

DIFFERENTIAL AND ANTAGONISTIC EFFECTS OF PALM TOCOTRIENOLS AND OTHER PHYTONUTRIENTS (carotenoids, squalene and coenzyme Q10) ON BREAST CANCER CELLS *in vitro*

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ABSTRACT

Palm vitamin E, tocotrienols in particular, are known to exert great anti-cancer effects on a variety of cell types. In this study, the effects of palm vitamin E, carotenoids, squalene and coenzyme Q10 were studied on two human breast cancer cell lines. All compounds caused anti-proliferative effect in vitro but tocotrienols (compounds and isomers) were generally more potent. The results show that the anti-cancer effects of palm vitamin E were more pronounced when these were used on their own rather than in combination with other phytonutrients (carotenoids, squalene and coenzyme Q10). The palm phytonutrient complex, which contains all the tested phytonutrients did not appear to exert better anti-proliferative effects compared to the individual compounds. Our results show that tocotrienols, as well as other phytonutrients (carotenoids, squalene and coenzyme Q10), have anti-proliferative effects on breast cancer cells but different and antagonistic mechanisms may be employed in combination.

Keywords: palm phytonutrients, tocotrienols, carotenoids, squalene, coenzyme Q10.

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INTRODUCTION

Breast cancer is the second leading cause of cancer related deaths in women after lung and bronchial cancer. Breast cancer is the most common cancer that affects Malaysian women from all ethnicities (Hisham and Yip, 2003; 2004) and world over (Parkin *et al.*, 2001). Bishop (1999) has reported that specific genes BRCA 1 and BRCA 2 are predisposed to only 10%-15% of breast cancer incidence. The remaining incidence accounts for unknown nutritional and

environmental factors. Most of the currently used chemotherapeutic drugs have adverse effects and cause damage to normal cells (Patel and Prajapati, 2011). Thus, nutritional strategies in the area of breast cancer treatment and prevention would be in great favour to reduce the risk and mortality rate.

The oil palm, *Elaeis guineensis* is the source of the tropical golden oil called palm oil. Palm oil could offer a wide range of culinary delights unrivalled by any other, whilst simultaneously promoting nutritional and health benefits (Loganathan *et al.*, 2010). The phytonutrients are present as 1% of its weight in crude palm oil. The most prevalent phytonutrients found in palm oil are vitamin E (600-1000 ppm), carotenes (500-700 ppm), phytosterols (300-620 ppm), squalene (250-540 ppm), coenzyme Q10 (10-80 ppm), polyphenols (40-70 ppm) and phospholipids (20-100 ppm) (Choo *et al.*, 2002).

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Seventy percent of vitamin E found in palm oil is in the form of tocotrienols and the remaining accounts for tocopherols (Nesaretnam *et al.*, 1995). Tocotrienols are unique in its ability to freely penetrate tissues with saturated fatty layers, thus performing a more efficient metabolic function, as compared to tocopherols. The accumulation of tocotrienols in tissue manifest supreme health benefits (Dass *et al.*, 2008). Studies have shown that tocotrienols could aid in the reduction of blood cholesterol (Qureshi *et al.*, 1991; Parker *et al.*, 1993; Song and Boyd, 2006) and arteriosclerosis functions (Tomeo *et al.*, 1995); encompass possible anti-angiogenic functions (Miyazawa *et al.*, 2008; Shibata *et al.*, 2008; Weng-Yew *et al.*, 2009); exhibit efficient anti-oxidant activity (Azlina *et al.*, 2005; Suzana *et al.*, 2005; Suarna *et al.*, 1993); possess anti-cancer activity (Nesaretnam *et al.*, 1995; 1998; 2002; 2008; Yu *et al.*, 2005; Srivastava and Gupta, 2006) and neuroprotective properties (Sen *et al.*, 2000).

Carotenoids are natural pigments responsible for the brilliant orange-red features of palm oil. About 600 types of naturally occurring carotenoids are known but only 13 different types are found in palm oil. Amongst them, the major carotenoids found are in the form of β -carotene, α -carotene, lycopene, phytoene and phytofluene. Crude palm oil is considered one of the world's richest sources of carotenoids. Carotenoids act as precursors of vitamin A that have been proven to prevent night blindness (Wattanapenpaiboon and Wahlqvist, 2003), improve vitamin A status of lactating women and their infants (Canfield and Kaminsky, 2000; Lietz *et al.*, 2000), improve serum retinol concentrations (Stuijvenberg and Benade, 2000) and combat vitamin A deficiency (Rao, 2000; Scrimshaw, 2000). Carotenoids can also protect against cardiovascular diseases (Rooyen *et al.*, 2008) and suppress the growth of various cancers such as breast (Nesaretnam *et al.*, 2004; Toniolo *et al.*, 2001; Zhang *et al.*, 1999; Tamimi *et al.*, 2005), lung and liver as well as colon tumour.

Coenzyme Q10 also known as ubiquinone, is a natural coenzyme found in palm oil. Coenzyme Q10 is claimed to possess 10 times greater antioxidant property as compared to vitamin E, however the presence of carotenes and vitamin E in greater concentration tend to mask its viability (Ng *et al.*, 2006). Besides being a powerful antioxidant and a free radical scavenger (Niklowitz *et al.*, 2007), coenzyme Q10 also plays a vital role in the mitochondrial electron transport chain and is considered to exhibit membrane stabilising properties. Coenzyme Q10 has been used in the treatment of many cardiovascular ailments (Verma *et al.*, 2007; Burke *et al.*, 1994) and studies have also reported its anti-cancer effects (Portakal *et al.*, 2000).

Squalene is a valuable triterpene enormously found in shark liver oil. It is present in trace amounts in palm oil. Squalene is an oxygen transmitter and

could aid cardiovascular health (Kelly, 1991; Verma *et al.*, 2007). Squalene has also been reported to possess the ability to promote anti-tumour activity in rodents (Smith *et al.*, 1998), and suppress hyperproliferation of cancer cells (Murakoshi *et al.*, 1992; Rao *et al.*, 1998; Smith *et al.*, 1998), in addition to exhibiting radioprotective effects (Smith *et al.*, 1998).

Unlike other dietary fats and oils, palm oil does not promote chemically-induced mammary tumours in rats (Sylvester *et al.*, 1986; Sundram *et al.*, 1989; Kritchevsky *et al.*, 1992). It is implied that this may correlate to the low linoleic acid content in palm oil. Linoleic acid has shown to promote cancer both *in vivo* and *in vitro* (Hopkins *et al.*, 1979; Clinton *et al.*, 1984; Gammal *et al.*, 1967; Rose and Connolly, 1989). In addition, most studies on the anti-carcinogenic potential of the palm oil's minor components have focussed primarily on the vitamin E. To date, several studies have reported that palm oil stripped of its vitamin E fraction can enhance tumourigenesis (Nesaretnam *et al.*, 1992; 1995). Our laboratory has conducted many studies on the tocotrienol component of palm oil, which has shown remarkable anti-proliferative effects on the growth of the human breast cancer cells (Nesaretnam *et al.*, 1992; 1995; 1998; 2004; 2008). Combinations of tocotrienols with the other phytonutrients present in palm oil such as squalene, coenzyme Q10 and carotenoids, which have also been reported to possess various therapeutic properties should also be considered to improve the efficacy of these micronutrients.

In the present study, the anti-proliferative effects of palm vitamin E (compounds and isomers), carotenoids, squalene and coenzyme Q10 were studied on oestrogen independent MDA-MB-231 and oestrogen dependent MCF-7 human breast cancer cell lines. Our results indicate that tocotrienols as well as other phytonutrients (carotenoids, squalene and coenzyme Q10) have anti-proliferative effects at different degrees *in vitro* on human breast cancer cells but different and antagonistic mechanisms may be employed in combination.

MATERIALS AND METHODS

Cell Lines

Oestrogen-independent MDA-MB-231 and oestrogen-dependent MCF-7 human breast cancer cells were obtained from the Malaysian Palm Oil Board (MPOB). The MDA-MB-231 cells were cultured as monolayer in T75 costar flasks in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic penicillin-streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C. The MCF-7 cells were grown at the same condition and medium

with the addition of 10^{-8} M of β -estradiol. The culture medium was changed routinely every two and three days. For treatment of MCF-7 and MDA-MB-231 cells, phenol red-free RPMI medium 1640 containing 5% dextran-charcoal treated fetal calf serum (DCFCS), 1% L-glutamine and 1% antibiotic penicillin-streptomycin was used.

Compounds

Tocotrienol-rich fraction (TRF) (Golden Hope Plantations, Malaysia), tocotrienol-enrich fraction (TEF) (Davos Life Sciences Ptd Ltd, Singapore), alpha-tocopherol (Sigma Aldrich Chemical Co., USA) and alpha-, delta-, gamma-tocotrienol isomers (Eisai Food & Chemicals Co. Ltd, Japan) of stock solutions of 10 mg ml^{-1} were prepared and stored at -20°C . Vitamin E tocotrienols stock treatments were quantified according to standard operating procedure using high performance liquid chromatography. Carotenoids in the form of 20% natural mixed carotenoids complex (Caromin 20%) was kind gift from Carotech Bhd. Squalene and Coenzyme-Q10 were purchased from (Sigma Aldrich Chemical Co., USA). Phytonutrients mixture was kindly provided by Dr Harrison Lau of Phytonutrients Laboratory, MPOB and phytonutrients analysis were conducted as per their established and patented method. Stock solutions of 10 mg ml^{-1} with 100% purity were prepared with appropriate vehicles and stored at -20°C .

Growth Inhibition Assay

The cells were trypsinised from the stock plates by treatment with 0.06% trypsin / 0.02% EDTA pH 7.3, added to an equal volume of phenol red-free RPMI medium 1640 and counted on haemocytometer. Cells were re-suspended in appropriate amounts of medium to a cell count of $0.5 \times 10^5 \text{ cells ml}^{-1}$ and plated in monolayer in 0.5 ml aliquots into 24 wells plastic tissue culture dishes. After 24 hr, the medium was changed to a medium supplemented with specified concentrations of required test compounds. In studies that involve just the palm vitamin E, the cells were cultured in the presence of varying concentrations ($0\text{-}20 \text{ }\mu\text{g ml}^{-1}$) of the test compound in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 hr. This assay was used to determine the IC_{50} values of the various isomers of palm vitamin E tested. The IC_{50} value indicates the treatment dose that inhibited 50% cell growth as compared to controls over the treatment period. In combination studies, the cells were treated with varying concentrations of palm phytonutrients ($0\text{-}20 \text{ }\mu\text{g ml}^{-1}$) and the IC_{50} concentration of the various isomers of palm vitamin E. Control cultures contained the same volume of medium alone. At the end of the 72 hr, the cells were washed with

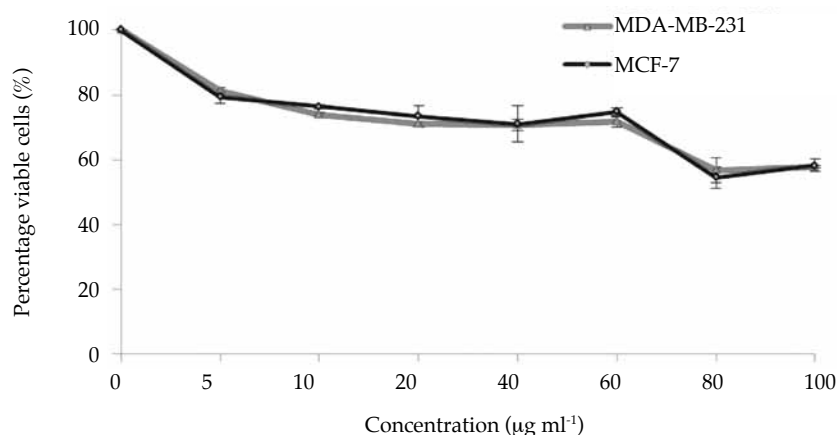
0.9% NaCl to remove non-adherent dead cells and lysed in 0.5 ml 2.5 mM HEPES buffer / 1.5 M MgCl_2 in addition to a drop of zap-oglobin II lytic reagent for 15 min. The released nuclei were suspended in isoton III and counted on a Coulter Particle Counter ZI with particle size set at $>5 \text{ }\mu\text{m}$. All cell counts were carried out in triplicate on triplicate well treatments. The results were calculated as the average of mean \pm standard deviation. One way analysis of variance (ANOVA) was used to assess the differences between groups. Differences among treatments were tested by Tukey HSD. Results were considered statistically significant when $p < 0.05$. The following formulae was used to calculate percentage cell viability:

$$\text{Percentage viability} = \frac{\text{Counts (treated)}}{\text{Counts (untreated)}} \times 100$$

RESULTS AND DISCUSSION

The phytonutrient mixture is present as 1% of its weight in crude palm oil. The most prevalent phytonutrients found in palm oil are vitamin E (600-1000 ppm), carotenes (500-700 ppm), phytosterols (300-620 ppm), squalene (250-540 ppm), coenzyme Q10 (10-80 ppm), polyphenols (40-70 ppm) and phospholipids (20-100 ppm) (Choo *et al.*, 2002). Our research hypothesis was that combining these phytonutrients as in palm phytonutrient complex would produce a more marked anti-proliferative effect as compared to the individual compounds at the same concentrations. Higher levels of anti-proliferative effects were observed when individual phytonutrients namely tocotrienols, carotenoids, squalene and coenzyme Q10 were used compared to the palm phytonutrient complex (results not shown). The palm phytonutrient complex exhibited reduction of growth in the exponential phase ($P < 0.05$) but the IC_{50} values could not be established (Figure 1). We show that tocotrienols (TRF, TEF and isomers α -, δ -, γ -T3), carotenoids, squalene and coenzyme Q10 have anti-proliferative on both the human breast cancer cell lines studies, *i.e.* the oestrogen-independent MDA-MB-231 and oestrogen-dependent MCF-7 cells.

However, a dose-dependent complete suppression of cell proliferation was only observed when tocotrienols were used. All other palm phytonutrients (carotenoids, squalene and coenzyme Q10) tested failed to induce complete suppression of cell growth in the concentration range tested. Instead these compounds formed a plateau after achieving its IC_{50} . We would like to propose that this may be the effect of ligand binding saturation based on the median effect principle and mass action law (Chou, 2006). This led to the next part of the study which was to test combinations of



Note: Points indicate triplicate the mean of triplicate counts/well \pm STD for triplicates in each treatment group. All data are significantly ($P < 0.05$) different from control.

Figure 1. The anti-proliferative effect of palm phytonutrients on oestrogen-independent (MDA-MB-231) and oestrogen-dependent (MCF-7) human breast cancer cells for 72 hr treatment.

palm vitamin E and phytonutrients. To date, there have been no reports of synergistic actions of palm vitamin E and the other phytonutrients. Strategies involving combinations of tocotrienols with the other palm phytonutrients should seriously be considered as this offers a wider range of possible mechanisms that could result in higher efficacy than a single agent alone. In addition, it has been proposed that multiple drug or compound applications may target multiple targets, multiple subpopulations or multiple diseases simultaneously (Chou, 2006). The application of multiple drug with different mechanisms or mode of action more efficiently and the multiple drug synergism could also increase the efficacy of the therapeutic effect, lower dose and toxicity, minimise and slow down the development of drug resistance and provide selective synergism.

In this study, the combination treatments of tocotrienols (Table 1) with any of the palm phytonutrients such as carotenoids (Table 2), squalene (Table 3), or coenzyme-Q10 (Table 4) resulted in anti-proliferation pattern that were significantly weaker than tocotrienols and analogous or weaker than the individual phytonutrient itself. These observations sug-

gest that tocotrienols and the phytonutrients (carotenoids, squalene and coenzyme Q10) use different mechanisms to achieve the decrease in cell number at the end of the treatment period and those pathways are antagonistic and competitive. It has been reported that synergism and antagonism may differ at different dose or effect levels (Chou, 2006). Hence, the combination effect was evaluated based on the dose response curve and their respective IC_{50} values. The potential mechanisms underlying the antagonism seen between tocotrienols and these phytonutrients are numerous. In the case of co-treatment, clearly direct receptor interactions may play a role. In order for these co-treated compounds to bind efficiently to enhance the anti-proliferative effect, they should have complementary or different binding sites. On the other hand if these compounds share the common binding sites, forms the basis for interactions and competition between the compounds. Another possible explanation for these antagonistic observations lies in the cell-cycle mechanism. It is likely that these compounds may regulate different pathways or genes in the cell cycle, *i.e.* these may have different effects on the gene products involved in cell dif-

TABLE 1. THE IC_{50} VALUES OF TOCOTRIENOL-RICH FRACTION (TRF), TOCOTRIENOL-ENRICH FRACTION (TEF) AND TOCOTRIENOL ISOMERS ON THE MDA-MB-231 AND MCF-7 HUMAN BREAST CANCER CELLS

Test compound	IC_{50}	
	MDA-MB-231 ($\mu\text{g ml}^{-1}$)	MCF-7 ($\mu\text{g ml}^{-1}$)
TRF	8.5 \pm 0.2	4.55 \pm 0.7
TEF	3.7	3.4 \pm 0.2
Alpha tocotrienol (α -T3)	9.55 \pm 1.1	11.05 \pm 0.45
Delta tocotrienol (δ -T3)	6.9 \pm 0.3	6.8 \pm 0.3
Gamma tocotrienol (γ -T3)	4.7 \pm 0.8	6.35 \pm 0.15

Note: Points indicate the mean of triplicate counts/well \pm STD for triplicates in each treatment group. The IC_{50} indicates treatment dose that induced a 50% cell growth inhibition as compared to controls over the three days treatment period.

TABLE 2. THE IC₅₀ VALUES OF COMBINATION TREATMENT USING VITAMIN E ISOMERS AT IC₅₀ CONCENTRATIONS AND CAROTENOIDS (0-20 µg ml⁻¹) ON THE MDA-MB-231 AND MCF-7 HUMAN BREAST CANCER CELLS

Test compound	IC ₅₀	
	MDA-MB-231 (µg ml ⁻¹)	MCF-7 (µg ml ⁻¹)
Carotenoids	3.8±0.2	3.9±0.0
TRF (IC ₅₀) + carotenoids	4.53	3.72
TEF (IC ₅₀) + carotenoids	3.2	5.61
α-T3 (IC ₅₀) + carotenoids	*	6.69±0.26
δ-T3 (IC ₅₀) + carotenoids	*	7.17±0.54
γ-T3 (IC ₅₀) + carotenoids	*	5.18±0.86

Note: Points indicate the mean of triplicate counts/well ± STD for triplicates in each treatment group. (*) IC₅₀ not achieved. TRF - tocotrienol-rich fraction. TEF - tocotrienol-enrich fraction.

TABLE 3. THE IC₅₀ VALUES OF COMBINATION TREATMENT USING VITAMIN E ISOMERS AT IC₅₀ CONCENTRATIONS AND SQUALENE (0-20 µg ml⁻¹) ON THE MDA-MB-231 AND MCF-7 HUMAN BREAST CANCER CELLS

Test compound	IC ₅₀	
	MDA-MB-231 (µg ml ⁻¹)	MCF-7 (µg ml ⁻¹)
Squalene	4.35±0.55	6.05±0.75
TRF (IC ₅₀) + squalene	4.44	4.72
TEF (IC ₅₀) + squalene	13.35	3.6
α-T3 (IC ₅₀) + squalene	*	6.72±0.34
δ-T3 (IC ₅₀) + squalene	1.43	11.32±0.4
γ-T3 (IC ₅₀) + squalene	*	*

Note: Points indicate the mean of triplicate counts/well ± STD for triplicates in each treatment group. (*)IC₅₀ not achieved. TRF - tocotrienol-rich fraction. TEF - tocotrienol-enrich fraction.

TABLE 4. THE IC₅₀ VALUES OF COMBINATION TREATMENT USING VITAMIN E ISOMERS AT IC₅₀ CONCENTRATIONS AND COENZYME Q10 (0-20 µg ml⁻¹) ON THE MDA-MB-231 AND MCF-7 HUMAN BREAST CANCER CELLS

Test compound	IC ₅₀	
	MDA-MB-231 (µg ml ⁻¹)	MCF-7 (µg ml ⁻¹)
Coenzyme Q10	7.5±0.4	5.65±0.5
TRF (IC ₅₀) + coenzyme Q10	3.49	3.75
TEF (IC ₅₀) + coenzyme Q10	6.6	6.81
α-T3 (IC ₅₀) + coenzyme Q10	*	2.99±0.64
δ-T3 (IC ₅₀) + coenzyme Q10	11.54	6.6±0.26
γ-T3 (IC ₅₀) + coenzyme Q10	*	7.39±0.16

Note: Points indicate the mean of triplicate counts/well ± STD for triplicates in each treatment group. (*)IC₅₀ not achieved. TRF - tocotrienol-rich fraction. TEF - tocotrienol-enrich fraction.

ferentiation, proliferation and cell death. It has been reported that the inhibition of MDA-MB-435 breast cancer cell proliferation involves a G0/G1 cell-cycle block, partly mediated by MAP2K1 and ERK1 as well as increase in the key cell-cycle regulatory protein p21^{waf1/cip1} (Yu *et al.*, 2002). The reduction in cell proliferation by terpenoid were found to be associ-

ated with S-G2/M phase cell cycle arrest and apoptosis induction; inhibition of cell cycle progression at the S-G2/M phase by up-regulation of p21/Cdc2 interaction and down-regulation of the expression of Cdc2, Cdc25C, cyclinB1 and cyclinA expression; as well as activation of p38 and ERK1/2 kinases and not JNKs, p53 or Fas/Fas ligand pathway (Hsu *et*

al., 2005). The anti-tumour property of carotenoids in colon cancer was reported to be via potentiation of the apoptosis pathway, in reduction the production of intracellular ROS and activation of ERK1/2 (Palozza *et al.*, 2005).

There are many reports to substantiate the fact that the compounds studied are involved in anti-proliferation, apoptosis, cell cycle arrest and cell regulation. The reason behind our observed antagonistic effect could also be due to the cell line being in different stage of differentiation or different cell regulation stage of the compounds may account for the contrasting observation.

CONCLUSION

Our study has shown that the individual compounds (tocotrienols, carotenoids, squalene and coenzyme Q10) possess anti-cancer effects but the effects of the compounds in combination are contradicting. In addition, tocotrienols were found to have potent anti-proliferative effect. On the other hand, carotenoids, squalene and coenzyme Q10 were found to slow down the cell growth and did not efficiently yield complete growth suppression at the dose and time period studied.

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