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Citrus reticulata peel oil inhibits non-small cell lung cancer cell proliferation in culture and implanted in nude mice

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Non-small cell lung cancer (NSCLC) accounts for most cases of lung cancer. The peel oil of mandarin *Citrus reticulata* Blanco cv. Dancy (MPO) is a natural source of essential oils and carotenoids. Volatile and non-volatile lipid compounds were characterized by chromatographic methods. We demonstrate that MPO causes a dose-dependent growth inhibition of NSCLC model cells (A549) in culture and tumour growth *in vivo* of the same cells implanted in nude mice fed with MPO-supplemented diets. MPO induced cell cycle arrest mainly at the G0/G1 phase and reduced the amount of membrane-bound Ras protein along with apoptosis induction. No toxicological effect was found in liver parameters analysed in treated mice and histopathological analyses of their organs did not show any morphological changes. In conclusion, the data suggest that MPO possesses significant antitumor activity without causing systemic toxicity, proposing it as a dietary supplement that may be helpful in cancer prevention.

Introduction

Cancer has become one of the most common causes of death in the last few decades. According to the World Health Organization, cancer disease is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015. The most common cause of cancer death is lung cancer (1.69 million deaths).[1] The two main types of lung cancer are small and non-small cell lung cancers, the latter accounting for approximately 85% of all cases of lung cancer.[2,3] Although numerous reports of phytocompounds with antitumoural activity provide grounds for promoting the intake of fruits and vegetables to prevent tumour development, the chemotherapeutic effects and mechanisms of action of the peel oils of one of the most commonly consumed fruits worldwide, such as *Citrus reticulata* (mandarin), have not yet been elucidated.

Citrus essential oil, the major component of citrus peel oil, has been used for centuries for relief from colds and flu and as a result, its anti-inflammatory mechanisms have been widely studied. Scientists have been studying this oil's wide variety of monoterpenes for decades, obtaining as a result a wide spectrum of biological activities displayed by these 10-carbon isoprenoids.[4,5] Limonene, a major constituent in several citrus essential oils (orange, lemon, mandarin, lime, and grapefruit), presents well-established chemopreventive activity against many types of cancer.[6,7] As an antiproliferative mechanism, it has been reported to inhibit the prenylation of small GTP-binding proteins[8] and the expression of cyclins that regulate the cell cycle.[7] However, numerous studies suggest a major role for a combined action of monoterpenes in cancer cell death.[9] Otherwise, citrus fruits are a complex source of carotenoids (natural fat-soluble pigments) accumulated mainly in the peel, many of them with proven anti-carcinogenic activity in several tissues.[10] Our group has previously demonstrated that mandarin peel oil (MPO) exhibits a greater antitumoural potential than limonene in cultured tumour cells.[11] In the present work we performed a characterization of volatile lipid compounds (essential oils) and non-volatile lipid compounds present in MPO, evaluating their effect on the cell cycle, on the anchoring of the Ras oncogenic protein to the membrane, and on apoptosis induction in A549 non-small cell lung cancer cells in culture and also implanted in a murine model. We also assessed morphometric parameters, and biochemical and histological markers of systemic toxicity in tumour-carrying mice and oral feeding with MPO with a view to gauging its potential as a food supplement with antitumoural properties.

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Experimental

Plant material and extraction of peel oil

Mandarins were collected from Mocoretá, Department of Monte Caseros, Province of Corrientes, Argentina and identified as *Citrus reticulata* Blanco (cultivar Dancy). Voucher specimen No F32 since it corresponds to the number of a specific classification is deposited in the Bank of Essential Oils, Laboratory of Phytochemistry, School of Forestry and Agricultural Sciences, National University of La Plata (UNLP), La Plata, Argentina.

The peels of fresh fruits were cold-pressed and the MPO was separated from the crude-extract by centrifugation at 9000*g* for 15 min at 4 °C. MPO was stored in a sealed dark glass bottle at 4 °C for 15 days and then centrifuged at 11 600*g* for 15 min at 4 °C to discard crystals. Finally, it was stored in the dark under a nitrogen atmosphere at −20 °C until further analysis.

Analysis of volatile compounds by gas chromatography–mass spectrometry

Quantitative analysis of volatile organic compounds (VOCs) of MPO (dissolved in ethyl acetate) was performed using a Hewlett Packard 6890 capillary gas chromatograph (CGC) employing a non-polar HP-5MS capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness) (J&W, Folsom, CA, USA) coupled to a Flame Ionization Detector (FID) set at 320 °C. The injector was operated in a splitless mode setting at 280 °C using helium as the carrier gas. The oven was programmed as follows: initial temperature: 50 °C, initial time: 1 min, final temperature: 300 °C, rate: 10 °C min^{-1}, final time: 10 min

VOCs were obtained under similar CGC conditions with a CGC HP 6890 coupled to a mass selective detector (Agilent 5975C VL) operated at 70 eV for further identification. The samples were obtained by head space-solid phase microextraction (HS-SPME). A 65 μm polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB, supplied by Supelco, Bellefonte, PA, USA) was conditioned and exposed for 10 min to 1 μl of MPO placed in a 2 mL glass vial sealed with a Teflon cover with a rubber septum at 40 °C. VOCs were identified by the interpretation of their mass spectral fragmentation; spectra were also compared to the data from a commercial mass database (NIST/EPA/NIH, NIST 05) and from the literature data.[12–14] VOC chain lengths were confirmed by calculating their Kovats index (KI).[15]

Analysis of non-volatile compounds

MPO was analysed by thin-layer chromatography (TLC) on silica gel G developed in two different solvent systems: hexane/diethyl ether/acetic acid 80/20/1 (v/v/v) and hexane/acetone 80/20 (v/v). Lipids were identified by comparison with appropriate standards run under identical conditions.

A sample of the MPO was saponified with 10% KOH in methanol at 80 °C for 1 h to eliminate unsaponifiable lipids and fatty acids were extracted from the methanolic phase with hexane after acidification with concentrated HCl. Fatty acids were transformed into methyl esters by treatment with a solution of 10% boron trifluoride in methanol. An inert atmosphere was maintained using N_2 to prevent lipid oxidation. CGC analyses of the fatty acid methyl esters (FAME) were performed on a Hewlett-Packard 6890 gas chromatograph, equipped with a Flame Ionization Detector (FID). The samples were analysed on a 30 m, 0.25 mm I.D., 0.25 μm film thickness Supelco Omegawax 250 (Alltech Associates, Arlington Heights, Il, USA) capillary column. Conditions of work were as follows: injector temperature: 260 °C, detector temperature: 270 °C, initial temperature: 175 °C, initial time: 3 minutes, rate: 3 °C min^{-1} to reach 230 °C, final time: 19 minutes. The injection was carried out in the split mode, using helium as the carrier gas. The fatty acid composition was obtained through a comparison of the relative retention times with commercial standards (Supelco 37 Component FAME mix (C4-C24)).

The fatty acid composition was confirmed by CGC-MSD chromatography using a Hewlett-Packard 6890 gas chromatograph coupled to a mass selective detector (Agilent 5975C VL). The column used to separate the sample was the Supelco Omegawax 250, and the identification of the methyl esters of the fatty acids was realized for comparison of the obtained spectra with the data from MS libraries (NIST/EPA/NIH, NIST 05). The conditions of the MS detector were: ionization energy 70 eV; scan mode in the range of masses 35–600 u; transference line at 260 °C; ionization chamber at 230 °C, and quadrupole at 150 °C.

The concentration of carotenoids in MPO was assessed by spectrophotometry (Beckman Coulter DTX 880 Microplate Reader, Fullerton, CA, USA) at 490 nm with a calibration curve of astaxanthin.

Cell culture and treatments

The non-small cell human pulmonary-carcinoma cell line A549 was obtained from Dr Amada Segal-Eiras (CINIBA, UNLP, Argentina). The cells were maintained in Eagle's Minimal Essential Medium (MEM) (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal-bovine serum (Natocor, Córdoba, Argentina) and 100 μg mL^{-1} streptomycin.

MTT assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St Louis, MO) assay. A549 cells were seeded in 24-well plates at a density of 12×10^3 cells per well, and the cultures were kept at 37 °C for 24 h for cell attachment before further additions. The cells were then incubated with 0–400 μg mL^{-1} MPO dissolved in a volume of dimethyl sulfoxide (DMSO) for 24 h, such that the final concentration of the vehicle was 0.2% (v/v). The same concentration of DMSO was added in parallel to the control cultures. After treatment, the cells were incubated with MTT in PBS (0.5 mg mL^{-1}) at 37 °C for 2 h. The MTT was then removed from the wells and acidified with 0.04 M HCl in isopropanol. The absorbance of the reaction product (the formazan) was measured at 560 nm with background subtraction at 640 nm with a Beckman Coulter DTX 880 Microplate Reader (Fullerton, CA, USA).

Cell cycle analysis by flow cytometry

The cells (1×10^5) were plated in 6 well plates, kept at 37 °C for 24 h for cell attachment and then incubated in serum-containing MEM supplemented with or without MPO for 24 h (as described above for MTT assay). After treatments, the cells were harvested by trypsinisation, centrifuged at 500*g* for 5 min, resuspended in PBS and fixed with 70% (v/v) aqueous ethanol at 4 °C overnight. Then, the cells were washed with cold PBS, and incubated with ribonuclease A (500 U mL^{-1}; Biodynamics, Argentina) at 37 °C for 15 min. Nuclei were stained with propidium iodide (25 μg mL^{-1} in PBS containing 0.1% (v/v) Triton X-100). The DNA content was determined with a FACSAria II flow cytometry cell sorter (BD Biosciences). Cell cycle distribution was calculated with the use of FlowJo software v. 7.6.2.

Animals and treatments

NIH female mice bearing the nu/nu genotype were obtained from the Animal Facility of the Ezeiza Atomic Centre and housed in a temperature-controlled room on a 12 h cycle of light and darkness. A549 cells were detached, centrifuged, resuspended in MEM and implanted subcutaneously in the back of nude mice (2×10^6 cells per mouse). Tumour growth was evident between 15–20 days following inoculation. Tumours weighing between 2–3 g were transplanted as previously described[16] to minimize both the lag time and growth rate variables in all the implanted tumours. In brief, tumours were surgically removed and washed with MEM, the necrotic regions were eliminated, and the remaining tissue was cut and dispersed by passing through a 1 mm^2 steel mesh to obtain a tissue suspension of 300 mg mL^{-1}. Finally, each animal was inoculated subcutaneously with 0.2 mL of the suspension. Host animals were maintained *ad libitum* on a gamma-irradiated chow diet and autoclaved water until the average tumour size reached 300 mm^3. Then mice were randomly separated into 3 groups, one used as a control without receiving MPO and the other 2 receiving 1.75 or 5.25 mg MPO per mouse per day, respectively. These concentrations were chosen based on preliminary assays with MPO and on data obtained in our previous studies performed with the commercial monoterpene geraniol.[17] Tumours were measured twice a week with callipers and their volume was calculated for an ellipsoid by the formula $V = a^2 \times b/2$, where "*a*" is the width and "*b*" is the length of the mass.

Mice were killed after three weeks by cervical dislocation in the middle of the light period.

All experiments were carried out in conformity with the Handbook of Laboratory Animal Management and Welfare.[18] The animal-use protocols used were approved by the Institutional Animal Care and Use Committee, School of Medical Sciences, Universidad Nacional de La Plata, Protocol number T09-02-2013.

Systemic toxicological evaluation, biochemical parameters and histological analysis

Daily food intake, body mass loss, organ weight alteration and liver function were evaluated. Mice were weighed every three days. After sacrifice, livers, kidneys, gut, spleens and tumours were also removed and weighed. Blood was obtained by cardiac puncture at sacrifice, collected and placed at 37 °C for 30 min. It was then centrifuged for 10 min at 3000*g* and the serum was stored at −20 °C until further analysis. As liver function parameters, cholesterol and triacylglycerols, which are synthesized and secreted by the liver, and the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes and albumin were measured using specific diagnostic kits (Wiener Lab, Argentina). Livers, kidneys, guts, spleens, and tumours were minced and immediately fixed in 10% (v/v) buffered formaldehyde. After 24 h the samples were dehydrated in graded alcohols and embedded in paraffin. Sections of 6 μm thickness were rehydrated, stained with hematoxylin–eosin and observed under a light microscope.

Western blot analysis

The cells were plated in 60 mm Petri dishes at a density of 2×10^5 and cultures kept at 37 °C for 24 h for cell attachment before treating with MPO for 24 h as described above (MTT assay). After treatments, the cells were washed twice in PBS and lysed in RIPA buffer [(50 mM Tris-HCl – pH 8.0, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, and 1 mM EDTA)] containing protease inhibitors for 30 min on ice and centrifuged for 15 min at 14 000*g* at 4 °C.

Membrane-bound and total Ras levels in tumour cells were determined as described by Galle and collaborators.[17] The protein content of each sample was determined by the method of Bradford with bovine serum albumin as a standard. The samples were subjected to western blot analysis using anti-cyclin E, anti-cyclin D1, anti-cyclin B1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the anti-pan-Ras antibody (Calbiochem, Darmstadt, Germany). Internal control was loaded with the anti-β-actin antibody (Sigma, St Louis, MO).

In situ detection of apoptosis

Apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL assay) using the *in situ* cell death detection kit, TMR red (Roche, Mannheim, Germany) in accordance with the manufacturer's protocol. The TUNEL-positive cells were evaluated under an Olympus BX51 fluorescence microscope (Tokyo, Japan) equipped with an Olympus DP70 digital camera and the results are analysed with the use of ImagePro Plus v.5.1 software (Media Cybernetics, Silver Spring, Md). The results were expressed as the percentage of TUNEL-positive cells relative to the total number of cells, the latter being determined by the uptake of the fluorescent dye DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Invitrogen by Life Technologies).

Statistical analysis

Statistical analyses were performed by means of the Student's *t* test. Differences in the data were considered statistically significant at $p < 0.05$. The software used to perform the statistical analysis was GraphPad InStat v3.05 (GraphPad Software, San Diego, CA, USA). The IC50 value (concentration that inhibited

the cell viability by 50%) was calculated by the nonlinear regression curve (SigmaPlot software; Systat Software, Inc., Point Richmond, CA, USA).

Results

MPO lipid composition

On a preliminary basis, general aspects such as yield extraction and MPO density were evaluated in order to schedule the assays. MPO yield extraction was 0.32% (w/w), whereas the MPO density obtained by gravimetry at 20 °C was 0.829 ± 0.004 g mL^{-1}. To evaluate the volatile lipid compounds present in MPO, the samples were obtained by HS-SPME, and CGC-MS analyses were performed. Among VOCs, monoterpene hydrocarbons are the major components of the citrus peel oil used in this study and limonene was the most abundant compound accounting for over 94.6%, followed by myrcene and α-pinene (Fig. 1 and Table 1). The presence of all other components amounted to less than 3%. Among non-volatile compounds, MPO contains carotenoids, carotenoid esters, and TG traces (data not shown). The calculated concentration of MPO was 330 μg carotenoid per mL MPO. The major fatty acids found were 16:0 (palmitic acid) and 18:2 n-6 (linoleic acid) with 29.26% and 33.71%, respectively. Other fatty acids detected were 14:0 (5.69%), 18:0 (6.61%), 18:1 (10.8%), and 18:3 (3.49%).

Inhibition of A549 cell proliferation in culture

In order to investigate the effect of MPO on A549 cells, the cell viability was evaluated by the MTT assay. The mitochondrial metabolic activity in the viable cells determined by the conversion of MTT to formazan demonstrated that MPO treatment (0–400 μg mL^{-1}) decreased the cell viability in a dose-dependent manner (Fig. 2A), and the IC50 value obtained was 96 μg mL^{-1}.

To deepen the knowledge and understanding of MPO effects on A549 cell proliferation, cell cycle progression was analysed by flow cytometry, and cyclin expression of cyclins B1, D1, and E was evaluated by western blotting. MPO was shown to block the A549 cell cycle progression after incubation for 24 h. As is shown in Fig. 2B, both evaluated concentrations of MPO (IC25 and IC50) caused an increase in G2/M cell population with a concomitant decrease of cells in the S phase compared to controls. However, the cells treated with the IC50 value of MPO showed significant changes at all cycle stages, mainly promoting a G0/G1 phase arrest (Fig. 2B).

In agreement with these results, the levels of cyclin E, which play a critical role in G1 progression, significantly decreased in the cells incubated with the IC50 value (Fig. 2C). In contrast, the levels of cyclin B1, which are still high in cells arrested in the G2/M phase, significantly increased in the cells treated with IC25 and IC50 values. Besides, no significant differences were found in cyclin D1 expression.

Tumour growth suppression

To evaluate the *in vivo* effects of MPO on tumour cell proliferation, human A549 cells were implanted in nude mice to establish a xenograft model frequently used for the evaluation of anti-cancer therapies.[19–21] Data obtained from the measurement of the tumour volume in A549-bearing mice (Fig. 3)

Table 1 Chemical compositions of volatile organic compounds of *Citrus reticulata* peel oil

GCG peak[a]	Volatile organic compound	Percent composition	Kovats indices
1	α-Pinene	0.55	939
2	β-Pinene	0.29	979
3	Myrcene	1.81	990
4	Undetermined	0.15	—
5	Limonene	94.59	1029
6	Terpinolene	0.09	1088
7	Linalool	0.51	1096
8	Citronellal		1153
9	Terpineol-α		1188
10	Decanal		1201
11	Citronellol		1225
12	Nerol		1229
13	Neral		1238
14	Carvone		1243
15	Decenal	2.01	1263
16	Geranial		1267
17	Perilla aldehyde		1271
18	Undecanal		1306
19	Decadienal		1316
20	Neryl acetate		1361
21	Dodecanal		1408
22	Farnesene		1505

[a] Numbers correspond to peaks from Fig. 1. CGC, capillary gas chromatograph.

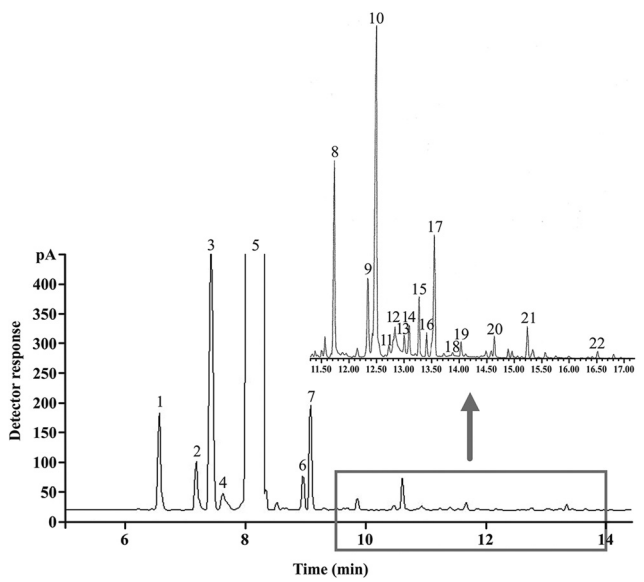

Fig. 1 Capillary gas chromatography profiles of volatile organic compounds of mandarin (*Citrus reticulata*) peel oil. The inset image represents the amplification of the minor components detected. All components were identified by CGC-MS; chromatographic conditions are described in Experimental. Numbers correspond to peak numbers from Table 1.

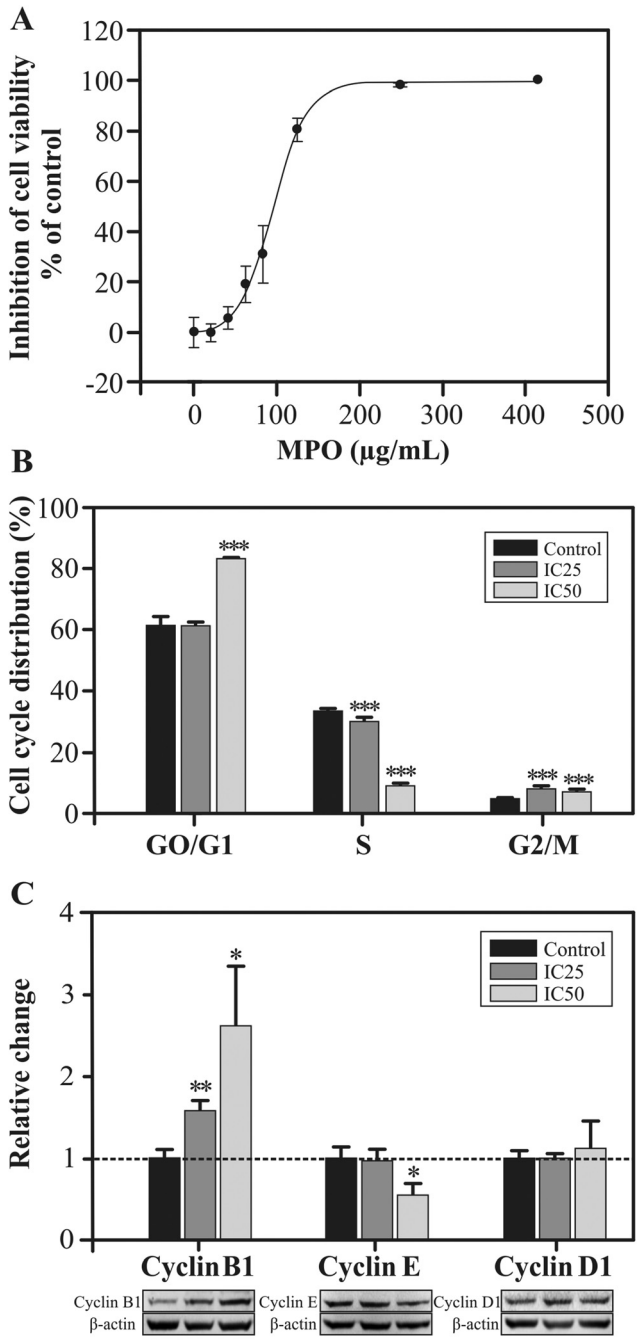

Fig. 2 Inhibition of A549-cell proliferation incubated with mandarin (*Citrus reticulata*) peel oil (MPO) for 24 h. (A) Dose–response curve expressed as a percentage inhibition of cell growth as assessed by the MTT test. Each point on the curve was calculated from the mean value ± SD of 8 replicate wells per dose performed in 3 separate experiments. (B) Cell cycle distribution evaluated by flow cytometry. Data are expressed as mean ± SD (n = 4). (C) Quantification of cyclin B1, E, and D1 levels by densitometric analysis and their representative immunoblots. β-Actin was used as a loading control. Data are expressed as the mean ± SEM (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001 *vs.* control group.

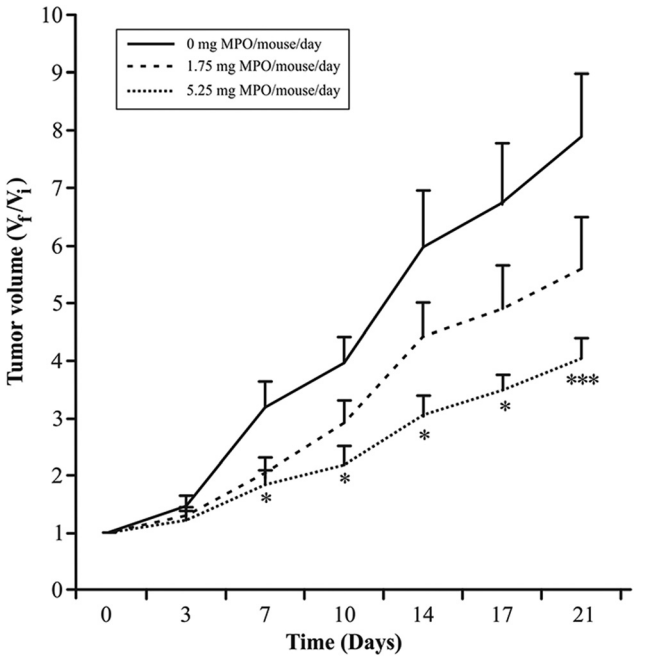

Fig. 3 Effect of mandarin (*Citrus reticulata*) peel oil (MPO) on tumour growth of A549 cells implanted in nude mice. Animals received supplemented diets containing 1.75 or 5.25 mg MPO per mouse per day for 21 days. Data are presented as means ± SEM for each group. Tumours of 5 mice per group were evaluated. *p < 0.05 *vs.* control, ***p < 0.001 *vs.* control.

demonstrated that MPO significantly decreased the tumour growth from day 7 in the groups receiving 5.25 mg MPO per mouse per day.

MPO effect on Ras protein levels

In order to evaluate a potential mechanism by which MPO inhibits tumour growth, we measured cell membrane Ras levels since Ras GTPase anchored to the membrane plays a central role in promoting cell proliferation.

Western blot analysis of the tumour-bearing mice indicated that mice treated with 5.25 mg MPO per mouse per day showed a statistically significant (p = 0.0142) decrease in the level of the membrane-bound Ras protein in the tumours compared to controls, whereas total Ras protein levels did not vary significantly in any of the MPO-treated groups (Fig. 4).

Increase in apoptosis after MPO treatment

As tumour growth depends on the balance between cell proliferation and cell death, the evaluation of both mechanisms is valuable to perform a more comprehensive analysis.

In order to evaluate apoptosis as one of the processes involved in tumour growth suppression by MPO, the TUNEL assay was performed. We observed that low as well as high concentrations of MPO significantly increased apoptosis in both *in vivo* and *in vitro* A549 cells (Fig. 5).

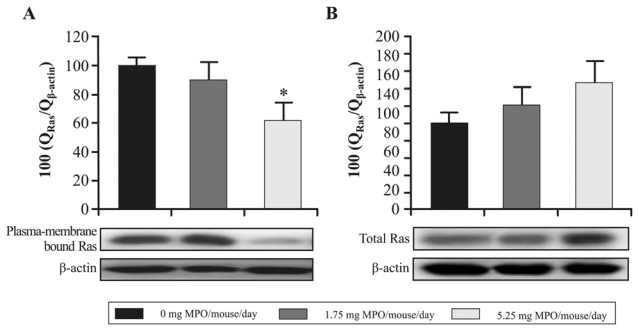

Fig. 4 Effect of mandarin (*Citrus reticulata*) peel oil (MPO) on the levels of total and membrane-bound Ras by western blot analysis. (A) Plasma-membrane bound Ras and (B) total Ras from the tumours of 5 mice per group. Upper panel: Density of Ras-immunoblot bands as a percentage of the control. Lower panels: Representative bands obtained by immunoblotting. β-Actin was used as a loading control. Values are expressed as means ± SEM. *$p < 0.05$ *vs.* control group.

Absence of toxicological effects

Side effects of most of the current chemotherapies promote the search for therapeutic products without systemic toxicological effects. The analysis of different biological parameters which determine potential toxicities in MPO-treated mice is important to be taken into account. Therefore, after treatment, mice were sacrificed and morphometric, histological, and biochemical parameters were evaluated. We observed that the supplemented diets containing 1.75 and 5.25 mg MPO per mouse per day did not appear to produce any adverse effects, with food consumption and body weight remaining the same throughout the treatment (data not shown), nor were any differences observed in the relationship between liver, kidney, and spleen weights with respect to the body weight of the three experimental groups of mice (Table 2). Histopathological analyses of livers, kidneys, gut, and spleens showed no marked changes in the morphology of their tissues (Fig. 6). In addition, there were no significant differences between the control and treated groups in terms of serum albumin, cholesterol, triacylglycerols and in most of the transaminases activities were found. Significant changes occurred only in AST activity in high-dose treated mice (Table 2).

Discussion

The major lipid components of MPO are monoterpenes and carotenoids. Both groups consist of natural chemical compounds that are generally recognized as safe for use in any foods (GRAS).[22,23] In the present work different parameters were assessed, showing that MPO concentrations that inhibit

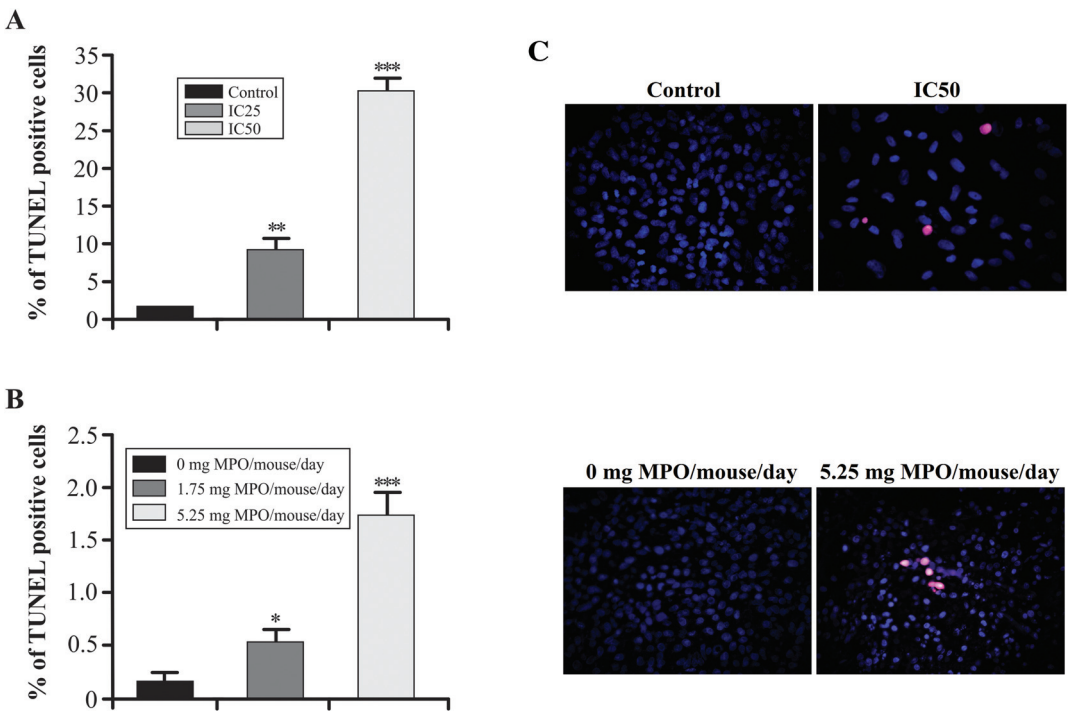

Fig. 5 Effect of mandarin (*Citrus reticulata*) peel oil (MPO) on apoptosis of A549 cells in culture and implanted in nude mice. The percentage of apoptotic cells was determined by the TUNEL assay. (A) The TUNEL-positive cells in culture cells incubated with a concentration of MPO corresponding to IC25 and IC50 values for 24 h were counted in 15 random fields with 1000 cells from 3 experimental situations. (B) The TUNEL-positive cells in tumour histological sections were counted in 10 random fields from each experimental situation (corresponding to tumours of 5 mice per lot). (C) Fluorescence micrographs of control cells (left fields) and treated cells (right fields) showing red-stained TUNEL positive cells (apoptotic cells) and nonapoptotic nuclei stained with DAPI. (600× magnification). Values are expressed as means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs.* control group.

Table 2 Effect of the *Citrus reticulata* peel oil intake on morphometric and blood biochemical parameters of different experimental groups

			mg MPO per mouse per day	
		Control	1.75	5.25
Biochemical parameters	Cholesterol (g L^{-1})	0.88 ± 0.09	0.90 ± 0.05	0.96 ± 0.06
	Triacylglycerols (g L^{-1})	0.78 ± 0.06	0.87 ± 0.04	0.85 ± 0.09
	AST activity (UI L^{-1})	206.9 ± 23.3	308.1 ± 72.1	328.1 ± 28.8*
	ALT activity (UI L^{-1})	72.1 ± 9.3	80.2 ± 22.6	95.4 ± 29.1
	Albumin (g dL^{-1})	3.5 ± 0.1	3.2 ± 0.1	3.6 ± 0.1
Morphometric parameters	LW/BW	$0.064 \pm 2 \times 10^{-3}$	$0.065 \pm 4 \times 10^{-3}$	$0.061 \pm 2 \times 10^{-3}$
	KW/BW	$0.015 \pm 3 \times 10^{-4}$	$0.015 \pm 4 \times 10^{-4}$	$0.015 \pm 2 \times 10^{-4}$
	SW/BW	$0.007 \pm 5 \times 10^{-4}$	$0.009 \pm 1.1 \times 10^{-3}$	$0.006 \pm 5 \times 10^{-4}$

Note: After exposure to the peel oil of mandarin *Citrus reticulata* (MPO) body weight (BW), liver weight (LW), kidney weight (KW), spleen weight (SW) and blood parameters were measured in the treated and control mice. AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are the means ± SEM ($n = 5$). *, $p < 0.05$ *vs.* control.

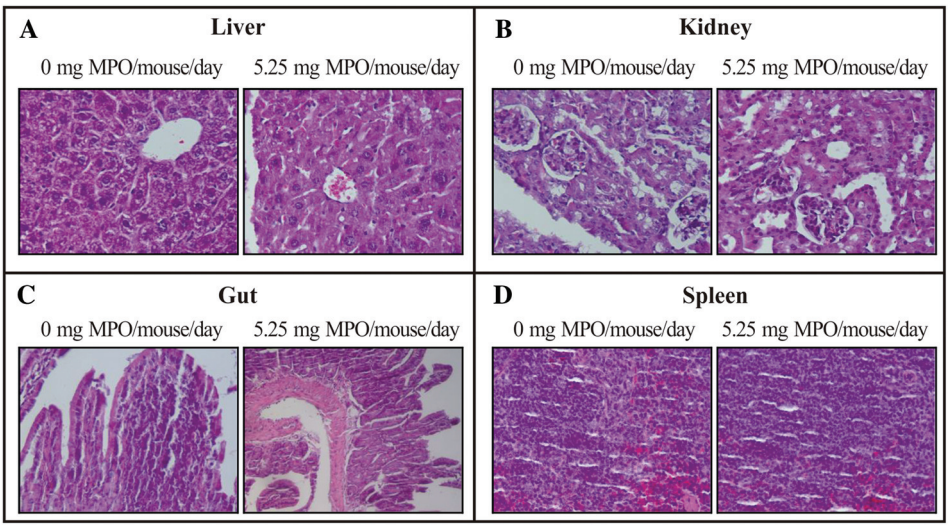

Fig. 6 Effect of mandarin (*Citrus reticulata*) peel oil (MPO) on liver (A), kidney (B), gut (C), and spleen (D) histology. Light microscopy images of hematoxylin–eosin staining of the organs of nude mice fed with the concentrations of MPO shown to inhibit tumour growth in A549 cells (5.25 mg MPO per mouse per day) compared to controls (400× magnification).

tumoural proliferation did not induce systemic toxicity in the evaluated murine model. It was demonstrated that the MPO did not produce changes in body weight, one of the most sensitive indicators of adverse effects[24,25] or in the weight, morphology, and histology of the different organs, nor did plasmatic cholesterol, triacylglycerols, albumin, and ALT as markers of liver function show differences with respect to control; only AST increased its activity in mice treated with the highest MPO concentration. However, the increase in this last parameter does not necessarily indicate a toxic effect of the oil, since this enzyme activity can be associated with other events such as sources of tissue necrosis in extrahepatic regions that could correspond to the tumoural regression exerted by MPO.

Carotenoids in Dancy mandarin are accumulated mainly in the peel (up to 94%)[26] and among them, hydroxylated carotenoids can be present in the free form or acylated with fatty acids, forming esters like cryptoxanthins,[26,27] vitamin A precursors.[28] Carotenoids, cryptoxanthins, and also vitamin A were reported to display anticancer activities.[10,29,30] In the present work we determined the presence of free and esterified carotenoids in the MPO, suggesting its potential contribution in the inhibition of tumour proliferation. Although the mechanisms of action could not be determined, the antioxidant activity of these compounds, widely reported in the bibliography[31–33] and observed in own experiments (data not shown), could play a key role in the aforementioned processes. Free radicals are generated as byproducts of normal metabolism but when there is an overgeneration, the resulting imbalance in the cellular redox state can induce oxidative stress and contribute to the initiation, promotion, and maintenance of tumour development.[34] In this context, the consumption of dietary antioxidants seems to play an important role in protecting cells and tissues from oxidative damage and consequently

preventing people from several pathological events.[35,36] Despite the fact that most of the studies available in the literature show the antioxidant effect of non-esterified carotenoids, some reports point out that –OH group esterification with fatty acids does not alter the antioxidant properties of carotenoids and even more stabilize them against heat degradation.[33]

In the present paper we determined the composition of monoterpenes, the major components in Dancy MPO, to be similar to that of other varieties previously reported,[37] where limonene was the most abundant component. The chemopreventive and chemotherapeutic activities of essential oils including those from citric fruits and pure monoterpenes are widely reported.[38–43] However, as with carotenoids, the mechanisms of action have not still been fully elucidated, and only a few monoterpenes and essential oils have reached clinical phases of investigation (phase I clinical trial).[6,38] Among these latter, limonene has demonstrated its bioavailability and safety, and there is pre-clinical evidence of its anti-cancer effects in humans, though studies to date have focused on its use in the prevention and treatment of breast cancer.[7,22] It was demonstrated that limonene induces apoptosis[44,45] and inhibits cell cycle progression through the inhibition of the isoprenylation of small G proteins such as Ras oncoproteins and the lowering of cyclin D1 mRNA levels.[8,46] As limonene is a main component in most of the citrus oils,[47] it is most likely that similar mechanisms are involved in the inhibition of tumour cell growth by MPO as well as by other citrus oils.

In fact, we herein demonstrate that MPO exerts growth inhibitory effects on human tumour A549 cells by inducing cell cycle arrest and apoptosis. From low concentrations (IC25), MPO arrested the cell cycle in the G2/M phase in parallel with the increased levels of cyclin B1. These results suggest that MPO arrests cells at some stage between metaphase and telophase, when cyclin B1 levels remain high. However, at a higher concentration (IC50) MPO also promoted an increase of G0/G1 cell population. The latter is accompanied by a decrease of cyclin E expression, a cell cycle regulator protein essential for G1 progression and transition to the S phase.[48,49] In both cases, the arrest was characterized by a significant decrease in S phase cells.

The inhibition of certain lipid synthesis arrests the cells in mitosis before ana/telophase,[50] and some pure monoterpenes and essential oils inhibit some enzymes involved in lipogenesis as reported in our previous studies.[51–53] Based on these facts, we propose that MPO could modulate lipid metabolic pathways that may affect cancer cell proliferation. Moreover, we demonstrated that some monoterpenes inhibit the metabolic pathway responsible for the synthesis of prenyl groups (mevalonate pathway) in A549 cells.[51,54] The prenyl groups are posttranslationally added to Ras GTPases by prenyltransferases, which is essential for their membrane anchorage and their intrinsic GTPase activity. Owing to its central role in oncogenesis,[55] Ras GTPase is considered a promising therapeutic target in several tumour cells.[56] Ras proteins operate in a complex signalling network. This network includes Ras-ERK and PI3K-Akt pathways that regulate many cellular functions such as cell proliferation by stimulating G1/S transition and apoptosis either suppressing the expression of pro-apoptotic genes or enhancing anti-apoptotic proteins.[57–59] Based on the herein obtained results and on our previous studies, we suggest that the reduced level of Ras GTPase prenylation could be one of the antiproliferative mechanisms exerted by MPO, leading to cell cycle arrest and apoptosis induction.

Conclusions

MPO exerts an antiproliferative effect on *in vivo* human tumour cells (implanted in a murine model) without exerting noxious effects on the host. These promising findings suggest that the consumption of foods prepared with MPO obtained from *Citrus reticulata*, one of the most representative species from the *Citrus* genus in terms of worldwide production and consumption,[60] may have significant health benefits.

Conflicts of interest

The authors have no conflicts of interest involving this study.

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