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Selective anti-cancer effects of palm phytonutrients on human breast cancer cells

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Abbreviations:

ADP; adenosine diphosphate

BSA; bovine serum albumin

CPO; crude palm oil

CoQ10; Co-enzyme Q10

DCFCS; dextran-charcoal treated fetal calf serum

DMEM; Dulbecco's modified eagle medium

EF; enrichment factor

FBS; fetal bovine serum

ELISA; enzyme-linked immunosorbent assay

HPLC; high performance liquid chromatography

HRP; horseradish peroxidase

IC_{50}; half maximal inhibitory capacity

NF-κB; nuclear factor kappa-light-chain-enhancer of activated B-cells

PARP; poly-(ADP-ribose) polymerase

PBS; phosphate-buffered saline

ppm; parts-per million

TMB; tetramethylbenzidine

TRF; tocotrienol-rich fraction

TNF-α; tumor necrosis factor-alpha

ABSTRACT

Palm oil contains a number of phytonutrients. To date, most of the studies using palm phytonutrients have focused on palm vitamin E. The objective of this study was to compare the anti-cancer activities of four major palm phytonutrients, namely tocotrienol-rich fraction, carotenoids, squalene and co-enzyme Q10 using some cell-based assays. Two human breast cancer cell lines; the highly aggressive triple-negative MDA-MB-231 and the estrogen-dependent MCF-7 cells were treated with these palm phytonutrients to determine their anti-cancer effects. Palm vitamin E exhibited higher ability to induce cell death by apoptosis both the human breast cancer cells compared to the other palm phytonutrients (carotenoids, squalene and co-enzyme Q10), which was absent in normal NIH/3T3 cells. In addition, there was higher cleavage of the poly-adenosine diphosphate-ribose polymerase (PARP) enzyme in palm vitamin E-treated MDA-MB-231 cells compared to the other phytonutrients. All the palm phytonutrients tested suppressed the expression of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) protein in the breast cancer cells exposed briefly to tumor necrosis factor-alpha (TNF-α). Palm phytonutrients possess varying extent of anti-cancer effects on the human breast cancer cells.

Key words: palm phytonutrients, tocotrienol-rich fraction, carotenoids, squalene, co-enzyme Q10, anti-cancer, anti-inflammation,

1. Introduction

Phytonutrients are phytochemicals or phyto-protectants substances that are derived from plant sources. The term phytonutrients covers all the natural vitamins, minerals or bioactive compounds obtained from plants.[1] In addition, some of the phytonutrients found in fruits and vegetables allow these to impart bright colors physiologically. Many of the phytonutrients exhibit diversified physiological and pharmacological effects. This, several agencies including the United States Department of Agriculture (USDA), had recommended consumption of diets rich in fruits and vegetables to garner various health benefits and to reduce the risk of developing cancer.[2] In addition, phytonutrients are preferred *in lieu* of synthetic compounds due to the perceived low or null adverse effects.

Palm oil offers several nutritional and health benefits. Phytonutrients make up approximately 1% of the weight of crude palm oil (CPO).[3] The most prevalent phytonutrients found in palm oil are vitamin E [600-1000 parts-per million (ppm)], carotenes (500-700 ppm), phytosterols (300-620 ppm), squalene (250-540 ppm), Co-enzyme Q10 (CoQ10) (10-80 ppm), polyphenols (40-70 ppm) and phospholipids (20-100 ppm).[3] These minor constituents also play a vital role in stabilizing and refining palm oil. About 70% of vitamin E found in the palm tocotrienol-rich fraction (TRF) is in the form of tocotrienols, while alpha-tocopherol accounts for the remaining 30%. It has been reported that tocotrienols are unique as these vitamers can freely penetrate tissues with saturated fatty layers, thus are able to perform more efficient metabolic functions as compared to tocopherols.[4] Studies have shown that accumulation of tocotrienols in tissues could provide a number of health benefits such as reduce blood cholesterol levels[5], reduce arteriosclerosis[6], exhibit anti-

angiogenic[7,8], antioxidant[9], radioprotector[10], anti-cancer[11,12] and neuroprotective[13] activities.

Carotenoids are natural pigments responsible for the brilliants orange-red feature of palm oil. Crude palm oil is considered to be one of the world's richest sources of carotenoids.[14] About 600 types of naturally occurring carotenoids are known but only 13 different types are found in palm oil; of which the major ones are β-carotene, α-carotene, lycopene, phytoene and phytofluene.[3] Carotenoids act as precursors of vitamin A, which has been reported to pay a role in preventing night blindness[15], improve vitamin A status of lactating women and their infants[16], improve serum retinol concentrations[17] and combat vitamin A deficiency.[14,18] Carotenoids can also protect against cardiovascular diseases[19] and suppress growth of various cancers. [20-23]

Co-enzyme Q10, also known as ubiquinone, is a natural co-enzyme found in CPO. Although palm CoQ10 has ten times greater antioxidant property as compared to vitamin E, its effects is masked by palm carotenes and vitamin E, which are present in greater abundance in the palm oil.[24] As CoQ10 has been found to be a good free radical scavenger[25], it plays a vital role in the mitochondrial electron transport chain as well as in stabilizing membranes.[26] This phytonutrient is reported to have anti-cancer effects[27] and it has also been used in the treatment of many cardiovascular diseases.[28,29]

Squalene is a valuable triterpene that is found in high quantities in shark liver oil.[30] It is present in trace amounts in palm oil.[24] Squalene is an oxygen transmitter and could aid cardiovascular health. This phytonutrient has also been reported to possess anti-tumor, anti-proliferative[31-33] and radio-protective effects.[31] Breast cancer is the second leading devastating cause of cancer-related death in women, after lung

and bronchus cancer. Breast cancer is the most common cancer that affects Malaysian women from all ethnicities[34,35] and world over[36]. The BRCA-1 and BRCA-2 genes predispose to only 10-15% of breast cancer incidence.[37] The remaining incidence of breast cancer accounts for unknown environmental and nutritional factors. Most of the currently used chemotherapeutic drugs have adverse effects and cause damage to normal cells.[38] Thus, nutritional strategies in the area of breast cancer treatment and prevention would be in great favor to reduce the risk and mortality rate. Currently, most studies on the anti-cancer potential of the palm phytonutrients have focused primarily its vitamin E component. To date, there has not been much focus on the anti-cancer effects of the other palm phytonutrients such as squalene, CoQ10 and carotenoids. Hence, in this study, we compared the anti-cancer effects of palm carotenoids, squalene and CoQ10, with that of palm vitamin E on two human breast cancer cell lines; the highly aggressive triple negative MDA-MB-231 and the estrogen-dependent MCF-7 human breast cancer cells. The

2. Methods and materials

2.1. Cell culture

Dulbecco's modified Eagle's medium (DMEM) with high glucose (GIBCO, Invitrogen, Grand Island, NY USA); TrypLE Express Stable Trypsin-like (GIBCO, Invitrogen, Grand Island, NY USA); L-glutamine (GIBCO, Invitrogen, Grand Island, NY USA); fetal bovine serum (FBS) (GIBCO, Invitrogen, Grand Island, NY USA); penicillin-streptomycin solution (GIBCO, Invitrogen, Grand Island, NY USA); β-estradiol (Sigma Chemicals, San Louis, USA); RPMI medium 1640 (GIBCO, Invitrogen, Grand Island, NY USA); phosphate-buffered saline (PBS) (Sigma Chemicals).

2.2. Test Compounds

Tocotrienol-rich fraction (TRF) is a standardized composition of palm vitamin E containing 32% α-tocopherol, 25% α-tocotrienol, 29% γ-tocotrienol and 14% δ-tocotrienol (Golden Hope Plantations, Selangor, Malaysia); Carotenoids in the form of 20% natural mixed carotenoids complex (Caromin 20%) (Carotech Bhd.); Squalene (Sigma Aldrich Chemical Co, USA) and CoQ10 (Sigma Aldrich Chemical Co, USA). Stock solutions of 10 mg/ml were prepared for each phytonutrient with appropriate vehicles and stored at -20°C.

2.3. Cell lines

The MCF-7 and the triple negative MDA-MB-231 human breast cancer cell lines as well as a primary mouse embryonic fibroblast (NIH/3T3) cells were purchased from the American Tissue Culture Collection (ATCC, USA). The MDA-MB-231 and NIH/3T3 cells were cultured as monolayers in culture flasks (Orange Scientific, Braine-l' Alleud, Belgium) in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin (complete medium) in a humidified atmosphere of 5% CO_2 at 37°C. The MCF-7 cells were cultured under identical conditions and medium, except that 10^{-8} M β-estradiol was added to the medium. Culture media were routinely changed every 2–3 days**.** For treatment of the cells, phenol red-free RPMI 1640 medium containing 5% dextran-charcoal treated fetal calf serum (DCFCS), 1% L-glutamine and 1% antibiotic penicillin-streptomycin was used.

2.4. Growth inhibition assay

The MCF-7, MDA-MB-231 and NIH/3T3 cells were harvested and counted using a hemocytometer. The cell numbers were adjusted to 5 x 10^6 cells/ml using complete

164 medium and 0.5 ml of this cell suspension was plated in 24-well tissue culture plates
165 (Orange Scientific, Braine-l' Alleud, Belgium). After 24 hours, the medium was
166 changed and the relevant test compounds (0–20 μg/ml) were added to cultures. The
167 concentration range of test compounds used in this study was chosen based on the
168 National Cancer Institute (NCI) criteria, which recommended that natural bioactive
169 compound with IC_{50} less than 20 μg/ml to be poor candidate for anti-cancer agents.[39]
170 The cells were cultured in presence of the test compounds at 37°C in humidified
171 atmosphere of 5% CO_2 for 72 hours. Cultures containing the cells and medium
172 served as control. After 72 h, the cells were washed in 0.9% NaCl to remove the
173 non-adherent dead cells. The cells were incubated in a lysis buffer [2.5 mM HEPES
174 (Calbiochem, San Diego, CA, USA) buffer, 1.5 M $MgCl_2$ (MERCK, Darmstadt,
175 Germany) and zapoglobin II lytic (Beckman Coulter, Brea, CA, USA)] for 15 min.
176 The released nuclei were suspended in isoton III (Beckman Coulter) and counted
177 using a Coulter particle counter ZI (Beckman Coulter) with particle size set at >5 μm.
178 All cell counts were carried out in triplicate on triplicate well treatments.

179

180 *2.5. Cell death mechanism*

181 A commercial cell death detection ELISA kit (Roche Diagnostic Gmbh, Mannheim,
182 Germany) was used to detect mechanisms of cell death as previously described.[40]
183 Briefly, the human breast cancer cells were seeded at 1 x 10^4 cells/well and
184 incubated overnight at 37 °C in a humidified 5% CO_2 incubator. Following this, 100
185 μl of the treatment compound (10 μg/ml) was added to the cells and incubated for 72
186 hours. Results are presented as enrichment factor (EF) of mono- and oligo-
187 nucleosomes calculated using the following formula, provided by the manufacturer:

EF = Absorbance of sample **(treated cells)** / Absorbance of corresponding control **(untreated cells)**

188

189 *2.6. Determination of PARP cleavage by ELISA*

190 Poly-adenosine diphosphate (ADP)-ribose polymerase (PARP)is a protein involved
191 in a number of cellular processes involving mainly DNA repair and programmed cell
192 death.[41] Cleavage of PARP indicates the presence of an apoptotic event. The
193 PARP cleavage activity was quantified using a commercial ELISA kit. This ELISA kit
194 detects the 89 kDa cleaved fragment that consist of the catalytic domain. A
195 commercial ELISA kit was used to determine levels of cleaved PARP-1 (Asp214),
196 according to the manufacturer's protocol (PathScan, Cell Signaling Technology Inc
197 Beverly, USA). This kit detects endogenous 89 kDa cleaved fragment of the PARP,
198 which consist of its' catalytic, using a sandwich immunoassay format. The MDA-MB-
199 231 and MCF-7 human breast cancer cells (1 X 10^7 cells) were harvested and
200 seeded in 10 cm petri dishes. The cells were incubated overnight and treated with
201 the respective test compounds (10 μg/ml) for 24 h. This was followed by treatment
202 with 1 nM tumor necrosis factor-alpha (TNF-α) for 30 min. Following this, the cells
203 were washed in ice-cold PBS and re-suspended in an ice-cold cell lysis buffer [20
204 mM Tris (pH 7.5), 150 mM NaCl,1 mM ethylene diamine tetraacetate (EDTA), 1 mM
205 ethylene glycol-bis(2 aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 1% Triton X-
206 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1
207 μg/ml leupeptin) containing 1 mM PMSF] (Calbiochem, San Diego, CA, USA). The
208 cell lysate was prepared according to the manufacturer's protocol. After the
209 sonication step, the cell lysate was recovered following centrifugation (*14,000 g* for
210 10 min at 4°C). Protein content in the cell lysate was estimated using the DC protein

assay according to the manufacturer recommended protocol (Bio-Rad Laboratories). Lysates with l μg/ml protein concentration were added to the wells of a 96-well plate coated with the cleaved PARP (Asp214) antibody (*provided with the kit*), and incubated overnight to allow binding of endogenous cleaved PARP to antibody-coated wells. After a washing step, PARP detection antibody (*provided with the kit*) was added and this was followed by the horse-radish peroxidase (HRP) conjugated detection antibody (*provided with the kit*). Finally, a chromagen (TMB substrate) (*provided with the kit*) was added to allow development of color, which was then stopped by the addition of the stop solution. Spectrometric absorbance of samples was measured at 450 nm; results are presented as percentage of cleaved PARP.

2.7. Expression of NF-κB p65 sub-unit

The two human breast cancer cells were seeded (2 x 10^6 cells/well) in 6-well plates and incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C. Following this, the cells were treated with the test compound (10 μg/ml) and incubated for 24 hours. Then, the cells were exposed to 1 nM TNF-α for 30 min. The media used for treatment was discarded and fresh media without treatment solutions were added to allow cells to stabilize and prevent cell stress. Following this, nuclear protein was extracted using a commercial nuclear extraction kit according to manufacturer's protocol (Panomics, Affymetrix Inc, Santa Clara, CA, USA). Protein estimation was performed using DC Protein Assay (Bio-Rad laboratories) according to manufacturer's protocol. The NF-κB transcription factor activity was analyzed using a commercial ELISA kit to quantify the NF-κB p65 as previously described.[42] Briefly, the activated NF-κB p65 molecules from 1 μg/μl nuclear protein were applied to NF-

κB p65 TF binding probe on biotinylated oligonucleotide *(provided with kit)* to form TF-DNA complexes for 30 min. To capture TF-DNA complexes, these samples were then transferred to streptavidin-coated plates *(provided with kit)* and incubated for an hour. Then, a primary antibody directed to NF-κB p65 *(provided with kit)* was added and the plate was incubated for another hour. Subsequently, horseradish-peroxidase (HRP) -conjugated secondary antibody *(provided with kit)* was left to react for another hour. Finally, the HRP substrate, tetramethylbenzidine (TMB) was added and incubated for 10-20 min for color change to take place. The TMB chromagen becomes blue in color upon oxidation with hydrogen peroxide catalyzed by the HRP. Yellow coloration is formed upon addition of a specific stop solution containing phosphoric acid *(provided with kit)* was added to the wells. Spectrometric absorbance of samples was measured at 450 nm; results are presented as fold change against control.

2.8. Statistical analysis

Results were calculated as average of mean ± standard deviation; one-way analysis of variance (ANOVA) was used to assess differences between groups. Differences among treatments were tested by the Tukey HSD post-hoc test. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Growth inhibition assay

Treatment with TRF inhibited the growth of the MDA-MB-231 and MCF-7 cells in a time- and dose-dependent manner (Fig 1). Carotenoids demonstrated comparable

inhibition pattern from day 1 to day 3 on both the human breast cancer cells. Carotenoids displayed a better half maximal inhibitory capacity (IC_{50}) values and growth inhibitory patterns on day 1 and 2 in the MDA-MB-231 cells (Fig 1). In the MCF-7 cells, the inhibitory effects were was observed later i.e. day 3. Squalene exhibited similar anti-proliferative pattern on the triple negative MDA-MB-231 cells from day 1 to day 3, until it reached a plateau at ~50% of cell growth after 4 μg/ml treatment (Fig 1). Squalene appeared to have a dose-dependent anti-proliferative activity, but the growth reduction was not very consistent. The CoQ10 treatment exhibited similar dose- and time-dependent activity on both human breast cancer cells where comparable IC_{50} values were observed (Fig 1). A plateau was achieved at ~50% of cell growth after 4 μg/ml from day 2 to day 3. Although the palm phytonutrients (carotenes, CoQ10 and squalene) suppressed the growth of the MDA-MB-231 and MCF-7 cells in a time- and dose-dependent manner, none of these phytonutrients were able to completely suppress the growth of both the human breast cancer cells at the concentrations tested (0 to 10 μg/ml); unlike the TRF. Table 1 shows a summary of the IC_{50} values obtained with both cell lines. The NIH/3T3 cells were chosen for this part of the study as these cells possess the unique property that mimics normal human fibroblast. The effect of co-culturing NIH/3T3 primary mouse embryonic fibroblast cells in the presence of varying concentrations (0-10 μg/ml) of TRF, carotenoids, squalene and coenzyme Q10 for 24, 48 and 72 hours is shown in Fig. 1. All the compounds tested here did not exhibit any time or dose dependent influence. There were no significant effects observed on the NIH/3T3 cells with all the treatment groups in the concentrations studied.

3.2. *Cell death mechanism*

Cell death can take place via various mechanisms such as apoptosis or necrosis. None of the treatments used showed any significant ($P > 0.05$) difference in the level of cell death induced through the apoptotic or necrotic pathways after the 72 hours of culture (Fig. 2).

3.3. *Determination of PARP cleavage by ELISA*

The relative ability of TRF, carotenoids, squalene and CoQ10 (10 μg/ml) to induce PARP cleavage in the two human breast cancer cells after 24 hours of exposure is shown in Figure 3. Exposure to TRF induced significant ($p<0.05$) PARP cleavage in the MDA-MB-231 human breast cancer cells but in the MCF-7 cells (Fig 3). In contrast, carotenoids and CoQ10 induced significant ($p<0.05$) PARP cleavage in the MCF-7 cells but not in the MDA-MB-231 cells (Fig 3). Squalene had no significant effects on PARP cleavage in both these cell lines.

3.4. *Expression of NF-κB p65 sub-unit*

There was a marked ($P<0.05$) increase in the expression of NF-κB in both the human breast cancers cells (MCF-7 and MDA-MB-231) when these cells were briefly exposed to 1 nM TNF-α (Fig 4). However, pre-treatment of these breast cancer cells with either of the four palm phytonutrients (10 μg/ml) prior to TNF-α exposure significantly ($P<0.05$) inhibited this expression of NF-κB in these cells (Fig. 4).

4. Discussion

This study evaluated the anti-cancer activity of vitamin E, carotenoids, squalene and CoQ10 on two human breast cancer cell lines; the highly aggressive triple negative MDA-MB-231 and estrogen-dependent MCF-7 cells.

Apoptosis is a reliable marker for the evaluation of potential agents for cancer prevention. In nature, many natural compounds possess cytotoxic and chemo-preventative effect, but not all of them can trigger apoptosis. No significant differences were observed in the level of apoptotic versus necrotic death in both human breast cancer cells lines treated with any of the palm phytonutrients (TRF, carotenoids, squalene and CoQ10). The PARP cleavage assay was used to elucidate how these compounds induce apoptosis in these cells. Majority of breast carcinomas were found to express high level of PARP-1.[43] Here, we found that TRF treatment induced apoptosis in both human breast cancer cells via the PARP cleavage pathway. This finding is consistent with what we had previously reported using the individual isoforms of tocotrienols.[40] Carotenoids and CoQ10 appear to induce PARP cleavage in the MCF-7 cells but not in the MDA-MB-231 cells whilst squalene had no significant effects on the PARP cleavage pathway in both cell lines.

PARP-1 is a major target protein for poly (ADP-ribosyl)ation.[44] However, there are also other acceptor proteins like p53, NF-κB, histones, DNA ligases, DNA polymerases and DNA-topoisomerases.[44-46] Activation of NF-κB has been implicated in carcinogenesis because of the critical roles of this transcription factor in cell proliferation, differentiation, survival, adhesion and inflammation.[44,46] Expression of NF-κB was induced in the human breast cancer cells by exposing these cells briefly to a low concentration (1 nM) of TNF-α. A marked inhibition in the expression

of NF-κB was observed in the cancer cells that were pre-treated low dose palm phytonutrients (TRF, carotenoids, squalene and CoQ10) prior to exposure to TNF-α.

There is growing evidence on the ability of carotenoids to confer protection against certain forms of cancer.[47] The natural mixed palm oil-based carotenoids complex used in this study contained a mixture of carotene isoforms (α-, β, γ-carotene and lycopene). Our findings show that carotenoids exerted anti-proliferative effects on both human breast cancer cell lines tested; most likely by inhibiting activation of NF-κB.

Parallel to our observation, there are many reported studies on carotenoids and positive effects on anti-cancer in many models of breast cancer. For instance, a significant inverse association was reported between plasma levels of α-carotene, β-carotene, β-cryptoxanthin, lutein and total carotenoids with incidence of breast cancer in humans.[23] The anti-tumor property of carotenoids was reported to be through down regulation of COX-2 in both heregulin-α induced and non-induced colon cancer cells.[48] Alpha-carotenes were found to down-regulate the activity of pro-carcinogen activator cytochrome, P450 1AA and this compound also has free radical scavenging activity, thus could protect lipid membranes.[49] Lycopene was reported to possess greater anti-proliferative activity when compared to α- or and β-carotenes on the MCF-7 cells.[50] Diet supplemented with lycopene suppressed development of breast cancer.[51]

Squalene is an immediate biological precursor for all triterpenoids, which is reported to be a lipophilic antioxidant with unique ability to attach to cell membranes.[30] Previous reports have found that triterpenoids inhibit proliferation of MDA-MB-231 and MCF-7 human breast cancer cells[52], a finding that is consistent

with the present report. Celastrol is a quinone methide triterpene derived from the medicinal plant *Tripterygium wilfordii* moderately inhibited the activation of the NF-κB.[53] Triterpenoids-induced Caspase-dependent apoptosis in MDA-MB-231 human breast cancer cells but not in non-tumorigenic human mammary epithelial cells.[54] In the present study, squalene was found to have an anti-proliferative effects on both the human breast cancer cells tested. This effect may be through the slowing down of growth and cell regulation due to the inhibition of NF-κB.

Co-enzyme Q10 is a naturally occurring compound that is structurally related to the lipid-soluble vitamins E and K.[24] Previous studies have found that CoQ10 has anti-inflammatory effects in patients with multiple sclerosis.[55] In breast cancer patients who underwent radical mastectomy, it was found that CoQ10 levels in tumor tissues were significantly lower than in normal tissues.[27] In the present study, we show that CoQ10 has anti-proliferative effect on MDA-MB-231 and MCF-7 human breast cancer cells. The inhibition could be through the inhibition of NF-κB, which is a pro-inflammatory factor that is implicated in carcinogenesis because of its many roles in many cancer-related processes.[46] All the palm phytonutrients included in this study inhibited the TNF-α induced expression of NF-κB in both MCF-7 and MDA-MB-231 cells.

Conclusion

All the palm phytonutrients studied have dose and time-dependent anti-proliferative activities on human breast cancer cells irrespective of their estrogen status without significant cytotoxic effect on normal cells. Palm vitamin E in comparison with other phytonutrients (carotenoids, squalene, CoQ10) has a higher anti-cancer activity.

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Competing Interests:

The authors declare that they have no competing interests.

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552
553 **Figure Captions**

554

555 **Figure 1:** Effect of palm (a) TRF, (b) carotenoids, (c) CoQ10 and (d) squalene on
556 MDA-MB-231 (■) and MCF-7 (□) human breast cancer cells or NIH/3T3 (■) primary
557 mouse embryonic fibroblast cells. The cells were treated with increasing
558 concentrations of 0-10 μg/mL of the various palm phytonutrients and cultured at
559 37°C in an atmosphere of 5% CO_2 for 24, 48 or 72 hours. Cell viability was assayed
560 using the coulter particle counter method. Points indicate percentage cell viability
561 based on control untreated cells (0 μg/ml) calculated from mean of triplicate samples
562 for each treatment group. *All values are significantly (P<0.005) different from control*
563 ***EXCEPT*** *those indicated with an asterisks (*)*

564

565 **Figure 2:** The (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells were
566 cultured for 72 hours at 37°C in a humidified 5% CO_2 incubator in the presence or
567 absence (control) of 10 μg/mL of various palm phytonutrients (TRF, carotenoids,
568 coenzyme Q10, squalene). The amount of cell death due to apoptosis or necrosis
569 was determined using a commercial cell death ELISA kit. Results are shown as the
570 mean ± STD from triplicate cultures.

571

572

573

574 **Figure 3:** The (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells were
575 cultured for 24 hours at 37°C in a humidified 5% CO_2 incubator in the presence or
576 absence (control) of 10 μg/mL of various palm phytonutrients (TRF, carotenoids,
577 coenzyme Q10, squalene). After 24 hours, the cells were exposed to TNF-α (1 nM)
578 for 30 minutes. Cell lysates were prepared from the cultured cells and the level of
579 PARP cleavage was determined using a commercial ELISA kit to detect this. *(*)*
580 *values are significantly different from control group, p < 0.05 (one-way ANOVA*

581

582 **Figure 4:** The (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells were
583 cultured for 24 hours at 37°C in a humidified 5% CO_2 incubator in the presence or
584 absence (control) of 10 μg/mL of various palm phytonutrients (TRF, carotenoids,
585 coenzyme Q10, squalene). After 24 hours, the cells were exposed to TNF-α (1 nM)
586 for 30 minutes. Then, nuclear extracts of the cultured cells were prepared and the
587 amount of NF-κB p65 express was determined using a commercial NF-κB p65
588 ELISA kit. Results are shown as the mean ± SE fold change compared to control
589 obtained from triplicate cultures. [*The control + TNF-α group was significantly (P <*
590 *0.05) higher than control group. All the palm phytonutrient pre-treated groups were*
591 *significantly (P < 0.05) different from control + TNF-α group (one-way ANOVA)]*

Palm TRF exhibited higher potential in the following activities compared to carotenoids, squalene and co-enzyme Q10:

- ***induce death by apoptosis human breast cancer cells***
- ***cleavage of the poly-(ADP-ribose) polymerase (PARP) enzyme***
- ***suppressed expression of NF-κB protein induced by exposure to TNF-α***

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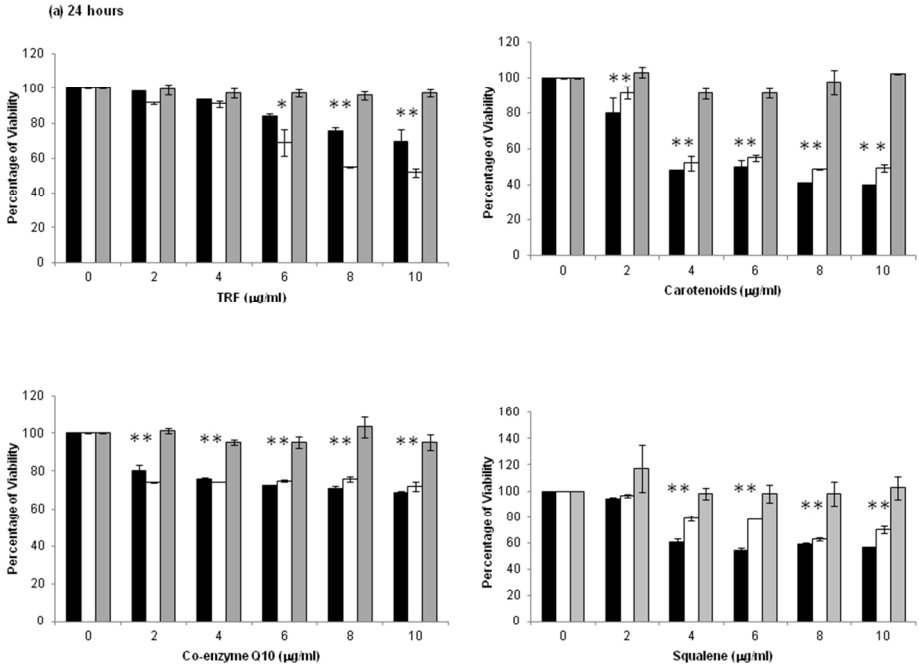

Figure 1: Effect of palm (a) TRF, (b) carotenoids, (c) CoQ10 and (d) squalene on MDA-MB-231 ( ) and MCF-7 ( ) human breast cancer cells or NIH/3T3 ( ) primary mouse embryonic fibroblast cells. The cells were treated with increasing concentrations of 0-10 μg/mL of the various palm phytonutrients and cultured at 37°C in an atmosphere of 5% CO2 for 24, 48 or 72 hours. Cell viability was assayed using the coulter particle counter method. Points indicate percentage cell viability based on control untreated cells (0 μg/ml) calculated from mean of triplicate samples for each treatment group. All values are significantly (P<0.005) different from control EXCEPT those indicated with an asterisks (*)
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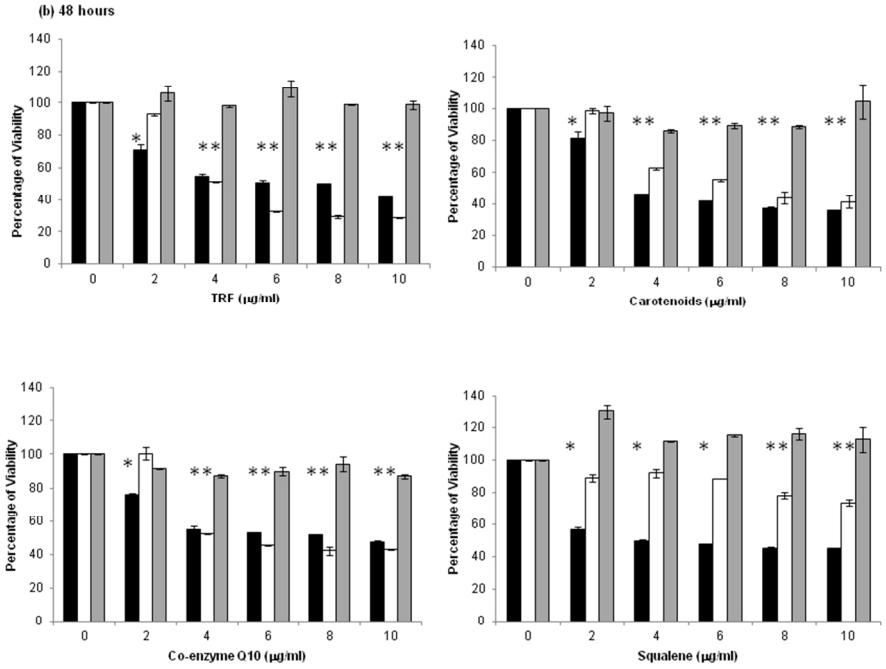

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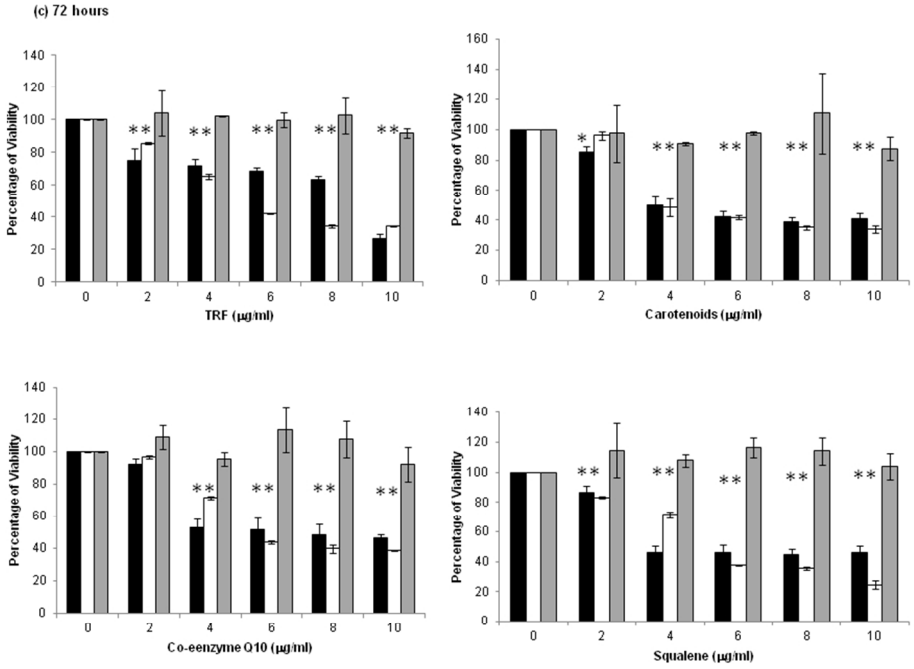

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Figure 2

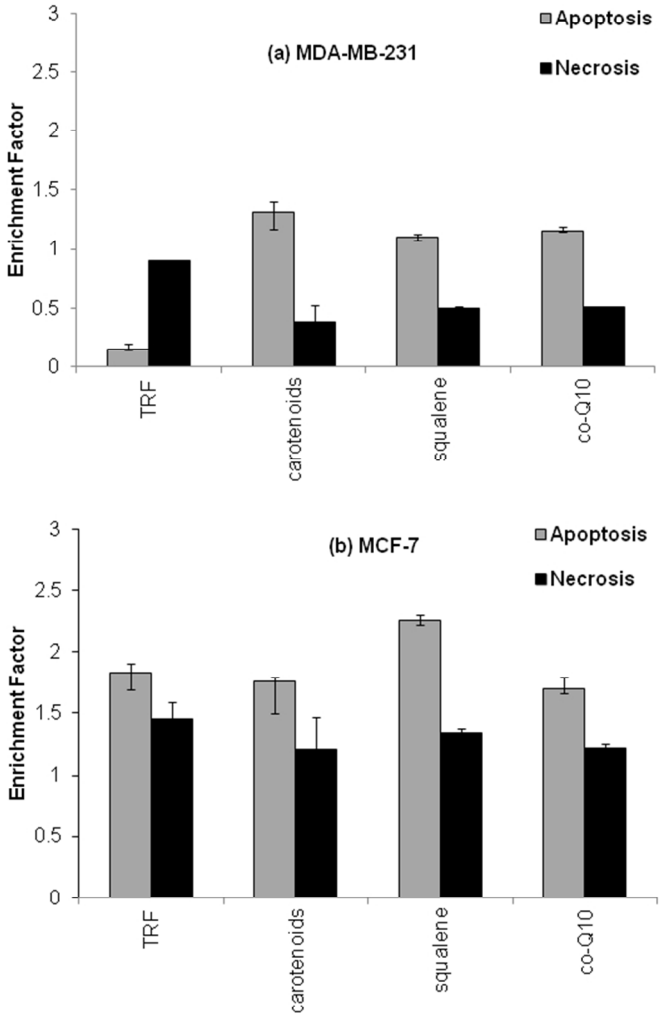

Figure 2: The (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells were cultured for 72 hours at 37oC in a humidified 5% CO2 incubator in the presence or absence (control) of 10 μg/mL of various palm phytonutrients (TRF, carotenoids, coenzyme Q10, squalene). The amount of cell death due to apoptosis or necrosis was determined using a commercial cell death ELISA kit. Results are shown as the mean ± STD from triplicate cultures.
91x121mm (200 x 200 DPI)

Figure 3

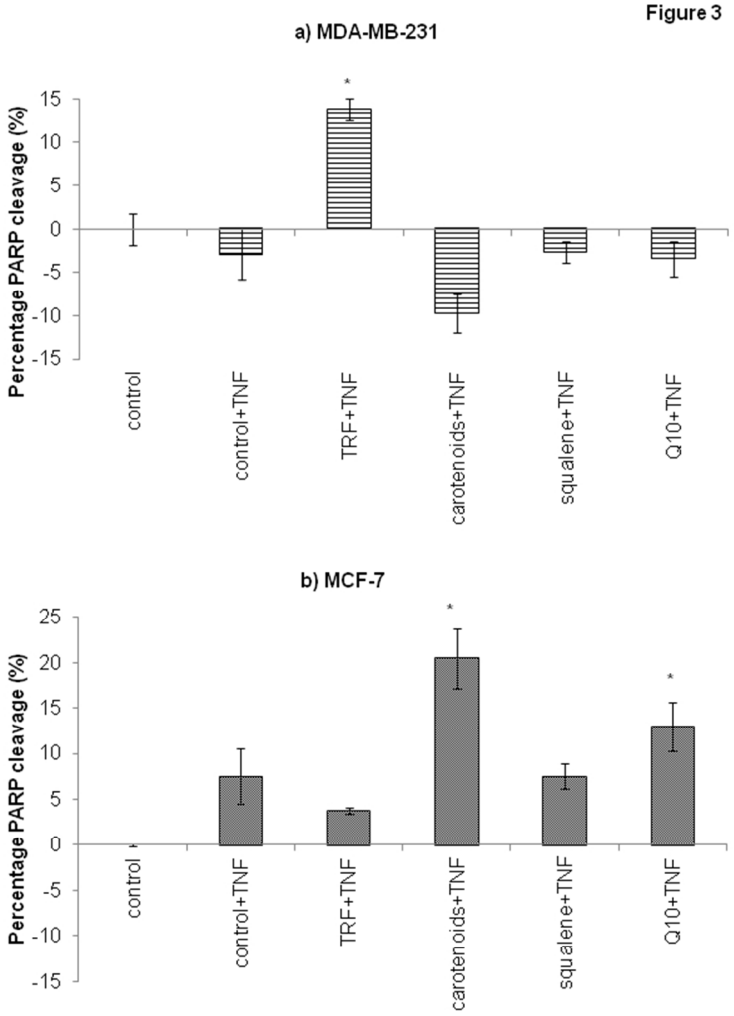

Figure 3: The (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells were cultured for 24 hours at 37oC in a humidified 5% CO2 incubator in the presence or absence (control) of 10 μg/mL of various palm phytonutrients (TRF, carotenoids, coenzyme Q10, squalene). After 24 hours, the cells were exposed to TNF-α (1 nM) for 30 minutes. Cell lysates were prepared from the cultured cells and the level of PARP cleavage was determined using a commercial ELISA kit to detect this. (*) values are significantly different from control group, $p < 0.05$ (one-way ANOVA
91x121mm (200 x 200 DPI)

Figure 4

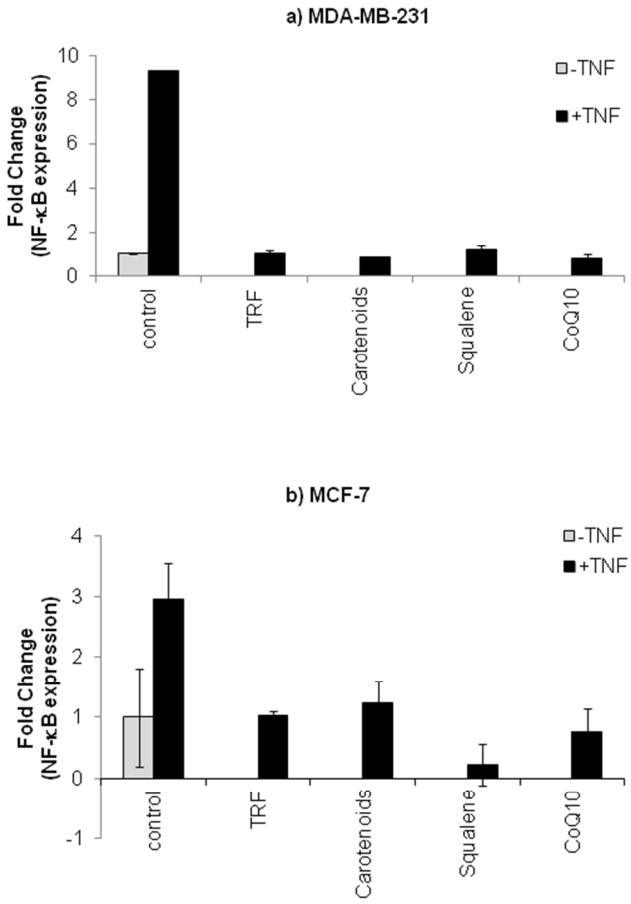

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Table 1: The IC_{50} values of palm phytonutrients against (a) MDA-MB-231 and (b) MCF-7 human breast cancer cells

Cell line	Test Compounds	IC_{50} (µg/ml)		
		Day 1	Day 2	Day 3
MDA-MB-231	TRF	18.05 ± 1.65	10.95 ± 1.95	8.5 ± 0.2
	Carotenoids	4.25 ± 0.35	3.85 ± 0.05	3.8 ± 0.2
	Squalene	16.8 ± 0.8	5.15 ± 1.15	4.35 ± 0.55
	Coenzyme Q10	*	9.0 ± 0.2	7.5 ± 0.4
MCF-7	TRF	7.25 ± 0.55	4.25 ± 0.15	4.55 ± 0.75
	Carotenoids	13.35 ± 1.25	6.3 ± 0.6	3.9
	Squalene	*	*	6.05 ± 0.75
	Coenzyme Q10	*	9.5 ± 0.7	5.65 ± 0.5

** not achieved*

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