JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Multiparametric evaluation of the cytoprotective effect of the *Mangifera indica* L. stem bark extract and mangiferin in HepG2 cells

Laia Tolosa[a], Idania Rodeiro[d], M. Teresa Donato[a,b,c], José A. Herrera[e], René Delgado[f], José V. Castell[a,b,c] and M. José Gómez-Lechón[a,c]

[a]Unidad de Hepatología Experimental, Centro de Investigación, Hospital La Fe, [b]Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Valencia, [c]CIBER de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain and [d]Departamento de Farmacología, Centro de Bioproductos Marinos (CEBIMAR), [e]Universidad de la Habana and [f]Departamento de Farmacología Centro de Investigaciones y Desarrollo de Medicamentos, (CIDEM), La Habana, Cuba

Keywords
cytoprotection; HepG2 cells; *Mangifera indica* L.; mangiferin; P-glycoprotein

Correspondence
Idania Rodeiro, Departamento de Farmacología, Centro de Bioproductos Marinos (CEBIMAR), Loma 37, Vedado, 10400, La Habana, Cuba.
E-mail: idania.rodeiro@infomed.sld.cu

Abbreviations
ABC, ATP binding cassette; AM, Amiodarone; DCF, 2′,7′-dichlorofluorescin; DCF-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, Dimethylsulphoxide; HCS, High-content screening; MDR-1, Multidrug resistance; MSBE, Mango (Mangifera indica L.) stem bark aqueous extract; MTT, 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; NR, Neutral red; t-BHP, t-butyl hydroperoxide; P-gp, P-glycoprotein; Rho-123, Rhodamine-123; ROS, Reactive oxygen species

Received August 9, 2012
Accepted March 18, 2013

doi: 10.1111/jphp.12071

Abstract

Objective Mango (Mangifera indica L.) stem bark extract (MSBE) is a natural product with biological properties and mangiferin is the major component. This paper reported the evaluation of the protective effects of MSBE and mangiferin against the toxicity induced in HepG2 cells by tert-butyl hydroperoxide or amiodarone.

Method Nuclear morphology, cell viability, intracellular calcium concentration and reactive oxygen species (ROS) production were measured by using a high-content screening multiparametric assay.

Key findings MSBE and mangiferin produced no toxicity below 500 mg/ml doses. A marked recovery in cell viability, which was reduced by the toxicants, was observed in cells pre-exposed to MSBE or mangiferin at 5–100 mg/ml doses. We also explored the possible interaction of both products over P-glycoprotein (P-gp). MSBE and mangiferin above 100 mg/ml inhibited the activity of P-gp in HepG2 cells.

Conclusions MSBE and mangiferin showed cytoprotective effects of against oxidative damage and mitochondrial toxicity induced by xenobiotics to human hepatic cells but it seemed that other constituents of the extract could contribute to MSBE protective properties. In addition, the drug efflux should be taken into account because of the inhibition of the P-gp function observed in those cells exposed to both natural products.

Introduction

Traditional medicine is based on the use of plants and plant extracts. Plants contain many classes of chemical constituents and they are extensively used worldwide for the treatment of different diseases. Polyphenols, which show strong antioxidant properties, have been identified as one of the main components of several medicinal herbs, foods and food supplements, and are responsible in part for their biological activities.[1–3]

The *Mangifera indica* L. (Anacardiaceae) stem bark aqueous extract (MSBE) has been used in Cuba for years in traditional medicine to improve the welfare of people suffering from different diseases, with emphasis placed on cancer patients. MSBE consists of a defined mixture of components (polyphenols, terpenoids, steroids, fatty acids and microelements).[4,5] Pharmacological screenings show MSBE to exhibit antioxidant, anti-inflammatory and

immunomodulatory properties.[5] Mangiferin is a xanthone and it is the major component in MSBE, representing up to 20% of the extract.[4] Several authors have identified mangiferin as the active principle of MSBE, and most of the extract biological activities have been attributed in part to this compound.[5] The pharmacological actions of mangiferin include antioxidant, analgesic,[6] antidiabetic,[7] anti-inflammatory,[8] antitumour, immunomodulatory and anti-HIV properties.[9,10]

Although natural medicines have beneficial effects, some of them may produce side effects. At present, it is accepted that the therapeutic intake of natural medicines should be sustained under a well-known risk–benefit balance, as for synthetic drugs. Toxicology preclinical studies performed to date show a broad margin of security for MSBE,[11,12] but since most MSBE components are polyphenolic structures that undergo extensive hepatic biotransformation, a more detailed evaluation of potentially harmful hepatic effects should be performed.

We previously examined the in-vitro effects of MSBE and mangiferin on hepatic cells using conventional cytotoxicity assays.[13] However, one major limitation of this kind of assay is its inability to measure a wide spectrum of potential early or late cytopathological changes involved in drug-induced toxic injury. Recently, multiparametric analyses of cytotoxicity at the single cell level using flow cytometry and cellular imaging-based approaches, such as high-content screening (HCS) technology, have been proposed as a new tool in the pre-lethal detection of toxicity and the classification of compounds based on their mechanisms of action.[14,15] Multiparametric and time-resolved assays are expected to greatly improve the prediction of toxicity as they evaluate complex toxicity mechanisms.[16,17] In order to study the toxicity effects of these products further, we examined the comparative evaluation of the effects of MSBE and mangiferin on the hepatic parameters in the human hepatoma HepG2 cell line. We used conventional assays and a HCS multiparametric approach that simultaneously measures nuclear morphology, cell viability, intracellular calcium concentration and reactive oxygen species (ROS). In addition, the potential cytoprotective effects of these natural products were evaluated by assessing their ability to reduce *in vitro* hepatotoxicity induced by tert-butyl hydroperoxide (t-BHP) and amiodarone, two model compounds known to produce oxidative stress and mitochondrial damage.

Finally, the effects of MSBE and mangiferin on the multidrug transporter P-glycoprotein (P-gp) were also evaluated. P-gp is acknowledged as being largely distributed in tumour and normal tissues, and it is particularly highly expressed in organs involved in the handling of chemicals, i.e. the liver, intestine and kidney. P-gp is encoded by the ABCB1 (MDR-1) gene and is the major determinant for the acquisition of the multidrug-resistant phenotype by cancer cells.[18] The potential interaction of phytoconstituents with hepatic P-gp could be determinant in their role in key pharmacodynamic events.

Intracellular calcium participates in many cellular functions and its levels are under highly regulated control, which can be altered by drugs.[19] In fact, Ca^{2+} activates proteases and endonucleases, and enhances ROS formation, thus leading to necrosis or apoptosis.[20] Additionally, ROS have been associated as second messengers in receptor tyrosine kinase signalling pathways, which may act as negative regulators of P-gp expression,[21] thus indicating that down-regulation of P-gp can be mediated via ROS.[22] The transport of P-gp substrates may therefore be affected by changing P-gp expression under conditions of chronic oxidative stress.[23] It was important to evaluate if MSBE and mangiferin exhibit antioxidant activity while inhibiting P-gp activity under similar experimental conditions. Our results provide rationale about the potential clinical use of these products as potential adjuvants to anticancer treatment for overcoming Pgp-mediated multidrug resistance in cancer patients.

Materials and Methods

Chemicals

Fluorescent probes Fluo-4 acetoxymethyl ester (Fluo-4 AM) and Rhodamine-123 were acquired from Molecular Probes, Invitrogen (Madrid, Spain); propidium iodide (PI), Hoechst 33342 and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were obtained from Sigma Aldrich (Madrid, Spain). Culture media and complements were purchased from Gibco (Gibco BRL, Paisley, UK). The other chemicals were purchased from Sigma Aldrich.

Plant material

Mangifera indica L. stem bark was collected from a cultivated field in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code 41722) were deposited at the Herbarium of Academy of Sciences, guarded by the Institute of Ecology and Systematics, Ministry of Science, Technology and Environment, Havana, Cuba. MSBE was prepared by decoction with water for 1 h and was later concentrated by evaporation and spray-dried to obtain a fine homogeneous brown powder with a particle size of 30–60 μm. The analytical analysis showed that the MSBE lot had the following content: moisture < 10%, water-soluble substances > 50%, total phenol (in an anhydrous base) > 30% according to the established quality specification. Mangiferin was isolated from the extract by extraction with methanol (95% purity). Characterization of the product's chemical composition was performed by using chro-

matographic methods, mass spectrometry and UV-vis spectrophotometry as described by Núñez *et al*.[4] Stock solutions of the products (1 mg/ml) were freshly prepared in culture medium and diluted to achieve the final concentrations used in each experiment.

Culture of HepG2 cells

HepG2 cells were cultured in Ham's F-12/Leibovitz L-15 (1 : 1 v/v) supplemented with 7% newborn calf serum, 50 U penicillin/ml and 50 μg streptomycin/ml. For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37°C. Cultures were used at 75% confluence. HepG2 cells were seeded into 96-well culture plates at a density of 5000 cells/well in order to study the hepatic effects of MSBE and mangiferin. Before treatments, cells were allowed to grow and equilibrate for 24 h.

Treatment of HepG2 cells with tested compounds

HepG2 cells were exposed to a wide range of concentrations of MSBE or mangiferin (10–1000 μg/ml) for 24 h in order to study the potential toxicity of both MSBE and mangiferin. Two experimental series were carried out for cytoprotection screening purposes. In the first one, cells were exposed for 24 h to MSBE or mangiferin at 5, 50 and 100 μg/ml (named here as pre-treatment experiments) prior to being exposed to toxic compounds, followed by either a further 3 h exposure to natural products and 5 μM t-BHP or a 24 h treatment with natural products and 10 μM amiodarone. In the second series, cells were exposed simultaneously for 24 h to t-BHP or amiodarone plus 5, 50 and 100 μg/ml of either MSBE or mangiferin but without a pre-incubation period (named here as the co-treatment experiments).

Cells were also incubated at increasing concentrations of MSBE or mangiferin (5–500 μg/ml) for 24 h in order to evaluate P-gp activity. Verapamil at 20 μM was used as a positive control; in this case, cells were incubated for 2 h in the presence of the inhibitor before measuring the protein function.

Cytotoxicity evaluation: the MTT test and the neutral red assay

Cytotoxicity of MSBE and mangiferin was evaluated in HepG2 cells by the MTT ([3–4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, Madrid, Spain) reduction test, as previously described.[24] The percentage of inhibition of the succinic dehydrogenase reduction of MTT was calculated in relation to the control cells in each experimental series.

The neutral red (NR) assay was performed according to the INVITTOX Protocol.[25] After exposure to natural products, the percentage of cell viability was calculated in relation to non-treated cells (assumed as 100% value).

The multiparametric assay using high-content screening

After treatments, HepG2 cells were washed and incubated with selected probes in order to measure multiple parameters indicative of cell toxicity: cytosolic-free calcium concentrations were detected using a Fluo-4 AM fluorescent probe; accumulation of the fluorescent compound 2′,7′-dichlorofluorescin (DCF), generated by intracellular oxidation of 2′,7′-dichlorofluorescin diacetate (DHCF-DA), was used as being indicative of ROS generation, primarily H_2O_2; cell viability was determined by propidium iodide (PI) exclusion; and Hoechst 33342 was used for nuclei staining and cell count. Fluorescent dyes were combined according to their optical compatibility in a standard flow cytometer. For each cell treatment (i.e. cells exposed to a concentration of a particular compound), three independent wells were simultaneously incubated with 1.5 μg/ml PI, 1.5 μg/ml Hoechst 33342, and 2 μg/ml DHCF-DA or 0.27 μg/ml Fluo-4 AM for 30 min.

After staining, samples were analysed with Scan^R (Olympus), an HCS system based on automated epifluorescence microscopy and image analyses of cells in a microtiter plate format. The 10X objective was used to collect images for the distinct fluorescence channels. Nine fields per well were imaged and analysed using the Scan^R analysis module, which allows not only the simultaneous quantification of the subcellular inclusions marked by different fluorescent probes, but also the measurement of fluorescence intensity associated with predefined nuclear and cytoplasmic compartments. Each measure was taken in individual cells and then averaged for the different treatments. The results of the compound-treated cells were expressed as a percentage of the corresponding parameter (intracellular calcium concentration, ROS generation or viability) in the non-treated cells (assumed as a 100% value).

The P-gp function assay

ABCB1/P-gp activity was evaluated by the Rhodamine-123 (Rho-123) accumulation assay, as described by Chieli *et al*.[26] Briefly, HepG2 cells treated for 24 h with MSBE or mangiferin were incubated for 2 h in the presence of 5 μg/ml Rho-123, then cells were lysed with 0.1% Triton X-100 to completely solubilise the probe. Aliquots of cell lysates were transferred to a 96-well plate and fluorescence intensity was measured at the 485 nm excitation and 535 nm emission wavelengths. Verapamil (20 μM), a known

modulator of P-gp, was used as an internal standard (positive control). Finally, data were expressed as a percentage of fluorescence accumulation in the control cells.

Statistical analysis

Each experimental procedure was performed in at least three cell preparations. Data were expressed as the mean ± SD. Statistical analyses of cell viability, nuclear changes, ROS and calcium concentration were performed by the Kruskal–Wallis ANOVA median test followed by the post-hoc Dunn's test using the Statistica for Windows software package (Release 4.5 F, Statsoft Inc., 1993). For the protection studies, the results were transformed into a percentage of controls (non-treated cells). The fluorescence accumulation percentage values of the P-gp study were analysed using the Mann–Whitney *U*-test. *P* values below 0.05 were considered statistically significant.

Results

Cytotoxicity evaluation of MSBE and mangiferin

The safety margin of MSBE and mangiferin was evaluated in first place by conventional MTT and NR assays and later their effects on the hepatic parameters were investigated using the HCS multiparametric assay. No cytotoxic effects were observed after exposing cells to MSBE and mangiferin for 24 h at any of the concentrations tested (10–1000 μg/ml) (Figure 1). The same range of concentrations was then selected to evaluate the potential hepatotoxicity of MSBE and mangiferin using the multiparametric HCS. Treatment of HepG2 cells with 500 μg/ml MSBE produced a drop of 20% in the nuclear area (an indicator of cell death), while 1000 μg/ml MSBE brought about 25% increase in cell viability and a 30% reduction in nuclear size (Figure 2). No significant changes in ROS production or intracellular calcium levels were induced by MSBE (Figure 2). The evaluated parameters showed no significant changes in those cells treated with up to 1000 μg/ml of mangiferin (Figure 2).

Cytoprotection evaluation of MSBE and mangiferin by HCS

In order to evaluate the potential cytoprotective properties of MSBE and mangiferin, we evaluated their effects on cell viability, intracellular calcium concentration and ROS production after pre-incubating or co-incubating HepG2 cells with MSBE or mangiferin. In the pre-incubation approach, cells were exposed to the products for 24 h, followed by another incubation lasting 3 or 24 h with 5 μM t-BHP or 10 μM amiodarone. In the co-incubation approach, cells were incubated simultaneously with MSBE or mangiferin plus one of the toxins for the same time periods. A range of MSBE and mangiferin concentrations of up to 100 μg/ml were used to evaluate their cytoprotective effects.

HepG2 cells exposed to 5 μM t-BHP for 3 h showed a 54% reduction in cell viability, a 7.5-fold increase in ROS production and a 2.7-fold increase in intracellular calcium levels when compared to non-treated cells (Figure 3). Noticeable dose-dependent recovery of cell viability (up to 85%), prevention of the changes in the intracellular calcium levels and ROS production (oxidative stress) induced by 5 μM t-BHP were observed in cells pre-exposed to MSBE before t-BHP treatment. A similar level of reduction in ROS generation was noted in the cells co-exposed to the extract and t-BHP, whereas the effects observed in cell viability and the intracellular calcium levels were milder than those in pre-treated cells (Figure 3). The pre- and co-treatments of mangiferin and t-BHP induced similar cytoprotective effects to MSBE at the intracellular calcium levels, whereas

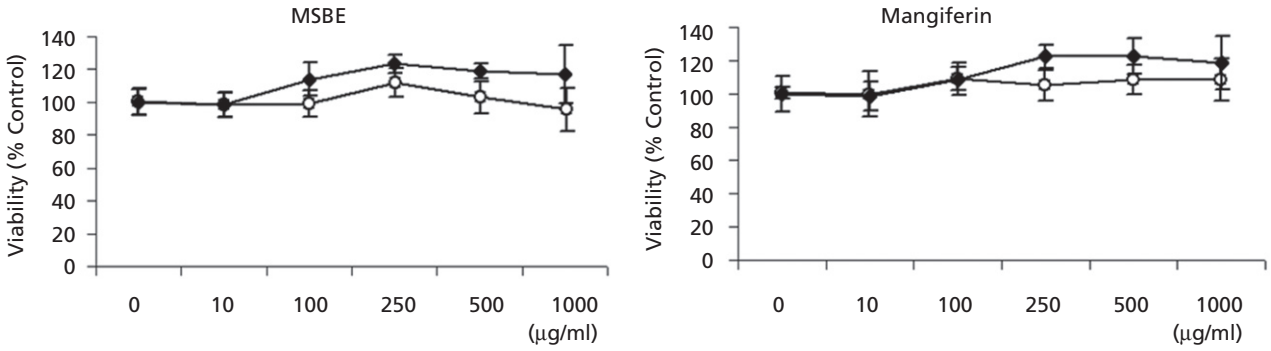

Figure 1 Cytotoxic study of the *Mangifera indica* L. extract (MSBE) and mangiferin on Hep G2 cells. Cells were exposed to increasing concentrations of the products, and cell viability was determined 24 h later by the MTT (solid rhombus) or Neutral Red (hollow circles) test. The results are expressed as the percentage in regard to controls (untreated cells). Each point represents the mean ± SD of three experiments with six replicates.

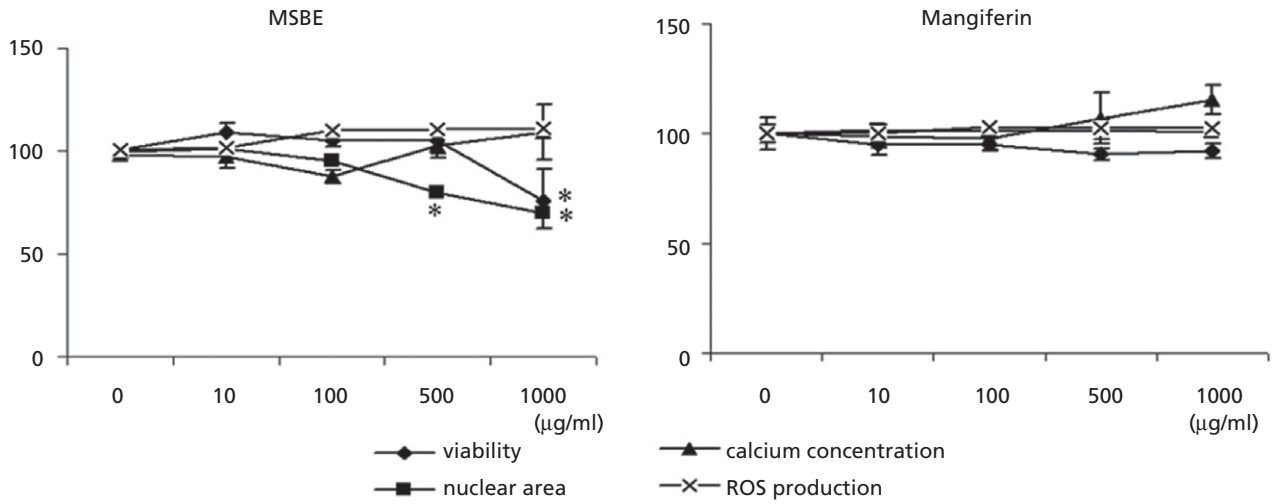

Figure 2 Cytotoxic evaluation of the *Mangifera indica* L. extract (MSBE) and mangiferin on HepG2 cells. Cells were exposed to increasing concentrations of the products and cell viability was determined 24 h later by the HCS multiparametric test. The results are expressed as the percentage in regard to controls (untreated cells). Each point represents the mean ± SD of three experiments with three replicates, $*P < 0.05$ (Kuskal–Wallis ANOVA median test followed by post-hoc Dunn's test).

mangiferin elicited milder effects on cell viability and ROS generation (Figure 3).

HepG2 cells treated with 10 μM amiodarone for 24 h showed significant reduction in cell viability (up to 65% of non-treated cells), increased intracellular calcium (3.4-fold of non-treated cells) and ROS production (2.5-fold) (Figure 4). MSBE and mangiferin also led to a general reduction in the toxic effects induced by amiodarone (Figure 4).

Effects of MSBE and mangiferin on the P-gp function

Intracellular fluorescence increased in a dose-dependent fashion in HepG2 cells exposed to MSBE or mangiferin for 24 h (Figure 5). Rho-123 is a fluorescent dye that passively enters cells and concentrates in mitochondria. As P-gp substrate, the intracellular loading of this probe is inversely proportional to P-gp activity. For both products, the inhibitory effects produced at the highest concentration tested (500 μg/ml) were greater than those produced by the positive control (20 μM verapamil).

Discussion

MSBE and mangiferin appeared to be safe under the MTT and NR assays at high concentrations (up to 1000 μg/ml) in human hepatic HepG2 cells (Figure 1) in agrement with our previous report in rat hepatic primary cultures.[13] However, HCS allowed us to detect slight changes produced by MSBE in cell viability at 1000 μg/ml and in nuclear size induced at 500 μg/ml (Figure 2), which were not detected with the single-parameter readouts. Multiparametric cell-based HCS protocol allowed us to perform a more sensitive evaluation of the potentially drug-induced hepatotoxicity than the conventional cytotoxicity assays do. Until now the latter were the only available data source regarding in-vitro toxicity of MSBE and mangiferin. Our findings supported the current thesis, which states that cytotoxicity assays based on the measure of lethal events usually offer poor sensitivity.[14] HCS allows the simultaneous evaluation of different pathways as well as detecting pre-lethal effects, which notably increase assay sensitivity.[15–17,27] Accordingly, we examined the potential cytoprotective effects of MSBE and mangiferin against t-BHP- and amiodarone-induced cell damage using the multiparametric HCS assay.

Two pathways have been described for t-BHP metabolism in hepatocytes. The first one is related to the microsomal P450 system, which produces ROS species and initiates lipid peroxidation. The second one involves the conversion of t-BHP by GSH peroxidase into *t*-butanol and oxidised glutathione disulphide (GSSG). GSSG is later reduced to GSH by GSH reductase, resulting in NAPDH oxidation. The drop in GSH and oxidised NADPH (NADP+) concentrations contribute to alter Ca^{2+} homeostasis, which is considered a major event in t-BHP toxicity.[28] Amiodarone induces mitochondrial impairment by inhibiting both mitochondrial beta-oxidation and oxidative phosphorylation, and produces mitochondria depolarization[29] and the disruption of intracellular Ca^{2+} homeostasis through direct action on the mitochondria.[30] Generation of ROS by the damaged respiratory chain can also be augmented which, in turn, induces lipid peroxidation.[31] Severe and prolonged impair-

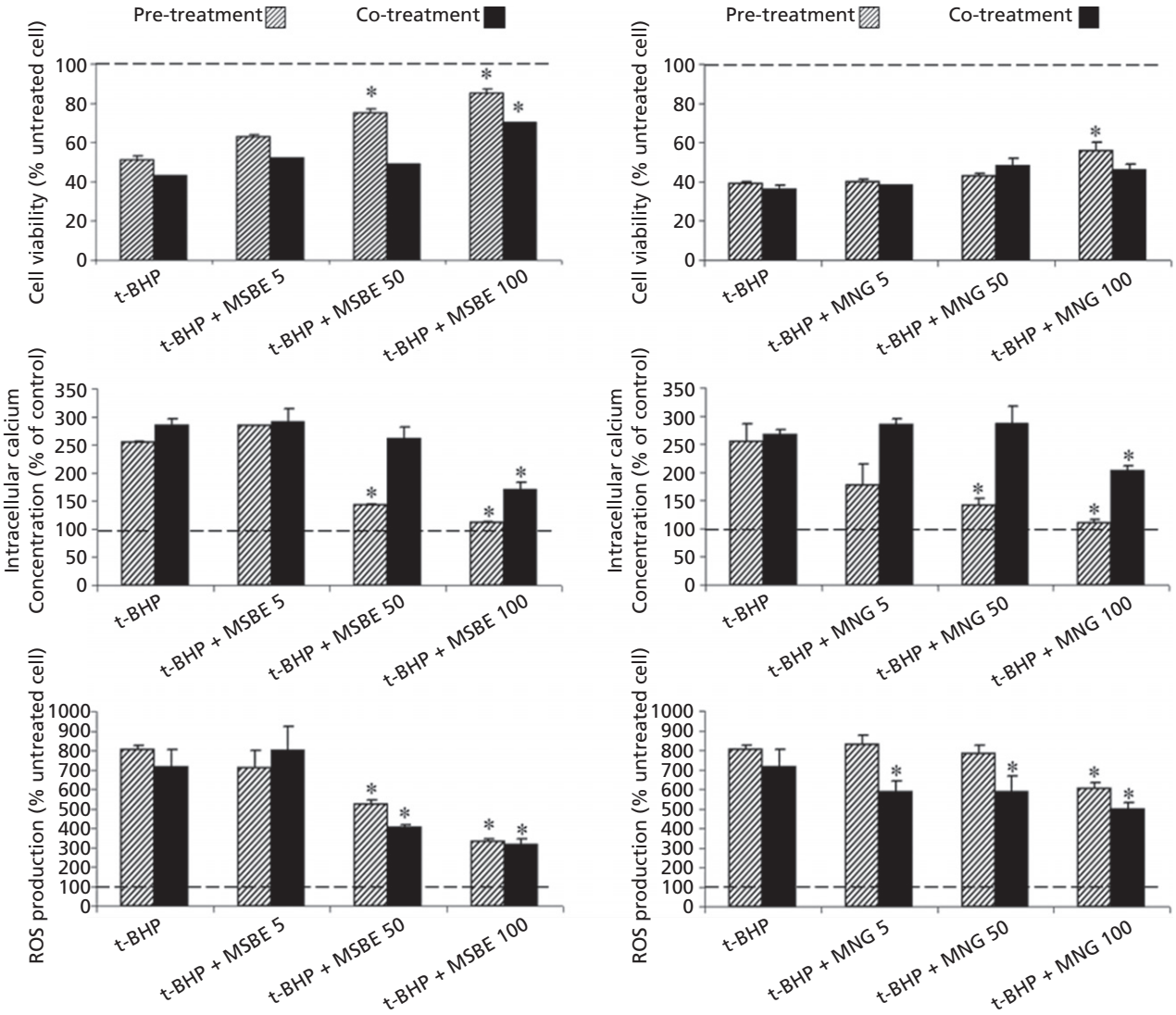

Figure 3 Effects of the *Mangifera indica* L. extract (MSBE) and mangiferin (MNG) on t-butyl hydroperoxide (t-BHP)-induced hepatotoxicity to Hep G2 cells. Pre-treatment: cells were exposed for 24 h to increasing concentrations of MSBE or MNG and later to a 3-h additional period in the presence of the natural product and 5 μM of t-BHP. Co-treatment: cells were exposed to 5 μM t-BHP for 24 h and to increasing concentrations of MSBE or MNG. Finally, cell viability, intracellular calcium concentration and ROS production were determined by the HCS multiparametric test. Results are expressed as the percentage in regard to control (untreated) cells. Each point represents the mean ± SD of three experiments with three replicates. $*P < 0.05$ in relation to cells treated with the toxin (Kuskal–Wallis ANOVA median test followed by post-hoc Dunn's test).

ment of mitochondrial beta-oxidation induced by amiodarone leads to microvesicular steatosis.[32] As expected, cell viability decreased, while ROS production and intracellular calcium levels increased after treatment with t-BHP and amiodarone.[27,33]

Pre-treatment with MSBE or mangiferin counteracted, in a dose-dependently fashion, the changes in cell viability, intracellular Ca^{2+} levels and ROS production in HepG2 cells (Figures 3 and 4). Similarly, MSBE and mangiferin showed protection against cell death in rat hepatocytes and lymphocytes.[34,35] Pre-treatment with mangiferin before cadmium chloride administration reduces lipid peroxidation in mice livers, whereas it increases the antioxidant defence mechanisms like hepatic GSH levels, glutathione-S-transferase, superoxide dismutase and catalase activities.[36] However, evaluating several biomarkers of damage within intact cells led us to support previous allegations that the in-vitro hepatoprotective role of MSBE and mangiferin is probably due to the scavenging of free radicals and the increased antioxidant status of cells against the insult of

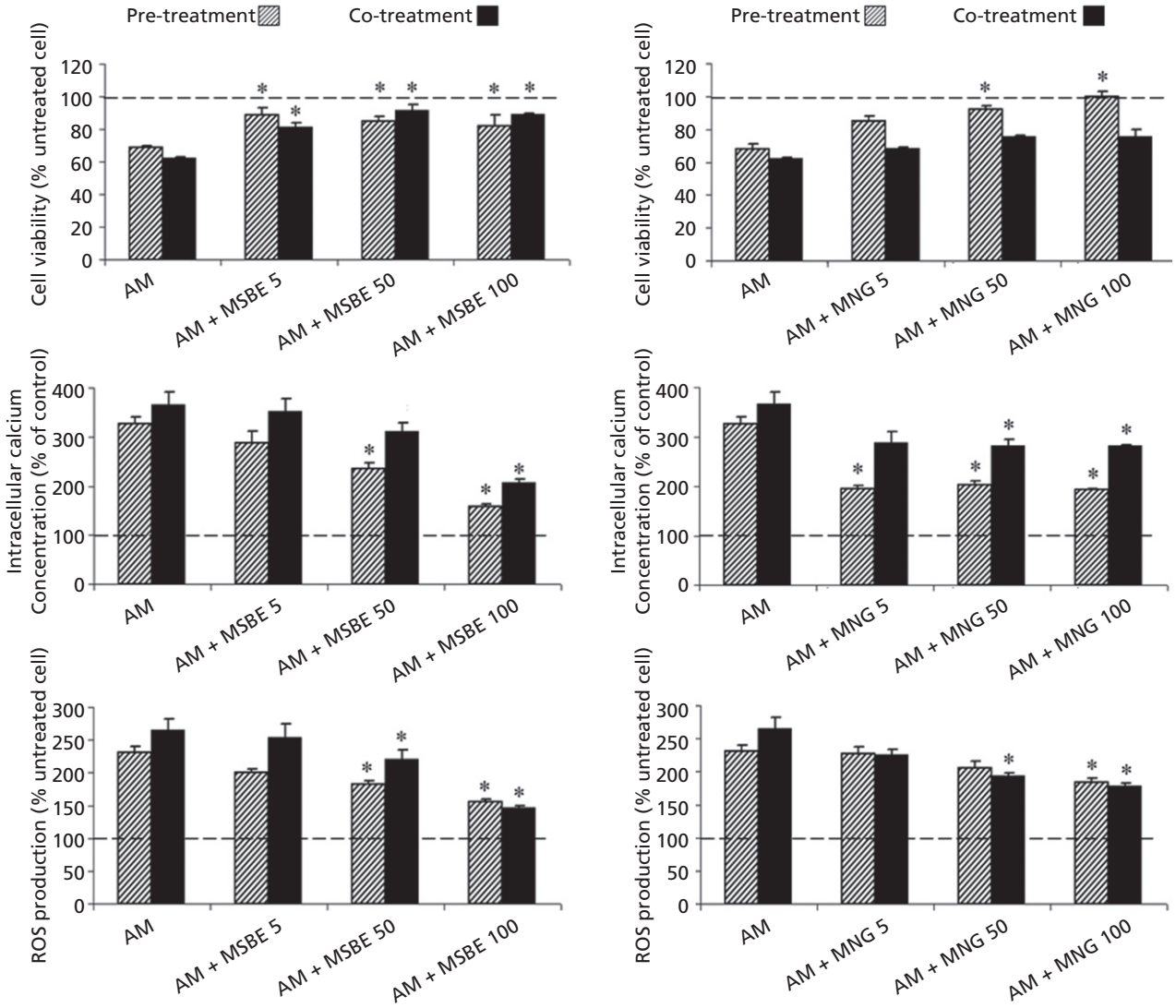

Figure 4 Effects of the *Mangifera indica* L. extract (MSBE) and mangiferin (MNG) on amiodarone (AM)-induced hepatotoxicity to Hep G2 cells. Pre-treatment: cells were exposed to increasing concentrations of MSBE or MNG for 24 h and later to a 24-h additional period with the natural product and 10 μM AM. Co-treatment: cells were exposed for 24 h to 10 μM of AM and to increasing concentrations of MSBE or MNG. Finally, cell viability, intracellular calcium concentration and ROS production were determined by the HCS multiparametric test. Results are expressed as the percentage in regard to control (non-treated) cells. Each point represents the mean ± SD of three experiments with three replicates. $*P < 0.05$ in relation to cells treated with the toxin (Kuskal–Wallis *ANOVA* median test followed by post-hoc Dunn's test).

toxicants.[34,36] In addition, our results suggest that pre-treatment with these natural products, rather than the co-treatment with hepatotoxins, seems to counteract the harmful effects of t-BHP and amiodarone in a more efficient way.

MSBE and mangiferin decreased P-gp activity in a strongly dose-dependent fashion, according to the Rho-123 cellular accumulation test, thus achieving about 2-fold fluorescence increase when compared to verapamil, a well-known P-gp inhibitor (Figure 5). It has been previously reported that these products inhibit P-gp activity/expression in HK and Caco cell lines,[26,37] which suggests that these effects are likely due to direct interference with the P-gp function. Several compounds have been identified as capable of overruling multidrug resistance by interfering with the P-gp-mediated export of the drugs used in the treatment of different diseases, like cancer.[38,39] The search for modulators which selectively inhibit P-gp activity without negative side effects is currently of great interest and hopes are pinned on the application of some plant polyphenols.[40] Our findings suggested that MSBE and mangiferin are potential inhibitors of the drug efflux

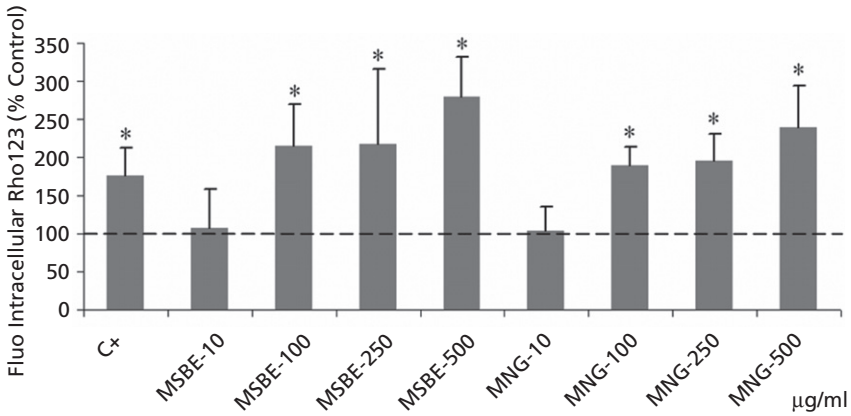

Figure 5 Influence of the *Mangifera indica* L. extract (MSBE) and mangiferin (MNG) on the P-gp activity of HepG2 cells. Cells were incubated with MSBE or MNG for 24 h. P-gp activity was assayed by the Rho 123 test. Results are expressed as percentage of fluorescence accumulation with regard to control (untreated) cells. Verapamil (20 μM) was used as an internal positive control. Bars represent the means ± SEM of three independent experiments with three replicates. $*P < 0.05$ in relation to controls (Mann–Whitney test).

mediated by hepatic P-gp. However, more in-vitro and in-vivo studies are needed to confirm the potential clinical relevance of this interaction.

Several authors propose mangiferin as the active principle of MSBE and suggest it is responsible for the biological activities of MSBE.[4,5,34,41] Our results show that mangiferin prevented the increase in calcium and ROS levels, but it also lowered the decrease in cell viability (Figures 3 and 4), thus suggesting that the cytoprotective effects of MSBE are due, at least in part, to its high mangiferin content. However, 100 μg/ml of MSBE (with an estimated 20 μg/ml mangiferin content) induced higher or similar hepatoprotective effects than 100 μg/ml mangiferin, which indicates that other constituents of the extract, for example gallic acid, benzoic acids and catechins, might contribute to these effects.

Concluding Remarks

In summary, we reported the cytoprotective effects of MSBE and mangiferin against oxidative damage and mitochondrial toxicity induced by xenobiotics to human hepatic cells. Mangiferin, the main constituent of the extract, and MSBE produced similar effects on cultured HepG2 cells, but it seemed that other constituents of the extract could contribute to its protective properties. In addition, our study suggested that the drug efflux should be taken into account because of the inhibition of the P-gp function observed in those cells exposed to both natural products.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work has been supported by the Spanish Ministry of Science (Grant Sab-2009/0187 to Dr I. Rodeiro), funds from the ALIVE Foundation and the Spanish Ministry of Health (Instituto de Salud Carlos III; IF08/3638 and PI10/00923). Dr L. Tolosa was a recipient of a Sara Borrell Contract from the Instituto de Salud Carlos III from the Spanish Ministry of Economy and Competitiveness.

Acknowledgements

The technical assistance of N. Jiménez and A. Negro is acknowledged.

References

1. Masella RT *et al*. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J Nutritional Biochem* 2005; 16: 577–586.
2. Chen D, Ping Q. Tea polyphenols and their roles in cancer prevention and chemotherapy. *Int J Mol Sci* 2008; 9: 1196–1206.
3. Link A *et al*. Cancer chemoprevention by dietary polyphenols: promising role for epigenetics. *Biochemical Pharmacol* 2010; 80: 1771–1792.
4. Núñez A *et al*. Isolation and quantitative analysis of phenolic antioxidants, free sugars and polyphenols from mango (*Mangifera indica* L.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *J Agric Food Chem* 2002; 50: 762–766.

5. Rivera D *et al*. *Mangifera indica* L. extract (Vimang) and mangiferin reduce the airway inflammation and Th2 cytokines in murine model of allergic asthma. *J Pharm Pharmacol* 2011; 63: 1336–1345.
6. Dar A *et al*. Analgesic and antioxidant activity of mangiferin and its derivatives: the structure activity relationship. *Biol Pharm Bull* 2005; 28: 596–600.
7. Muruganandan S *et al*. Effect of mangiferin on hyperglycemia and atherogenicity in streptozotocin diabetic rats. *J Ethnopharmacol* 2005; 97: 497–501.
8. Ojewole O. Anti-inflammatory, analgesic and hypoglycemic effects of *Mangifera indica* Linn. (Anacardiaceae) stem-bark aqueous extract. *Methods Find Exp Clin Pharmacol* 2005; 27: 547–554.
9. Zheng MS, Lu ZY. Antiviral effect of mangiferin and isomangiferin on herpes simplex virus. *Chin Med J* 1990; 103: 160–165.
10. Rajendran P *et al*. Protective role of mangiferin against benzo(a)pyrene induced lung carcinogenesis in experimental animals. *Biol Pharm Bull* 2008; 31: 1053–1058.
11. Rodeiro I *et al*. Evaluation of the genotoxic potential of *Mangifera indica* L. extract (Vimang), a new natural product with antioxidant activity. *Food Chem Toxicol* 2006; 44: 1707–1713.
12. Garrido G *et al*. In vivo toxicological studies of an antioxidant extract from *Mangifera indica* L. (Vimang). *Drug Chem Toxicol* 2009; 32: 52–58.
13. Rodeiro I *et al*. Modulation of P450 enzymes by Cuban natural products rich in polyphenolic compounds in rat hepatocytes. *Chem Biol Interact* 2007; 172: 1–10.
14. Abraham VC *et al*. Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans. *J Biomol Screen* 2008; 13: 527–537.
15. Tolosa L *et al*. Development of a multiparametric cell-based protocol to screen and classify the hepatotoxicity potential of drugs. *Toxicol Sci* 2012; 127: 187–198.
16. Dambach DM *et al*. New technologies and screening strategies for hepatotoxicity: use of in vitro models. *Toxicol Pathol* 2005; 33: 17–26.
17. O'Brien PJ *et al*. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Arch Toxicol* 2006; 80: 580–604.
18. Gillet JP *et al*. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochim Biophys Acta* 2007; 1775: 237–262.
19. Dong Z *et al*. Calcium in cell injury and death. *Annu Rev Pathol* 2006; 1: 405–434.
20. Grattagliano I *et al*. Biochemical mechanisms in drug-induced liver injury: certainties and doubts. *World J Gastroenterol* 2009; 15: 4865–4876.
21. Wartenberg M *et al*. Down-regulation of intrinsic P-glycoprotein expression in multicellular prostate tumor spheroids by reactive oxygen species. *J Biol Chem* 2001; 276: 17420–174208.
22. Wartenberg M *et al*. Redox regulation of P-glycoprotein-mediated multidrug resistance in multicellular prostate tumor spheroids. *Int J Cancer* 2000; 85: 267–274.
23. Hong H *et al*. Up-regulation of P-glycoprotein expression by glutathione depletion-induced oxidative stress in rat brain microvessel endothelial cells. *J Neurochem* 2006; 98: 1465–1473.
24. Jover R *et al*. Evaluation of the cytotoxicity of ten chemicals on human cultured hepatocytes: predictability of human toxicity and comparison with rodent cell culture systems. *Toxicol In Vitro* 1992; 6: 47–52.
25. INVITTOX Protocol. The FRAME modified neutral red uptake cytotoxicity test. 1990, 3a.
26. Chieli E *et al*. In vitro effects of *Mangifera indica* and polyphenols derived on ABCB1/P-glycoprotein activity. *Food Chem Toxicol* 2009; 47: 2703–2710.
27. Xu JJ *et al*. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chem Biol Interact* 2004; 150: 115–128.
28. Lee HS *et al*. Antioxidant effects of aqueous extract of *Terminalia chebula* in vivo and in vitro. *Biol Pharm* 2005; 28: 1639–1644.
29. Donato MT *et al*. High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay. *J Biomol Screen* 2012; 17: 394–400.
30. Himmel HM *et al*. N-desethylamiodarone modulates intracellular calcium concentration in endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol* 2000; 362: 489–496.
31. Begriche K *et al*. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 2006; 6: 1–28.
32. Fromenty B, Pessayre D. Impaired mitochondrial function in microvesicular steatosis. Effects of drugs, ethanol, hormones and cytokines. *J Hepatol* 1997; 26: 43–53.
33. Kikkawa R *et al*. In vivo hepatotoxicity study of rats in comparison with in vitro hepatotoxicity screening system. *J Toxicol Sci* 2006; 31: 23–34.
34. Rodeiro I *et al*. Potential hepatoprotective effects of new Cuban natural products in rat hepatocytes culture. *Toxicol In Vitro* 2007; 22: 1242–1249.
35. Hernandez P *et al*. Protective effect of *Mangifera indica* L. polyphenols on human T lymphocytes against activation-induced cell death. *Pharmacol Res* 2007; 7: 173–176.
36. Viswanadh EK *et al*. Antigenotoxic effect of mangiferin and changes in antioxidant enzyme levels of Swiss albino mice treated with cadmium chloride. *Hum Exp Toxicol* 2010; 29: 409–418.
37. Chieli E *et al*. In vitro modulation of ABCB1/P-glycoprotein expression by polyphenols from *Mangifera indica*. *Chemico Biol Interact* 2010; 186: 287–294.
38. Pu W *et al*. Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of

current strategies. *Curr Mol Pharmacol* 2008; 1: 93–105.

39. Takano M *et al*. Paclitaxel-resistance conferred by altered expression of efflux and influx transporters for paclitaxel in the human hepatoma cell line, HepG2. *Drug Metab Pharmacokinet* 2009; 24: 418–427.
40. Bansal T *et al*. Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy. *J Pharm Pharm Sci* 2009; 12: 46–78.
41. Pardo G *et al*. *Mangifera indica* L. (Vimang) protection against serum oxidative stress in elderly humans. *Arch Med Res* 2006; 37: 158–161.