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Research Article

Apoptosis-inducing activity of hydroxylated polymethoxyflavones and polymethoxyflavones from orange peel in human breast cancer cells

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Sweet orange (*Citrus sinensis* L.) peel is a rich resource of flavonoids, especially polymethoxyflavones (PMFs). Citrus flavonoids exert a broad spectrum of biological activity, including antiproliferative and proapoptotic effects in cancer cells. We have recently shown that individual PMFs from orange peel induce Ca^{2+}-mediated apoptosis in human breast cancer cells and that hydroxylation of PMFs is critical for enhancing their proapoptotic activaty. Here, we report that the fraction of orange peel extract containing a mixture of non-hydroxylated PMFs (75.1%) and hydroxylated PMFs (5.44%) and the fraction containing only hydroxylated PMFs (97.2%) induce apoptosis in those cells as well. Treatment of MCF-7 breast cancer cells with these fractions inhibited growth and induced apoptosis associated with an increase in the basal level of intracellular Ca^{2+}. Effective concentrations of the hydroxylated PMFs fraction in inhibiting growth, inducing apoptosis, and increasing intracellular Ca^{2+} were lower than those of the non-hydroxylated PMFs fraction. Our results strongly imply that bioactive PMFs from orange peel exert proapoptotic activity in human breast cancer cells, which depends on their ability to induce an increase in intracellular Ca^{2+} and thus, activate Ca^{2+}-dependent apoptotic proteases.

Keywords: Apoptosis / Breast cancer / Flavones / Flavonoids / Hydroxylated polymethoxyflavones

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1 Introduction

The sweet orange (*C. sinensis* L.) peel oil is a rich resource of flavonoids with polymethoxyflavones (PMFs) as major constituents [1, 2]. In recent years, there was a marked interest in citrus flavonoids due to their broad spectrum of biological activity, including anti-inflammatory, anti-carcinogenic, anti-viral, antioxidant, anti-thrombogenic and anti-atherogenic properties [2 – 4]. Citrus flavonoids, including PMFs, have been shown to exert antiproliferative activity in cancer cells [1, 4 – 8], however the mechanisms involved remain largely undefined. We have recently demonstrated [9] that PMFs derived from the sweet orange peel induce cell death via Ca^{2+}-mediated apoptotic mechanism.

Induction of apoptotic cell death is emerging as a promising strategy for chemoprevention and treatment of breast cancer, because it may allow for selective elimination of cancer cells [10 – 12]. Our findings [10, 13] suggest that regulation of apoptosis in normal and cancer human mammary epithelial cells by the key cellular signal, Ca^{2+}, is different. Therefore, plant bioactive compounds (*e.g.* PMFs) that trigger Ca^{2+}-mediated apoptosis in cancer cells, but not normal cells, would be of tremendous importance for breast cancer prevention and treatment.

Cellular Ca^{2+} has been strongly implicated in regulation of apoptotic pathways [10, 12, 13 – 17]. We have shown that the apoptotic Ca^{2+} signal represents a sustained increase in the basal concentration of intracellular Ca^{2+}, reaching elevated, but not cytotoxic levels [12, 13, 18]. We have also

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Abbreviations: OPE, orange peel extract; **PMFs,** polymethoxyflavones; **PMF-OH,** hydroxylated PMFs

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demonstrated that such an increase in intracellular Ca^{2+} activates Ca^{2+}-dependent apoptotic proteases, calpain and caspase-12 [12, 13, 16, 19]. It is noteworthy to note that Ca^{2+} -mediated apoptosis accompanied by activation of calpain and caspase-12 can be induced, besides PMFs, by the soy isoflavone genistein [20] and the Ca^{2+} regulatory steroid hormone 1,25-dihydroxyvitamin D_3 [12, 13].

In this study, we evaluated efficacy of two PMF fractions of orange peel extract (OPE); namely, the fraction containing mainly non-hydroxylated PMFs (75.1%) and the purified fraction containing hydroxylated PMFs (PMF-OH) (97.2%), to inhibit growth and induce apoptosis in human breast cancer cells. The hypothesis was that the OPE PMF fractions induce apoptosis in breast cancer cells via Ca^{2+}-mediated mechanism. The findings obtained indicate that the PMF fractions trigger a concentration- and time-dependent increase in the basal level of intracellular Ca^{2+}, associated with inhibition of cell growth and induction of apoptosis, and that the fraction of PMF-OH is significantly more active, than the non-hydroxylated PMFs fraction, in inducing apoptotic death in breast cancer cells.

2 Materials and methods

2.1 Materials

Sweet OPE from cold-pressed orange peel oil, containing approximately 30% PMFs, was obtained from Florida Flavors (Lakeland, FL). This extract was used for preparation of the PMF-OH fraction . Pre-packed silica gel (60Å, 32–63 μm) column (120 g) for normal phase chromatography was purchased from Teledyne Isco (Lincoln, NE). Octadecyl (C_{18}) derivatized silica gel (60 Å) RP analytical and preparative columns for HPLC were purchased from YMC (Kyoto, Japan). Seventy percent PMF fraction was obtained from Danisco (Lakeland, FL). This fraction, labeled as PMF fraction, was analyzed and directly used in biological activity studies.

2.2 Analytical procedures for PMF fractions

2.2.1 Flash column system

An automated flash chromatography system (Model Foxy 200, Teledyne Isco, Lincoln, NE) equipped with a pre-packed silica gel flash column was used. The mobile phase for normal phase flash column consisted of hexanes and ethyl acetate in varying proportions; the flow rate was set at 96 mL/min. The eluent was monitored with a single channel UV detector at a wavelength of 254 nm.

2.2.2 Preparative HPLC system

An automated HPLC from Gilson (Middleton, WI) was used for preparative purpose. This semi-preparative HPLC system was equipped with two pumps (322 HPLC pump with H2 pump heads), a UV-Vis (diode array) detector (155) and an automated injection system (215 liquid handler with syringe pump and 819 injection module). The mobile phase for the HPLC system was ACN and water with a flow rate set at 20 mL/min. The eluent was detected with dual UV wavelength at 326 nm and 254 nm.

2.2.3 Mass spectrometer

EI-MS spectra were obtained on a MicroMass AutoSpec HF (Micromass, Beverly, MA). MS conditions: mass scan range, 100–1500 amu, 250°C (EI-MS).

2.2.4 LC-ESI-MS

A HPLC-MS system was composed of an auto-sampler injector, an HP1090 system controller, with a variable UV wavelength (190–500 nm) detector, an Evaporizing Laser Scattered Deposition detector and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass, Beverly, MA). ESI-MS conditions were as following: acquisition mode, ESI-positive; mass scan range, 100–800 amu; scan rate, 0.4 s; cone voltage, 25 V; source temperature, 150°C; probe temperature, 550°C. Analytical HPLC conditions on HPLC-MS: column, Chromeabond WR C_{18}, 3 μm, 120 Å; length and od, 30×3.2 mm; injection volume, 15 μL; flow rate, 2 mL/min; run time, 3 min. Mobile phase consisted of ACN and water with 0.05% TFA, typical gradient of 10–90% ACN.

2.2.5 Analytical HPLC analysis of composition of PMF and PMF-OH fractions

HPLC was utilized to analyze the individual PMF and PMF-OH composition in the PMF and PMF-OH fractions. The dissolved solutions were analyzed on an HPLC system (Shimadzu Scientific Instruments, Columbia, MD) with vendor-provided auto injector (SIL-10 AD vp), UV-Vis detector (SPD-10A vp), dual pumps (LC-10 AT vp) and system controller (SCL-10A vp) components with a NOVA-PAK silica (3.9×150 mm, 5 μm) analytical column (Waters Corp., Milford, MA). The analytical method was a gradient method, from 15% ethyl acetate-85% hexanes to 50% ethyl acetate-50% hexanes in a 15 min run with a flow rate of 2 mL/min and the monitoring UV absorbance set at 280 nm.

2.3 Preparation of the PMF-OH

2.3.1 General separation procedures from crude sweet OPE (cold-pressed oil)

The commercial OPE mixture (10 g, 30% PMFs) from Florida Flavors was dissolved in a mixture of methylene chloride (2 mL) and hexanes (2 mL) and loaded onto a 120 g pre-conditioned silica gel flash column. The stepped gradient was started with 100% hexanes for 10 min, and went to 60% ethyl acetate and 40% hexanes within 40 min. The isocratic mobile phase (40% ethyl acetate-60% hexanes) was applied for another 10 min (total run of 60 min). The

fractions that had UV absorbance at 254 nm were analyzed by LC-ESI-MS and the PMF-OH containing fractions characterized by LC and MS were combined and concentrated. Further separation was done using RP HPLC.

2.3.2 Separation procedures of PMF-OH by RP HPLC

The concentrated residue that contains PMF-OH characterized by LC-ESI-MS was dissolved in ACN (4 mL) and DMSO (1 mL). The dissolved solution was loaded onto a C_{18} RP HPLC system. A gradient method was used from 25% ACN-75% water to 60% ACN-40% water in 25 min with a flow rate of 20 mL/min. The fractions were analyzed by LC-ESI-MS. The fractions containing PMF-OH were combined, concentrated and lyophilized to dryness. The dried fraction was yellow solid and weighed 0.26 g. Analysis by LC-MS showed the total content of PMF-OH being greater than 90%. This fraction, labeled as PMF-OH fraction, was used in biological activity studies.

2.4 Cell culture

The human breast carcinoma cell line MCF-7 (ATTC, Manassas, VA) was used in this study. MCF-7 cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were treated with the PMF or PMF-OH fractions (at six concentrations ranging from 1.16 μg/mL to 37.0 μg/mL) or vehicle, DMSO (0.1%), for 1, 3 or 6 days. Stock solutions of the fractions were prepared in DMSO. The treatments of cells started 24 h after seeding. Cell growth was evaluated by counting cell numbers in a hemacytometer using Trypan Blue exclusion assay and by measuring cellular total nucleic acid content with the fluorescent probe CyQuant GR (485 nm excitation, 530 nm emission; CyQuant Cell Proliferation Assay Kit, Invitrogen/Molecular Probes, Eugene, OR). For these assays, cells were grown in 12-well plates or 96-well black-walled plates, respectively; fluorescence intensity was measured in the FLx800 plate reader with KC software (BioTek, Woburn, MA).

2.5 Apoptosis

Apoptosis was evaluated by the plasma membrane and nuclear changes. Annexin V assay (Alexa Fluor 488 Annexin V Assay Kit; Molecular Probes) was used for detection of the apoptotic plasma membrane (loss of membrane asymmetry due to phosphatidylserine translocation). Fluorescence (485 nm excitation, 530 nm emission) of the Annexin V-labeled cells grown in 96-well plates was measured in the FL × 800 plate reader and expressed in relative fluorescence units (RFU) per 1×10^3 cells. Propidium iodide uptake was also used to evaluate cell death (the dye permeates the plasma membrane and stains nucleic acids of dead cells, including late apoptotic cells with the compromised plasma membrane, but not early apoptotic cells). Fluorescence (530 nm excitation, 620 nm emission) of the propidium iodide-labeled cells was measured in the FL × 800 reader.

Additionally, Hoechst 33342 and Alexa Fluor 488 Annexin V were employed to visualize apoptotic nuclei (nuclear fragmentation) and the apoptotic plasma membrane, respectively. Fluorescence microscopy of Hoechst 33342- and Annexin V-labeled cells was performed with cells in the microincubation chamber (37.0 ± 0.2°C) on an Eclipse TE-300 inverted microscope (Nikon, Tokyo, Japan) equipped for epifluorescence digital imaging. The images were captured using SuperFluor 40 × 1.3 NA oil-immersion objective lens (Nikon) and CoolSnapFX CCD camera (Photometrics, Tucson, AZ) (360 nm or 485 nm excitation for Hoechst 33342- and Annexin V, respectively, 530 nm emission barrier filter). Image analysis [13, 21] was performed

Table 1. Composition of the PMF and PMF-OH fractions

Compound name	Content (%)	
	PMF fraction	PMF-OH fraction
Heptamethoxyflavone	10.43 ± 1.15	
Tangeretin	17.60 ± 1.75	
5,6,7,4′-Tetramethoxyflavone	12.02 ± 1.16	
Nobiletin	29.63 ± 2.83	
Sinesetin	5.41 ± 1.21	
Total PMFs	75.09	
5-Demethyltangeretin		0.68 ± 0.02
5-Hydroxy-6,7,4′-trimethoxyflavone	1.11 ± 0.10	19.00 ± 0.55
5-Hydroxy-3,6,7,8,3‘,4′-hexamethoxyflavone	<0.10	28.56 ± 4.90
5-Demethylnobiletin	2.82 ± 1.34	38.4 ± 7.04
5-Hydroxy-3,6,7,3′,4′-pentamethoxyflavone	1.25 ± 0.25	5.96 ± 0.08
5-Hydroxy-6,7,3′,4′-tetramethoxyflavone	0.26 ± 0.08	4.64 ± 0.88
Total PMF-OH	5.44	97.24

Table 2. Antiproliferative and proapoptotic activities of PMF and PMF-OH fractions in MCF-7 cells.[a)]

Fraction	Inhibition of cell proliferation (IC_{50}, μg/mL)	Induction of cell apoptosis (EC_{min}, μg/mL)	Induction of cell death (EC_{min}, μg/mL)	Increase in intracellular Ca^{2+} (EC_{min}, μg/mL)
PMF	17.9	9.25	18.5	4.62
PMF-OH	10.2	4.62	18.5	2.31

a) IC_{50}, the concentration inhibiting cell growth by 50%, is presented as the average of the day 3 and day 6 determinations. The minimal effective concentrations, EC_{min}, for induction of cell death, apoptosis, and increase in $[Ca^{2+}]_i$ are also indicated. Effects of the fractions on proliferation, apoptosis, death, and intracellular Ca^{2+} levels in MCF-7 cells were evaluated as described in Section 2.

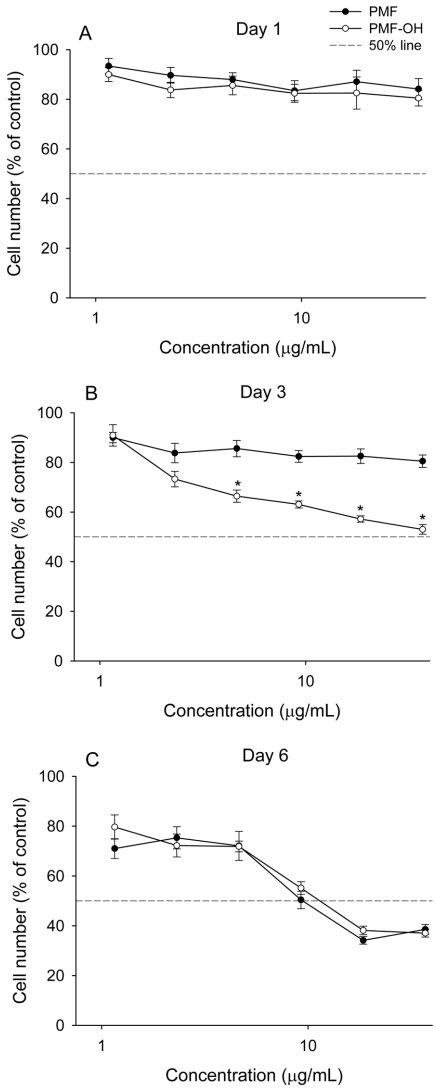

Figure 1. Effect of PMF and PMF-OH fractions on cell number. The cell numbers, presented as percent of control, were determined as described in Section 2. Here and in Figs. 2, 4, and 5, MCF-7 cells were treated with fractions or vehicle for 1 (A), 3 (B) or 6 (C) days. Results are means ± SE of triplicate determinations of two independent experiments; (*), indicates statistically significant differences ($p < 0.05$) between PMF-OH and PMF treatments at the same concentrations. *X* axis is log (common).

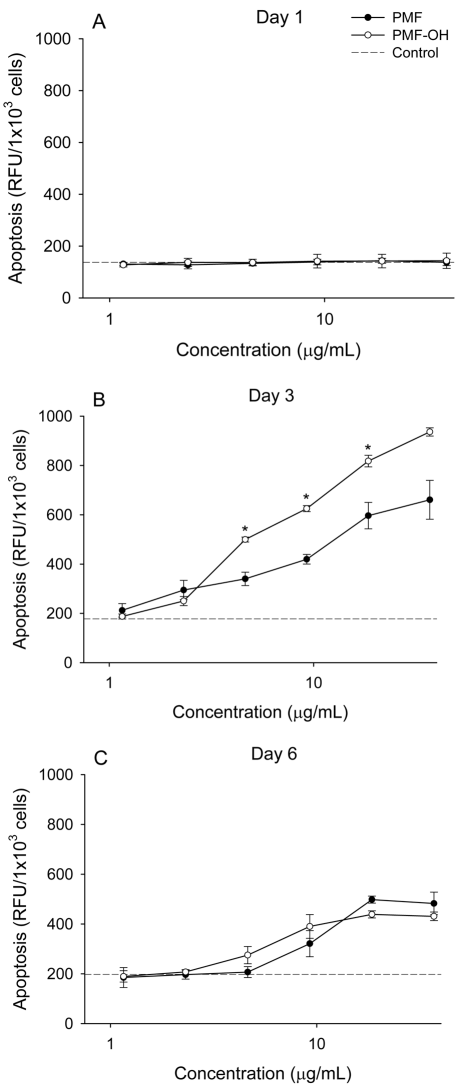

Figure 2. Apoptosis-inducing activity of PMF and PMF-OH fractions. Apoptosis was measured with Annexin V, as described in Section 2. Here and in Figs. 4 and 5, results are presented as relative fluorescence units (RFU) per 1×10^3 cells.

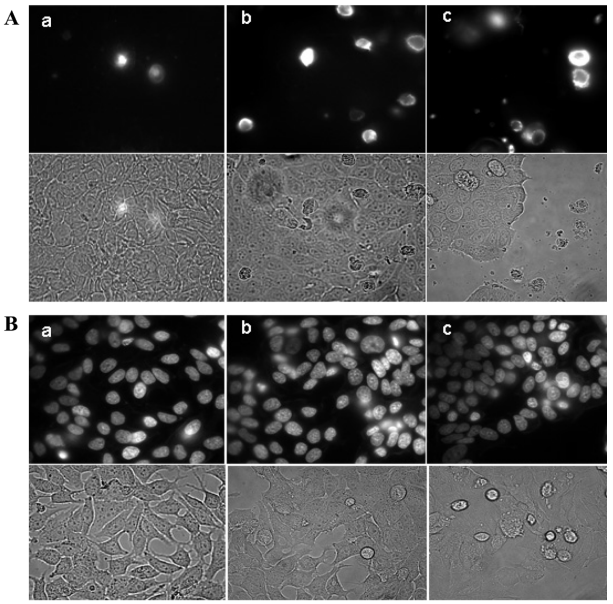

Figure 3. Apoptotic plasma membrane and nuclear fragmentation in MCF-7 cells treated with the PMF and PMF-OH fractions. (A), Annexin V-labeled cells; (B), Hoesht 33342-labeled cells. On panels A and B, upper rows are fluorescence images, lower rows are phase-contrast images; a, control; b, PMF; c, PMF-OH. Cells were treated with the PMF or PMF-OH fractions at concentrations of 18.5 μg/mL and 9.25 μg/mL, respectively, for 3 days (Annexin V) or 6 days (Hoesht 33342). Note the labeling of, and nuclear fragmentation/DNA condensation in, the apoptotic (round and bright fluorescent) cells. See Sections 2 and 3 for additional explanations.

using MetaMorph 6.3 software (Molecular Devices/Universal Imaging, Downingtown, PA).

2.6 Intracellular calcium

Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in MCF-7 cells were measured with the Ca^{2+} indicator fluo-3, as described in detail previously [9, 13, 22]. Cells grown in the 96-well black-walled plates were loaded with 2 μM of fluo-3/AM (Molecular Probes) in Dulbecco's PBS (D-PBS) supplemented with 0.1% DMSO for 40 min at 37°C. Fluorescence (485 nm excitation, 530 nm emission) was measured in the FL × 800 plate reader.

3 Results

3.1 Composition of OPE fractions

Composition of the OPE PMF and PMF-OH fractions is presented in Table 1. PMF fraction contained mainly non-hydroxylated PMFs (75.1%) and a small amount (5.44%) of PMF-OH, while total content of PMFs in the PMF-OH fraction was 97.2% as PMF-OH.

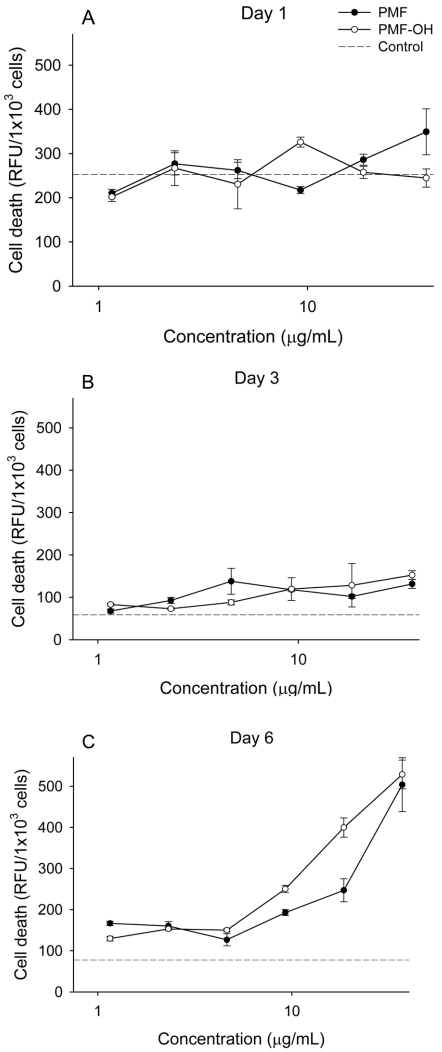

Figure 4. Cell death induced by PMF and PMF-OH fractions. Cell death was evaluated with propidium iodide, as described in Section 2. The elevated fluorescence intensity levels at day 1, as compared with day 3 and 6, indicate higher permeability of the plasma membrane to propidium iodide of the recovering after trypsinization cells at day 1.

3.2 Cell growth

PMF and PMF-OH fractions inhibited proliferation of MCF-7 breast cancer cells in a concentration- and time-dependent fashion, as evaluated by counting cell numbers (Fig. 1). Similar results were obtained by measuring cell proliferation (cellular nucleic acid content) with the CyQuant GR probe (not shown). The IC_{50} values for inhibition of cell proliferation are presented in Table 2. The PMF-OH fraction exhibited significantly higher antiproliferative activity, as compared with the PMF fraction, at day 3 of treatment (see Fig. 1). The tested fractions did not exert cytotoxic effect, as evident by no changes in the numbers of viable cells after a 1 day treatment.

3.3 Apoptosis and cell death

PMF and PMF-OH fractions induced apoptosis in MCF-7 breast cancer cells at day 3 and 6 of treatment in a concentration-dependent manner (as measured with the fluorescent probe Alexa Fluor 488 Annexin V; Fig. 2, also see Table 2). The effective concentrations were similar to those for antiproliferative activity. Again, the PMF-OH fraction demonstrated higher proapototic activity, as compared with the PMF fraction, at day 3 of treatment. Fluorescence microscopy of the PMF and PMF-OH treated cells labeled with Annexin V and Hoechst 33342 confirmed apoptotic changes of the plasma membrane and nuclei (Fig. 3).

PMF and PMF-OH fractions induced death of MCF-7 breast cancer cells, characterized by compromised (permeable) plasma membrane (as evaluated with the propidium iodide; Fig. 4, also see Table 2). It was particularly evident at day 6 of treatment, and there was no difference between PMF and PMF-OH fractions in inducing cell death at this time point.

3.4 Intracellular Ca^{2+}

PMF and PMF-OH fractions induced an increase in the basal level of intracellular Ca^{2+} in breast cancer cells at day 3 and 6 of treatment in a concentration-dependent fashion (as evaluated with fluo-3; Fig. 5, also see Table 2). Effective concentrations were similar to those for the antiproliferative and proapoptotic activities. At higher concentrations, the PMF-OH fraction was more effective than the PMF fraction in inducing $[Ca^{2+}]_i$ increases at day 3 and 6 of treatment. However, there were no significant differences in inducing cell death and inhibiting cell growth between PMF-OH and PMF fractions at day 6 of treatment (see Fig. 1(C), 2(C), and 4(C)). This may indicate that activation of Ca^{2+}-dependent apoptotic proteases with the PMF fraction is delayed, as compared with the PMF-OH fraction.

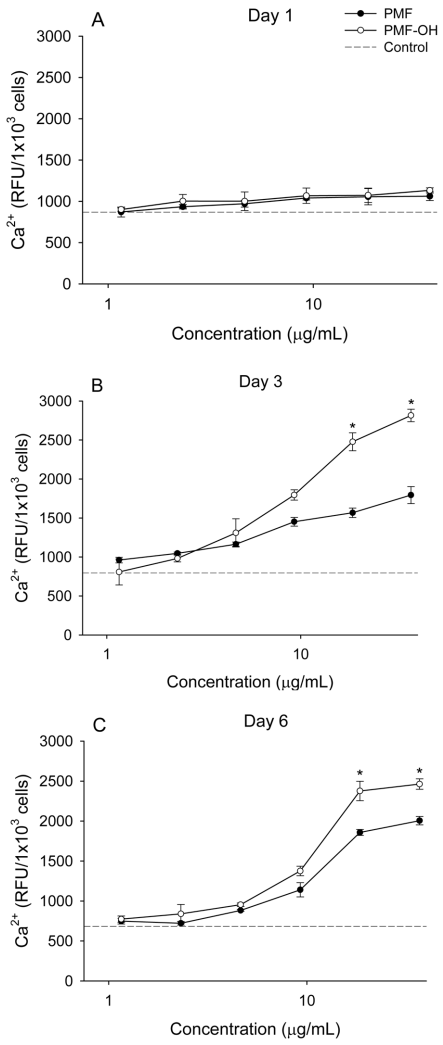

Figure 5. Effect of PMF and PMF-OH fractions on intracellular Ca^{2+} levels. $[Ca^{2+}]_i$ was measured with fluo-3/AM, as described in Section 2.

4 Discussion

The results presented in this paper demonstrate for the first time that the OPE PMF and PMF-OH fractions exert antiproliferative and proapoptotic activity in human breast cancer cells *in vitro* and support the hypothesis that the PMF-induced increase in $[Ca^{2+}]_i$ is associated with induction of apoptosis in these cells. Our recent findings demonstrate that individual PMFs, contained in the PMF and PMF-OH fractions, induce apoptosis by triggering an increase in $[Ca^{2+}]_i$ followed by activation of Ca^{2+}-dependent apoptotic proteases, μ-calpain and caspase-12, and that hydroxylation of PMFs, particularly the C-5 hydroxyl group, is critical for enhancing their proapoptotic activity [9]. As we showed here, the OPE PMF and PMF-OH fractions induce a similar increase in the basal level of intracellular Ca^{2+}, which is required for activation of Ca^{2+}-dependent apoptotic proteases.

It is important to note that the PMF-OH fraction appears to be more effective than the PMF fraction in inhibiting proliferation and inducing apoptosis in breast cancer cells. This was evident at day 3 of the cell culture, when cells are at the rapidly proliferating phase and when induction of apoptosis would be particularly effective for suppressing cell enumeration. The data obtained are in agreement with our previous findings demonstrating higher proapoptotic activity of PMF-OH, as compared with non-hydroxylated PMFs [9]. Noteworthy, the study of antiproliferative activity of *Citrus* flavonoids by Kawaii *et al.* [23] ranked low the non-hydroxylated PMFs, nobiletin and heptamethoxyflavone, which are the major components of the PMF fraction used in our study. Importantly, Kawaii *et al.* [23] also found

that the C-3 hydroxyl group seems to enhance the antiproliferative potency of PMFs.

An increase of intracellular Ca^{2+} induces apoptosis in various cell types [13, 14, 16, 17]. We have shown earlier that such a $[Ca^{2+}]_i$ increase results from both Ca^{2+} influx from the extracellular space and Ca^{2+} mobilization from the endoplasmic reticulum stores and that it is associated with activation of apoptotic proteases, the Ca^{2+}-dependent μ-calpain and the Ca^{2+}/calpain-dependent caspase-12 [12, 13, 20]. We have also demonstrated that PMFs can utilize Ca^{2+}-mediated signaling to induce apoptosis; namely, PMFs trigger influx of Ca^{2+} and mobilization of intracellular Ca^{2+} stores, accompanied by activation/cleavage of calpain and caspase-12 [9]. The mechanism of generating the apoptotic Ca^{2+} signal with PMFs might include their interactions with membrane receptors coupled to the low-conductance Ca^{2+} channels [21]; additionally, hydroxylation of PMFs appears to be critical for conferring their proapoptotic activity, while position and number of methoxy groups may be important for modulating this activity [9].

In summary, results presented here further support the hypothesis that an increase in basal levels of intracellular Ca^{2+} is associated with induction of apoptosis. Ca^{2+}-mediated apoptosis triggered by PMFs can be exploited for induction of cell death in prevention of certain diseases. For example, differences in regulation of intracellular Ca^{2+} in normal *vs*. cancer human mammary epithelial cells may allow selective elimination of cancer cells via Ca^{2+}-mediated apoptosis [12, 13]. OPE fractions enriched with PMF-OH may prove to be useful as dietary supplements with apoptosis-inducing activity for breast cancer prevention.

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