from the intactness of their plasma membrane as determined by the trypan blue (0.1% w/v) exclusion assay. Aliquots of the hepatocyte incubate were taken at different time points during the 2 h incubation period. At least 80-90% of the control cells were still viable after 3 h.

**Determination of reactive oxygen species (ROS)**

To determine the amount of hepatocyte ROS generation, DCFH-diacetate was used as it penetrates the cells and becomes hydrolyzed by an intracellular esterase to form DCFH. The latter reacts with intracellular ROS to form the highly fluorescent 2′,7′-dichloro-fluorescein, which effluxes the cell /1/. The hepatocytes were incubated for 3 hours under three conditions: 1. hepatocytes plus the $H_2O_2$ generating system glucose/glucose oxidase (Glu/GO) (10 mM/ 1 U/ml); 2. hepatocytes plus Vimang at different concentrations (20, 50 and 100 μg/ml) plus Glu/GO; and 3. hepatocytes plus Vimang alone. Aliquots (1 ml) were taken at 1 hour for ROS determination. The sample was centrifuged for 30 sec at 50 *g*, the supernatant was discarded, and 1 ml of DCFH-diacetate was added to the pellet. This suspension was then incubated for 10 min at 37°C. 100 μl of the incubate was then resuspended in 2 ml of purified $H_2O$ in a quartz cuvette and the fluorescence intensity of the 2′,7′-dichlorofluorescein formed was monitored at 500 nm (excitation) and at 520 nm (emission). The results were expressed as fluorescent intensity per $10^6$ cells /4/.

**Lipid peroxidation**

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU-7 spectrophotometer. The samples were incubated for 2 hours under three conditions: 1. hepatocytes plus cumene hydroperoxide (CHP) 120 μM; 2. hepatocytes plus CHP plus Vimang at different concentrations; and 3. hepatocytes plus Vimang alone. After 10 and 40 min 1.0 ml aliquots of hepatocyte suspension ($10^6$ cells/ml) were removed and assayed for TBARS with trichloroacetic acid (70% w/v) and the suspension was boiled with thiobarbituric acid (0.8% w/v) for 20 min /17/.

**Superoxide radical-scavenging activity measurement**

Vimang (30 and 100 μg/ml), propyl gallate (10 μM) and gallic acid (10 μM) were compared for their superoxide radical scavenging activity by their ability at inhibiting nitroblue tetrazolium (50 μM)

reduction by hypoxanthine (3.5 mM) and xanthine oxidase (25 munit/ml) in Tris buffer (0.1 M, pH 7.4). The total volume of the mixture was 1 ml. The absorbance of the reaction mixture was monitored at 560 nm for 10 min using a Pharmacia Ultraspec model 1000 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) /18/.

**Statistical analysis**

Statistically significant differences between control and test compounds were determined using Student's t-test. Values of $p < 0.05$ were regarded as significant.

## RESULTS

**Effectiveness of Vimang versus gallates in preventing ROS induced hepatocyte cytotoxicity**

As shown in Table 1, $H_2O_2$ generated by glucose/glucose oxidase markedly increased hepatocyte DCFH oxidation at 60 min and induced cytotoxicity at 120 min which was prevented significantly in a dose dependent manner by Vimang (100, 50 and 20 μg/ml). Hepatocytes incubated with only Vimang did not show significant DCFH production or cytotoxicity when compared with hepatocytes alone. Propyl gallate was more effective than gallic acid.

**Effectiveness of Vimang versus gallates in preventing cumene hydroperoxide induced lipid peroxidation (LPO) and hepatocyte cytotoxicity**

As shown in Table 2 cumene hydroperoxide produced a marked increase of lipid peroxidation measured at 532 nm 10 and 40 min following hydroperoxide addition before cytotoxicity ensued at 60 min (not shown) and 120 min. Vimang at concentrations of 100, 50 and 20 μg/ml significantly decreased the lipid peroxidation and cytotoxicity induced by cumene hydroperoxide in a concentration and time dependent manner. Propyl gallate was more effective than gallic acid and both were more effective at inhibiting LPO (Table 2) than ROS (Table 1).

**TABLE 1**

Effect of Vimang on endogenous ROS formation by isolated hepatocytes

| Addition | Concentration | ROS formation (fluorescence units) 60′ | Cytotoxicity (% trypan blue uptake) 120′ |
|---|---|---|---|
| **Hepatocytes** | | 85 ± 7 | 20 ± 3 |
| **+ Vimang** | 100 μg/ml | 83 ± 6 | 21 ± 3 |
| **+ Glu/GO** | 10 mM/1 U/ml | 320 ± 8 | 58 ± 5 |
| **+ Vimang** | 100 μg/ml | 99 ± 7* | 30 ± 3 |
| **+ Vimang** | 50 μg/ml | 142 ± 8* | 38 ± 3 |
| **+ Vimang** | 20 μg/ml | 245 ± 9* | 48 ± 4 |
| **+ Propyl gallate** | 70 μM | 103 ± 8* | 31 ± 3 |
| **+ Gallic acid** | 150 μM | 109 ± 9* | 35 ± 4 |

Hepatocytes ($10^6$ cells/ml) were incubated in Krebs-Hensleit buffer, pH 7.4, at 37°C under 10% $O_2$, 5% $CO_2$, 85% $N_2$. DCFH oxidation to fluorescent dichlorofluorescein was used to determine ROS formation, as described in Materials and Methods. $H_2O_2$ was generated with 10 mM glucose and 1 U/ml glucose oxidase. ROS formation was expressed as fluorescence intensity units. Cytotoxicity was determined as the percentage of cells that take up trypan blue.
Values are expressed as means ± SD from three separate experiments.
* $p < 0.05$.

**Superoxide radical-scavenging (SRS) activity of Vimang and gallates**

The SRS activities of Vimang, propyl gallate and gallic acid (the two last compounds are components of Vimang) were compared using an enzymatic superoxide radical-generating system (hypoxanthine/ xanthine oxidase) and nitroblue tetrazolium. As shown in Table 3, the SRS activity of Vimang was dose dependent. Propyl gallate and gallic acid at 10 μM were more effective in scavenging superoxide radicals than 100 or 50 μg/ml Vimang.

**TABLE 2**

Effects of Vimang on cumene hydroperoxide induced lipid peroxidation (LPO) measured at 532 nm

| Addition | Concentration | LPO (nmol/$10^6$cells) 10′ | LPO (nmol/$10^6$cells) 40′ | Cytotoxicity % TB uptake 120′ |
|---|---|---|---|---|
| **Hepatocytes** | | 2.8 ± 0.2 | 3.1 ± 0.2 | 19 ± 2 |
| **+ CHP** | 120 μM | 15.1 ± 1 | 19.1 ± 1 | 56 ± 6 |
| **+ Vimang** | 100 μg/ml | 6.4 ± 0.3* | 8.2 ± 0.4* | 34 ± 3 |
| **+ Vimang** | 50 μg/ml | 8.2 ± 0.4* | 10.1 ± 0.3* | 42 ± 4 |
| **+ Vimang** | 20 μg/ml | 13.1 ± 0.8 | 16.1 ± 0.3 | 51 ± 5 |
| **+ Propyl gallate** | 25 μM | 6.1 ± 0.2* | 8.4 ± 0.3* | 32 ± 3 |
| **+ Gallic acid** | 100 μM | 7.3 ± 0.3* | 9.5 ± 0.2* | 43 ± 4 |

Hepatocytes ($10^6$ cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C under 10% $O_2$, 5% $CO_2$, 85% $N_2$. Cumene hydroperoxide (CHP) was used for inducing lipid peroxidation, as described in Materials and Methods. LPO formation was determined by measuring TBARS as described in Material and Methods, and expressed as UV absorbance at $\lambda_{max}$ 532 nm, expressed as nmol/$10^6$ cells. Cytotoxicity was expressed as % trypan blue (TB) uptake. Values are expressed as means ± SD from three separate experiments.

* Significant difference ($p < 0.05$) compared with hepatocytes treated with only CHP.

**TABLE 3**

Superoxide radical-scavenging (SRS) activity of Vimang

| Addition | Concentration | SRS activity (%) |
|---|---|---|
| **Hypoxanthine/XO + NBT** | 3.5 mM + 25 mU + 50 μM | 100 |
| **+ Vimang** | 100 μg/ml | 48 ± 3* |
| **+ Vimang** | 30 μg/ml | 59 ± 5* |
| **+ Propyl gallate** | 10 μM | 50 ± 4* |
| **+ Gallic acid** | 10 μM | 61 ± 5* |

The reaction mixture contained the compounds for testing at different concentrations and xanthine oxidase (25 mU/ml) with nitroblue tetrazolium (NBT) (50 μM) in Tris buffer (0.1 M, pH 7.4) to which hypoxanthine (3.5 mM) was added. The absorbance of the reaction mixture was monitored spectrophotometrically at 560 nm for 10 min. Values are expressed as means ± SD from three separate experiments.
* $p < 0.05$.

## DISCUSSION

In this study the antioxidant activity of a standard *M. indica* extract (used in Cuba as a food supplement) was evaluated in some *in vitro* experimental models.

Previously, it was reported that a standard aqueous Vimang extract administered to rats prevented hepatic injury and lipid peroxidaition after right-lobe blood occlusion for 45 min followed by 45 min of reperfusion /6/. Vimang administered to mice also decreased reactive oxygen species formation by peritoneal macrophages and prevented the increase in liver microsomal lipid peroxidation induced by tetradecanoylphorbol-13-acetate administration. In this *in vivo* experiment, Vimang was a better scavenger of ROS and inhibitor of oxidation tissue damage than vitamin C, vitamin E, a combination of vitamin C plus vitamin E, mangiferin, or β-carotene /7/.

In the study described here, Vimang prevented hepatocyte ROS formation and cytotoxicity induced by the $H_2O_2$ generating system (glucose/glucose oxidase) in a concentration dependent manner (Table 1). Vimang was not cytotoxic to intact rat hepatocytes and there was

no loss of cell viability over a 3 h incubation period, as assessed by trypan blue exclusion. Propyl gallate was more effective than gallic acid at inhibiting hepatocyte ROS formation and preventing hepatocyte cytotoxicity. Previously we showed that hepatocyte ROS formation induced by glucose/glucose oxidase was also prevented by hydroxyl radical scanvegers such as dimethylsulfoxide and mannitol /19/. Vimang also scavenged hydroxyl radicals or hypochlorous acid and acted as an iron chelator in *in vitro* experiments in the absence of hepatocytes /20/.

In order to determine the pro-oxidant activity of Vimang, hepatocytes were incubated with non-toxic concentrations of glucose/glucose-oxidase/horseradish peroxidase. However, Vimang at 50 μg/ml did not become cytotoxic when oxidized by $H_2O_2$/peroxidase (results not shown), as occurs with polyphenolic antioxidants /21/.

Lipid peroxidation (LPO) is probably the most extensively investigated process induced by free radicals. This process and subsequent cellular damage are regarded as an important mechanism underlying the toxicity of several xenobiotics /22/. Cumene hydroperoxide induced microsomal LPO is a common model for evaluating the effectiveness of antioxidants /23/. In this study, Vimang was able to inhibit the LPO and cytotoxicity induced by cumene hydroperoxide in a dose and time dependent manner. These results are also in accord with the Vimang inhibition of liver microsomal LPO induced by NADPH /24/. Propyl gallate was also more effective than gallic acid at preventing cumene hydroperoxide induced hepatocyte LPO and cytotoxicity.

Vimang was also tested in a superoxide-generating system. Two of its components, gallic acid and propyl gallate, were also evaluated, and propyl gallate was found to be as effective as Vimang, whereas gallic acid was less effective (Table 3). Propyl gallate is the propyl ester of the naturally occurring gallic acid, 3,4,5-trihydroxybenzoic acid, and is used as a synthetic antioxidant in processed foods, cosmetics and food packing materials to prevent rancidity induced by LPO /21/.The superoxide radical scavenging effect of Vimang could also be due at least in part to the presence of propyl gallate and gallic acid. This scavenger effect could also be due to the inhibition of xanthine oxidase, but Vimang at 100 μg/ml or propyl gallate at 10 μM was shown not to affect uric acid formation (at 340 nm) by xanthine oxidase and hypoxanthine (results not shown).

The main antioxidant component of the Vimang formulation is likely the gallate polyphenolics /21/, but the C-glucosylxanthone, mangiferin (40-60%), has also been shown to exhibit an antioxidant action in *in vitro* systems /25/.

In summary, these findings show that Vimang has antioxidant and superoxide radical scavenging properties in the models assayed. To our knowledge, this is the first report of the cytoprotective activity of Vimang in these experimental models.

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