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Scavenger effect of a mango (*Mangifera indica* L.) food supplement's active ingredient on free radicals produced by human polymorphonuclear cells and hypoxanthine–xanthine oxidase chemiluminescence systems

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Abstract

The *in vitro* antioxidant and free radical scavenging properties of a stem bark aqueous extract of mango tree (*Mangifera indica* L.), whose formulations are used in Cuba as food supplements under the brand name of Vimang®, were studied. Luminol-enhanced chemiluminescence was used to elucidate the effect of this extract on the generation of reactive oxygen species in PMA- or zymosan-stimulated human polymorphonuclear leukocytes and on superoxide radicals generated in the hypoxanthine–xanthine oxidase reaction. Chemiluminescence was reduced in a dose-dependent manner at extract concentrations from 5 to 100 μg/ml, most probably by inhibiting the superoxide generation reaction. Part of this *M. indica* extract antioxidant activity could be ascribed to the presence of mangiferin as its main component.

Keywords: Antioxidant activity; Chemiluminescence; *Mangifera indica*; Vimang; Mangiferin; Hypoxanthine–xanthine oxidase

1. Introduction

Oxygen radicals and others activated oxygen species are common products of cellular metabolism (Clapperton, McMurray, Fisher, & Dargie, 1995; Pantano, Reynaert, van der Vliet, & Janssen-Heininger, 2006). Formation of activated oxygen compounds occurs during the respiratory burst of phagocytic cells including neutrophils, monocytes and macrophages, with the generation of photons of very weak light signal, which may be amplified and measured as chemiluminescence (CL). The generation of CL has been reported to involve superoxide, singlet oxygen, hydrogen peroxide, hydroxyl radicals and myeloperoxidase-catalysed reactions (Catala, 2006; Hsu, Raung, Tsao, Lin, & Wang, 1997; Meretey et al., 1987). However, most of the radicals detected by the luminol-CL (LCL) system originated primarily from the dismutation of superoxide. Therefore, different oxygen species formed during the activation of leukocytes and assessed by means of LCL have been used to evaluate different agents, which affect free radical production, e.g. non-steroidal anti-inflammatory drugs (Paino et al., 2005).

On the other hand, much evidence has supported the involvement of reactive oxygen species (ROS) in some pathological conditions like ischemia-reperfusion injury, inflammatory and degenerative diseases, cancer, DNA damage and ageing, among others. Substances that have antioxidant or free radical scavenger properties can interfere with these pathological processes, leading to the

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improvement of the quality of life (Aruoma, 2003; Maxwell, 1997). The active principles in extracts from a variety of plant sources continue to be of interest for use in complementary medicine supplements. Mainly, most of the extracts contain important vitamins, flavonoids, terpenoids and other polyphenols with antioxidant activity (Aruoma, 2003).

It is for that reason an aqueous extract from the stem bark of *Mangifera indica* L. (Anacardiaceae) has been used in Cuban traditional medicine for decades, on patients suffering from several types of stress (Guevara et al., 2002) and their formulations have been registered under the brand name of Vimang®. The chemical composition of this extract has been reported elsewhere (Center of Pharmaceutical Chemistry, 2002; Núñez-Sellés et al., 2002; Núñez-Sellés et al., 2007) and has enabled the isolation and identification of phenolic acids (gallic acid, 3,4 dihydroxy benzoic acid, benzoic acid), phenolic esters (gallic acid methyl ester, gallic acid propyl ester, benzoic acid propyl ester), flavan-3-ols (catechin and epicatechin) and the xanthone mangiferin as the major component (10–20%). Biologically active terpenoids like beta-elemene, beta-selinene, alpha-guaiene, hinesol and beta-eudesmol have been also identified. Calcium, copper, zinc and selenium have been quantified, the latter in quantities to fulfill the daily recommended allowance given by nutritional regulatory bodies. Other components such as free sugars, polyalcohols, sterols and unsaturated fatty acids (as myristic, palmitic, stearic, oleic–linoleic and eicosatrienoic) have been also reported and quantified.

Recently, the first analgesic and anti-inflammatory effects from this *M. indica* aqueous stem bark extract (MSBE) was reported. This extract inhibited the abdominal constriction induced by acetic acid and formalin-induced licking in mice, and the edema induced by carrageenan and formalin in mice, rats and guinea-pigs (Garrido et al., 2001). Additionally, it has been demonstrated that the extract has a powerful scavenger activity against hydroxyl radicals and hypochlorous acid. This extract also presented a significant inhibitory effect on the peroxidation of rat-brain phospholipid and inhibited DNA damage by bleomycin or copper phenanthroline systems (Martínez et al., 2000). It was compared the protective abilities of the extract, mangiferin and other well-known antioxidants, against the tetradecanoylphorbol acetate-induced oxidative damage in serum, liver, brain as well as in the hyper-production of ROS by peritoneal macrophages. It could be concluded that the stem bark extract of *M. indica* prevented the production of ROS and the oxidative tissue damage *in vivo* and it was more active than vitamin C, vitamin E, mangiferin and β-carotene (Sánchez et al., 2000).

In the present study the effect of the MSBE and mangiferin on the luminol-amplified luminous signal obtained from polymorfonuclear leukocytes (PMN) activated with particulate (zymosan) and soluble (phorbol-myristate-acetate, PMA) initiators and the hypoxanthine–xanthine oxidase reaction was evaluated.

An inhibitory effect of MSBE in both systems was demonstrated, which support previous findings about the antioxidant and anti-inflammatory properties of this natural product. The major component of this extract, the xanthone mangiferin, is involved in only part of these effects.

2. Materials and methods

2.1. Drugs

M. indica was collected from a cultivated field located 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 Systematic from Ministry of Science, Technology and Environmental, La Habana, Cuba. Stem bark extract of *M. indica* was prepared by decoction for 1 h. The extract was concentrated by evaporation and spray dried to obtain a fine brown powder, which was used as the standardised active ingredient of Vimang® formulations. It melts at 210–215 °C, with decomposition. The chemical composition of this extract has been characterised by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV/VIS spectrophotometry (Núñez-Sellés et al., 2002). The solid extract was dissolved in distilled water for pharmacological studies.

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetra-hydroxy-9H-xanthen-9-one) was supplied by the Laboratory of Analytical Chemistry, Center of Pharmaceutical Chemistry (Cuba). It was purified from *M. indica* stem bark standardised extract by extraction with methanol and its purity (90%) was assessed (Núñez-Sellés et al., 2002).

2.2. Reagents

Luminol, hipoxanthine, dimethyl sulphoxide (DMSO), phorbol-myristate-acetate (PMA), xanthine oxidase (0.19 U/mg) from milk, superoxide dismutase (5333 U/mg protein) from bovine erythrocytes and zymosan A were purchased by Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline solution (PBS) was made with the following composition (mM): 140 NaCl, 2.7 KCl, 12 Na_2HPO_4, 1.5 KH_2PO_4, 0.9 $CaCl_2$ and 0.49 $MgCl_2$, pH 7.4.

A stock solution of PMA (2 mg/ml) in DMSO was prepared. Before use, it was diluted to give a final concentration of 16 μM. Luminol stock solution (10^{-2} M) in DMSO was prepared and it was further diluted to 10^{-4} M in PBS prior to use. All other reagents were of analytical grade.

2.3. PMN leukocytes isolation

PMN were isolated from 10 ml heparinised (20 U/ml) venous blood obtained, from healthy volunteers who had not taken any drug during the two weeks before blood sampling (Pascual, González, & Romay, 1991). The blood was mixed with an equivalent volume of dextran T500 (6%

w/v in NaCl 0.9%). After mixing well and allowing to stand for 45–60 min at room temperature, the upper layer was removed by aspiration. Then, it was mixed with an equivalent volume of PBS and centrifuged for 10 min at 500*g* at 4 °C. After that, 5 ml of distilled water was added to the pellet to lyse red cells and the centrifugation step was repeated after addition of 15 ml PBS. The pellet was resuspended in 1 ml of PBS and the polymorphs counted and diluted to a concentration of 5×10^6 cells/ml in PBS. Cell suspensions containing more than 95% viable PMN, assessed by the trypan blue exclusion test, were used within 2 h of cell isolation.

The studies were performed according to the guidelines of the Ethical Committee of the Center of Pharmaceutical Chemistry, which are in accordance with international regulations.

2.4. Opsonisation of particles

Zymosan was opsonised immediately prior to the experiment by suspending 50 mg in 3 ml fresh human serum and 1 ml PBS according to Romay et al. (1998). The mixture was incubated for 30 min at 37 °C and then it was centrifuged for 10 min at 1500*g*. The supernatant was removed and the pellet washed with 4 ml PBS. After a second centrifugation step, the pellet was re-suspended in PBS at a concentration of 12.5 mg/ml.

2.5. Chemiluminescence assay of leukocytes

It was performed as described by Pascual et al. (1991) with minor modifications. One hundred μl of leukocyte suspension (5×10^6 cell/ml), 450 μl PBS buffer and 200 μl luminol 10^{-4} M were incubated for 5 min with 50 μl of MSBE (in a range of 1–100 μg/ml) in the measuring cuvette. Just before the assay, 200 μl of opsonised zymosan or PMA were added and the light intensity was measured at 37 °C. The reaction was observed for 8 min (PMA) or 20 min (zymosan) in a LKB Wallac 1250 luminometer, which was coupled to an LKB 2210 two channel recorder. In a system without cells, there was no interaction between luminol and MSBE. The viability of PMN after being exposed to the higher MSBE concentrations for 40 min at 37 °C was 94%, which was assessed by the trypan blue exclusion test. The results were expressed as the means of the areas under the curve (AUC) obtained for each MSBE concentration and control.

2.6. Chemiluminescence produced by the hypoxanthine–xanthine oxidase reaction

The superoxide radical scavenging activity of MSBE was determined as described by Pascual and Romay (1992). The reaction consisted of 800 μl of a mixture containing 68 mM glycine buffer at pH 8.6, 10 μM luminol and 5 μM *p*-iodophenol. Distilled water (50 μl) or MSBE (1–100 μg/ml) aqueous solution were added. Then 2 μl of xanthine oxidase (2 U/ml) were added and the reaction was initiated with 10 μl of hypoxanthine 1 mM. The intensity was registered immediately. Enzyme superoxide dismutase (SOD) was used in the system as the reference substance.

2.7. Statistical analysis

The results are presented as the mean ± S.D. of at least three independent experiments. Statistical significance between each group with respect to the control group was determined by means of one-way analysis of variance (ANOVA) followed by unpaired Student's *t*-test to determine statistical significance. p values less than 0.05 were considered as indicative of significance. Regression analysis was used to calculate the inhibitory concentration 50 (IC_{50} value), defined as the concentration of each drug necessary to produce 50% inhibition of each experiment.

3. Results

3.1. Effect of M. indica extract and mangiferin on PMA- and zymosan-stimulated-PMN chemiluminescence system

The effects of MSBE and mangiferin on the PMA-stimulated-PMN CL system are shown in Table 1. Both the extract (5–100 μg/ml) and mangiferin (1–20 μg/ml) exhibited a dose-dependent inhibitory effect on the CL signal. The IC_{50} values were 24.8 μg/ml (MSBE) and 4.4 μg/ml (mangiferin).

The inhibitory effect of MSBE was also observed in zymosan-stimulated PMN. Human leukocytes stimulated with opsonised zymosan produced a typical dose-dependent response (Table 1). The CL signal (AUC) was significantly reduced regarding the control with an $IC_{50} =$ 25.7 μg/ml for MSBE. Mangiferin had a weak activity in this model.

3.2. Effect of M. indica extract and mangiferin on hypoxanthine–xanthine oxidase system

As can be seen on Table 2, the effects of MSBE and mangiferin on the CL produced by the hypoxanthine–xanthine oxidase reaction in a cell-free system were evaluated. MSBE (1–100 μg/ml) and mangiferin (1–20 μg/ml) were able to capture the superoxide generated by the hypoxanthine–xantine oxidase system with an IC_{50}s= 19.4 μg/ml (MSBE) and 3.4 μg/ml (mangiferin). SOD enzyme (1/1000 U), used as a reference control, also inhibited the generation of superoxide.

4. Discussion

The measurement of LCL response of activated PMN is a valuable method to determine the oxidative burst of these cells and reflects primarily myeloperoxidase (MPO) activity. Various PMN stimulants can be selected to sepa-

Table 1
Effect of *Mangifera indica* extract (MSBE) and mangiferin on the chemiluminescence response of human leukocytes stimulated with PMA or zymosan

Treatments	Doses (μg/ml)	PMA		Zymosan	
		AUC (mean ± S.D.)	Inhibition (%)	AUC (mean ± S.D.)	Inhibition (%)
MSBE	0	467 ± 29.5	–	353 ± 20.2	–
	1	453 ± 21.3	2.9	310 ± 16.4	12.0
	5	324 ± 10.7*	30.7	229 ± 15.2*	35.0
	10	204 ± 30.8*	56.4	168 ± 10.7*	52.5
	50	15.1 ± 2.1*	96.7	54.8 ± 2.4*	84.4
	100	7.9 ± 0.8*	98.3	26.4 ± 1.9*	92.5
	200	7.7 ± 0.9*	98.4	26.8 ± 1.2*	92.4
Mangiferin	1	373 ± 9.7*	20	344 ± 10.1	2.3
	2	226 ± 4.1*	51.7	343 ± 8.5	2.8
	10	61.2 ± 1.8*	86.9	329 ± 14.7	6.7
	20	32.2 ± 2.6*	93.1	285 ± 8.2*	19.1
	40	31.8 ± 1.7*	93.2	286 ± 9.1*	18.9

The reaction was observed for 8 min (PMA) or 20 min (zymosan) and chemiluminescence was recorded in square centimetres (cm^2) for the integral response. Values are expressed as means ± S.D. of triplicate experiments.
* $p < 0.05$ as compared to control values. AUC-area under the curve.

Table 2
Effect of *M. indica* extract (MSBE), mangiferin and superoxide dismutase (SOD) on chemiluminescence produced in the luminol–hypoxanthine–xanthine oxidase system as the light intensity (*I*) produced by the chemiluminescence system was recorded in millivolts (mV)

Treatments	Doses	*I* (mV) (mean ± S.D.)	Inhibition (%)
MSBE	0	67.3 ± 5.1	–
	1 μg/ml	42.6 ± 5.0*	36.7
	10 μg/ml	30.8 ± 1.3*	54.2
	100 μg/ml	15.0 ± 2.6*	77.7
	200 μg/ml	16.1 ± 1.5 *	76.1
Mangiferin	1 μg/ml	38.1 ± 3.6*	43.4
	10 μg/ml	23.0 ± 5.2*	65.8
	20 μg/ml	18.6 ± 3.6*	72.4
	40 μg/ml	19.0 ± 3.3*	71.8
SOD	1/1000 U	20.5 ± 1.5*	69.5

Each point represents the means ± S.D. of three determinations.
* $p < 0.05$ as compared to control values.

rate receptor-related events from those not requiring receptors on the cell surface (Kannan, 2002).

The origin of neutrophilic luminol-dependent CL stimulated by zymosan, was studied by Cheung, Archibald, and Robinson (1983). They studied a variety of inhibitors such as superoxide dismutase, catalase, benzoate and inhibitors of arachidonate metabolism. They postulated that metabolism of arachidonate via lipoxygenase and cyclooxygenase is the source of CL after phagocytosis. It is likely that both lipoxygenase–cyclooxygenase and MPO play important roles in zymosan activated luminol-dependent CL.

PMA is a strong activator of luminol-dependent CL. As part of its action, hexose monophosphate activity is stimulated with an increase in the oxidative burst by the action of NADPH oxidase producing superoxide. This activation of NADPH oxidase and phospholipase A_2 producing arachidonic acid (which is metabolised by cyclooxygenase and lipoxygenase) may be a consequence of conformational changes in the neutrophilic membrane or of a kinase cascade initiated by protein kinase C (Karlsson & Dahlgren, 2002).

The release of arachidonic acid by phospholipase A_2 and of diacylglicerol and inositol trisphosphate by phospholipase C, the metabolism of arachidonic acid by the cyclooxygenase and lipoxygenase pathways, the activation of membrane NADPH oxidase by diacylglicerol and the calcium mobilisation by inositol triphosphate, are necessary for CL reaction. Inhibition of any of those mechanisms suppressed the CL response (Lindena, Burkhardt, & Dwenger, 1987). Cheung et al. (1983) postulated that the lipoxygenase and cyclooxygenase pathway is the source of zymosan-induced and luminol-enhanced CL. Since luminol-dependent CL is related to a great extent to oxidative MPO activity, it would not be surprising that those drugs, which interfere with oxidation (antioxidants), could be important CL inhibitors. Non-steroidal anti-inflammatory drugs have been found to be excellent inhibitors of luminol dependent CL from human granulocytes (Aam & Fonnum, 2006; Symons & King, 2003; Van Dyke, Van Dyke, Udernya, Brister, & Wilson, 1979).

The effects of MSBE and mangiferin on the PMA-stimulated-PMN CL system (Table 1) can be attributed to a scavenger activity of HOCl reported for MSBE in systems without cells (Martínez et al., 2000) and to the MSBE's anti-inflammatory activity on a PMA model of mice-ear edema in which MPO activity and the respiratory burst elicited in mouse neutrophils by PMA were inhibited dose-dependently by MSBE (Garrido et al., 2004). It has been well established that polyphenols, the main fraction of the extract (Núñez-Sellés et al., 2002), can inhibit the activity of NADPH-oxidase and MPO in human neutrophils (Tauber, Fay, & Marletta, 1984) and their antioxidant activity has also been reported (Catapano, 1997).

The inhibitory effect of MSBE observed in zymosan-stimulated PMN, suggested that the antioxidant activity

described might play an important role in the anti-inflammatory activity. The mangiferin is into the MSBE at a concentration of 10–20% (Núñez-Sellés et al., 2002). However, the weak activity of mangiferin in this model, suggest that other components inside the extract also exercise its antioxidant action attributed to all the extract.

The weak activity of mangiferin in this experiment could be explained because of the intensity and kinetic profile of luminol-enhanced CL and is dependent on the type of activator. The CL response of zymosan-activated samples is slowest when compared with the other activators but had the highest maximum. Opsonised particulate stimuli, such as zymosan, activates an oxidative burst of phagocytes by binding itself to complement and immunoglobulin receptors, which induces signal transduction that leads to the activation of protein kinase C (PKC) and a consequent activation of NADPH-oxidase, the key enzyme of oxidative burst. Therefore, zymosan induces long-term ROS production, starting from zymosan binding onto the phagocyte surface up to the final processing of zymosan in phagolysosomes (Khan, 2004). In contrast, soluble stimuli PMA directly activates molecules involved in this signal transduction pathway without needing to bind to the membrane receptor. PMA directly activates PKC and evokes a faster and more short-term response than zymosan (Pavelkova & Kubala, 2004). In this case, it could be thought that mangiferin acts at the level of receptors.

Recently, MSBE has been also tested on arachidonic acid metabolite production, demonstrating that it is effective on prostaglandin E_2 and leukotriene B_4 in stimulated macrophages (Garrido et al., 2004). A number of studies have shown that the oxidation of arachidonic acid to eicosanoids requires oxygen free radicals, and that several anti-inflammatory agents act either by inhibiting the production of free radicals or by scavenging them (Kirkova et al., 2007).

The reports of the H_2O_2 scavenger activity of the extract (Sánchez et al., 2000) and the mangiferin capacity to react with superoxide (Leiro, Alvarez, Arranz, Siso, & Orallo, 2003), may explain the CL produced by the hypoxanthine–xanthine oxidase reaction in a cell-free system. The reported anti-inflammatory activity of MSBE can also contribute to the decrease in the observed LCL response.

CL of PMNs is the final stage of their oxidation by luminol. The major and most active oxidants are free radicals and superoxide radicals that originate from enzymatic processes i.e. superoxide anion, hydroxyl radical, singlet oxide, hydrogen peroxide and hypochlorous acid. Flavonoids, cinnamic acid and acrylic acid derivatives have the ability to scavenge free radicals and superoxides and inhibit some enzymatic systems that generate free radicals and superoxides (Lahouel et al., 2006; Limasset et al., 1993).

It has been established that polyphenols inhibit the activity of NADPH-oxidase and MPO and that the inhibition of CL is correlated with the number of free hydroxyl groups on the active molecule. It has been established that this structural requirement is the single most dominant factor in defining the hydrophilic/lipophilic balance of these compounds, as it affects their membrane transport and affinity to active intracellular binding sites. (Tauber et al., 1984).

The antioxidant effect of MSBE, exhibited in the models described herein, could be attributed in part to its main component, mangiferin, that has a catechol moiety on its structure (6,7-dihidroxylated), which has been assumed as extremely important for the antioxidant activity of many polyphenol derivatives (Jha, Recklinghausen, & Zilliken, 1985; Rodrigo & Bosco, 2006). Nevertheless, when mangiferin has been tested in other *in vitro* and *in vivo* experiments against the whole extract (Sánchez et al., 2000), its antioxidant activity was less than MSBE, which suggests that other components, probably through a synergistic effect, are also playing an important role in such tested activity. Some of the main components of MSBE such as terpenoids, cathechin, fatty acids and microelements have also been reported to exhibit antioxidant properties (Beltz, Bayer, Moss, & Simet, 2006; Cholbi, Paya, & Alcaraz, 1991; Grassmann, 2005). Recently, we showed that major polyphenols from MSBE (mangiferin, catechin and epicatechin) attenuated the increase in intracellular Ca^{2+} influx, induced by T cell receptor (TCR) activation (Hernandez, Rodriguez, Delgado, & Walczak, 2007). These data demonstrate that the major polyphenols from MSBE may stabilise Ca^{2+} homeostasis and decrease ROS levels induced by TCR triggering and consequently reduce the ratio of activation-induced cell death (AICD) in human T cells. Although the major polyphenols of MSBE inhibits AICD, particularly mangiferin and catechin, none of them reached the inhibitory level achieved by the whole extract at equivalent concentration. The possibility of the synergistic effects between the compounds, that might explain the high activity of the extract, persuades us to combine the three polyphenols trying to reproduce the polyphenolic fraction in the extract. In our experiments, this pool resulted neither in an additive effect nor reached the inhibitory level achieved by the whole extract. In this regard, a study reports that the synergistic additive or potentiated effects shown by the plant extract usually exceed the effects of single compounds, or mixtures of them at equivalent concentrations (Paquay et al., 2000). Previous reports also indicate that the high activity in protective effect, found in the polyphenolic fraction of the green tea extract, could not be explained by the mixture of catechins (Wagner, 2004).

In the present study, the IC_{50} values of mangiferin in PMA-stimulated PMN and in the hypoxanthine–xantine oxidase system were lowest than the IC_{50} value of MSBE. This could indicate that the other constituents in MSBE exert antagonist action with respect to mangiferin for these experimental conditions. We also showed this contradictory effect on LTB_4 in calcium ionophore-stimulated macrophages with the IC_{50} of mangiferin ten fold higher than MSBE (Garrido, González, Lemus, Delporte, & Delgado, 2006).

Further experiments will be carried out in due course to possibly correlate the potential therapeutic benefits of MSBE on inflammatory events, where free radicals generated by PMN play an important role.

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