

## Anti-proliferation Effect of Palm Oil $\gamma$ -tocotrienol and $\alpha$ -tocopherol on Cervical Carcinoma and Hepatoma Cell Apoptosis

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### Abstract

Gamma-tocotrienol has been reported to exhibit a protective effect on hepatocarcinogenesis through the enhancement of immune response and anti-proliferation effect on breast cancer cell lines such as MDA-MB-435 and MCF7. The anti-proliferation mechanism was suggested to be via an increase in apoptosis. The effects of palm oil  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol on the cell proliferation were determined using 5-Bromo-2'-deoxy-uridine (BrdU) detection method. CaSki and Alexander cells were sensitive to  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol-anti proliferation effects with 93.5 to 97.8% ( $P$  less than 0.01,  $n$  is equal to 4) and 59.7 to 69.1% ( $P$  less than 0.001,  $n$  is equal to four) inhibition, respectively beginning with a concentration of 100 $\mu$ M and above. Treatment with  $\alpha$ -tocopherol showed a lesser inhibition in the proliferation activity of CaSki cell by 19.7 to 39.4% ( $P$  less than 0.01,  $n$  is equal to four) and Alexander cell by 16.9 to 19.6% ( $P$  less than 0.001,  $n$  is equal to four) beginning with a concentration of 50 $\mu$ M and 200 $\mu$ M, respectively. However, both the compounds had no effect on Chang cell. Gamma-tocotrienol showed an  $IC_{50}$  inhibition of CaSki and Alexander cells growth at a dose of 75 $\mu$ M ( $P$  less than 0.05,  $n$  is equal to four) and 66 $\mu$ M ( $P < 0.05$ ,  $n = 4$ ), respectively. Alpha-tocopherol showed a lesser growth inhibition of CaSki cell at  $IC_{40}$  value of a concentration of 75 $\mu$ M ( $P$  less than 0.05,  $n$  is equal to four) and Alexander cell at  $IC_{20}$  value of a concentration of 300 $\mu$ M ( $P$  less than 0.05,  $n$  is equal to four), respectively. Gamma-tocotrienol induced maximum apoptotic activity of both CaSki and Alexander cells at a concentration 150 $\mu$ M of treatment as compared to  $\alpha$ -tocopherol at a concentration 300 $\mu$ M and 500 $\mu$ M, respectively. Evidence of the cellular DNA laddering fragments induced by both the compounds at similar doses as above was detected using electrophoresis. The results suggested that palm oil  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol exhibit antiproliferation effect on CaSki and Alexander cells via selective induction of apoptosis.

**Key words:** Cancer cell apoptosis,  $\gamma$ -tocotrienol,  $\alpha$ -tocopherol

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### Introduction

Vitamin E refers to a group of naturally occurring compounds called  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienols, as well as acetate and succinate derivatives of both natural and synthetic  $\alpha$ -tocopherol [1].

Palm oil vitamin E consists of a mixture of 78 to 82% of  $\alpha$ -,  $\gamma$ -,  $\delta$ -tocotrienols and 18 to 22% of  $\alpha$ -,  $\beta$ -,  $\delta$ -tocopherols [2]. Each of these molecules shares a great

similarity in their molecular structures that consist of a chromanol ring and an isoprenoid tail. Both the tocopherol and tocotrienol isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -) slightly differ in the number and position of the methyl groups on the chromanol ring. Tocopherols have saturated tails, whereas tocotrienols have three double bonds at 3', 7' and 11' position in their isoprenoid tails [1]. Scientists believed that these unique differences enable tocotrienols to display efficient anti-cancer activity [3]. Biological activities of these isomers depend on their structures. The chromanol ring carries an active

antioxidant group and therefore, both tocotrienols and tocopherols are excellent antioxidant [1]. However, tocotrienols are 40 to 50 times more effective in their antioxidant capabilities [4]. The reason may be due to effective uptake of tocotrienols by cells and better distribution throughout cell membranes as compared to tocopherols [5].

The protective effect of vitamin E in cancer has been postulated through the inhibition of cancer formation by the quenching of free radical and control of tumor growth via the induction of differentiation, cell cycle inhibition at G<sub>1</sub>-S transition phase and apoptosis [6].

*In vitro* studies have demonstrated that  $\alpha$ -,  $\gamma$ -,  $\delta$ -tocotrienols effectively inhibit the growth of human cervical carcinoma, HeLa and breast cancer cells (non-estrogen-responsive, MDA-MB-435 and estrogen-responsive, MCF-7) by inducing apoptosis [7]. Another study reported that  $\alpha$ -tocopherol inhibits the growth of several human cancer cell lines, including prostate (androgen-resistant, PC-3 and androgen-sensitive, LNCaP) and lung (A549) cells. However, it has no effect on normal prostate epithelial (PrEC) cells [8].

Vitamin E succinate acts as a potent apoptotic inducer for an extensive variety of both epithelial and lymphoid human cancer cells that comprise of more than 90% of all human malignancies [9]. Studies indicated that  $\alpha$ -tocopheryl succinate induces apoptosis in human breast cancer cell via the translocation of Bax from the cytosol to the mitochondria and the releases cytochrome c from the mitochondria to the cytosol [10]. Another study has demonstrated that  $\alpha$ -tocopheryl succinate also induces apoptosis in prostate cancer cell through the inhibition of Bcl-xL/Bcl-2 function [6].

The mode of action of anti-cancer agents indicates that regardless of the diverse nature of anti-cancer drugs, most of them elicit apoptosis in the target cells [11]. Apoptosis is a natural cell death that complements proliferation [12] to maintain the homeostasis of cells [13]. The ability of tumor cells to detect cellular damage and activate the apoptotic response may determine the ultimate success of cancer chemotherapy treatment [14].

Our study intends to demonstrate that palm oil  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol could effectively inhibit the cell proliferation of human cancer cells (CaSki and Alexander cells) via the selective induction of apoptosis.

## Material and Methods

### Cell culture

CaSki cell was cultured in RPMI, while Alexander (PLC/

PRF/5) and Chang cells were cultured in EMEM with Earle's balanced salts and supplemented with 10% fetal bovine serum, 20mM Hepes, 20mM sodium bicarbonate, 2mM L-glutamine and 1% penicillin and streptomycin. Cells (American Type Cell Collection, Manassas, VA, USA) were grown on culture flask (Falcon, Becton Dickinson, NJ, USA) as monolayer to approximately 80% of confluence in five per cent CO<sub>2</sub>, at 37°C. Culture media and the above chemicals were purchased from FLOWLAB, Sydney, Australia.

### Vitamin E treatment

Palm oil  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol were obtained as 80% concentration (single peak by HPLC) from the Palm Oil Research Institute of Malaysia, Kuala Lumpur. Stock solutions of both the vitamins were dissolved in absolute alcohol at 500 $\mu$ M and then diluted so that the final concentration of alcohol in the culture media was less than 0.1%.

### Cell proliferation assay

The 2 x 10<sup>4</sup> cells were treated separately with different concentrations of  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol (0, 10 $\mu$ M, 50 $\mu$ M, 100 $\mu$ M, 150 $\mu$ M, 200 $\mu$ M and 300 $\mu$ M) and incubated in 5% CO<sub>2</sub> at 37°C for 48 hours. The effect of both compounds on the cell proliferation was determined using a 5-Bromo-2'-deoxy-uridine (BrdU) labelling and detection method (Bohrienger Mannheim, Mannheim, Germany).

### Cell growth assay

2 x 10<sup>4</sup> of CaSki and Alexander cells were treated separately with  $\gamma$ -tocotrienol at IC<sub>50</sub> value (a concentration of 75 $\mu$ M and 66 $\mu$ M, respectively) and  $\alpha$ -tocopherol at IC<sub>40</sub> and IC<sub>20</sub> values (a concentration of 300 $\mu$ M), respectively on different days (0, 2, 4, 6, 8, 10, 12, 14). Cells were stained with 10% trypan blue (Flow General Company, McLean, VA, USA) and counted on different days of treatment as above. Cells without test compound were also cultured as controls.

### Apoptosis assay

#### (i) Analysis of DNA fragmentation activity

The 2 x 10<sup>4</sup> cells were treated separately with  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol at different concentrations (0, 10 $\mu$ M, 50 $\mu$ M, 100 $\mu$ M, 150 $\mu$ M, 200 $\mu$ M, 300 $\mu$ M and 500 $\mu$ M) and incubated in 5% CO<sub>2</sub> at 37°C for 24 hours. The cellular DNA fragmentation induced by both compounds was measured using a Cellular DNA Fragmentation-ELISA method (Bohrienger Mannheim, Mannheim, Germany).

#### (ii) Detection of DNA laddering via electrophoresis

The 10<sup>6</sup> cells were treated separately with  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol at a concentration of 150 $\mu$ M and 300 $\mu$ M, respectively (values obtained from the maximum apoptotic activity assay) for 24 hours. Cells were treated with 10 $\mu$ L

of 0.5mg/mL Protenaise K containing 10mM EDTA (pH 8.0), 0.5% SDS and 50mM Tris-HCl (pH 8.0) at 50°C for 1 hour. After 1 hour of incubation in 10 $\mu$ L of RNase A [0.5mg/mL RNase A, 10mM EDTA (pH 8.0), 50mM Tris-HCl (pH 8.0)] at 50°C for 1 hour, DNA samples and loading buffer [10 $\mu$ L 10mM EDTA (pH 8.0), one per cent (w/v) low-gelling-temperature agarose, 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose] were loaded into two per cent agarose gel stained with 0.5 $\mu$ g/mL ethidium bromide and electrophoresis in 10 x Tris-phosphate buffer [0.9M Tris-fosfat, EDTA 0.2M pH (8.0)] at 75 volts for three hours. All the above chemicals were obtained from Sigma Chemical Co, St. Louis, MO, USA.

#### **Cellular uptake of $\gamma$ -tocotrienol and $\alpha$ -tocopherol**

The 10 x 10<sup>6</sup> cells were incubated separately with  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol at different concentrations (0, 50 $\mu$ M, 100 $\mu$ M, 200 $\mu$ M and 300 $\mu$ M) in 5% CO<sub>2</sub> at 37°C for 24 hours. At the end of incubation, the cells were washed thrice with ice-cold phosphate buffer saline (10mM NaF, 0.9% NaCl, pH 7.2) and 50 $\mu$ L of 10mg/mL  $\beta$ -hydroxytoluene was added to prevent oxidation of vitamin E's isomers. After the final wash with phosphate buffer saline, 100 $\mu$ L ethanol and  $\beta$ -hydroxytoluene were added and the cells were saponificated for 40 seconds. The samples were dissolved into 100 $\mu$ L HPLC grade-hexane containing  $\beta$ -hydroxytoluene, vortex and centrifuged at 300g for three minutes to separate the phases. Aliquots of the top phase were air dried using a vacuum concentrator and again dissolved in 100 $\mu$ L of hexane. The samples were filtered with 0.4 $\mu$ m nitrocellulose membrane and injected into a 150 x 4.6mm normal silica column with a mobile phase of hexane:methanol (99.75:0.25) at a rate of 1.50mL/min. Vitamin E compounds were quantified with a fluorescence detector at 294nm and emission detection at 330nm. All the above chemicals were obtained from Sigma Chemical Co, St. Louis, MO, USA.

#### **Statistical analysis**

Paired Student's *t*-test was used to compare between control and different levels of each treatment on the measured parameters. Significance was set up at *P* less than 0.05.

## **Results**

#### **Effects of $\gamma$ -tocotrienol and $\alpha$ -tocopherol on cell proliferation**

To determine the effects of both the vitamin E compounds, on cell proliferation, we examined the incorporation of BrdU into freshly synthesized cellular DNA. Gamma-tocotrienol, at the lowest dose of 10 $\mu$ M slightly enhanced the proliferation of CaSki cells by 3.8% (*P* less than 0.01, *n* is equal to four), however it slightly inhibited the proliferation of Alexander cells by 9.7% (*P* less than 0.001,

*n* is equal to four). At 50 $\mu$ M,  $\gamma$ -tocotrienol enhanced the proliferation of both CaSki and Alexander cells by 22.0 and 9.9% (*P* less than 0.01, *n* is equal to four), respectively. Interestingly, at concentrations of 100 $\mu$ M and above,  $\gamma$ -tocotrienol effectively suppressed the proliferation of both cells by 93.5 to 97.8% (*P* less than 0.01, *n* is equal to four) and 59.7 to 69.1% (*P* is less than 0.001, *n* is equal to four) with IC<sub>50</sub> values of 75 $\mu$ M and 66 $\mu$ M, respectively. Alpha-tocopherol reduced the proliferation of CaSki cells by 19.7 to 39.4% (*P* is less than 0.01, *n* is equal to four) beginning at a concentration of 50 $\mu$ M, followed by 16.9% to 19.6% (*P* is less than 0.001, *n* is equal to four) reduction of Alexander cells beginning at a concentration of 200 $\mu$ M with IC<sub>40</sub> value of 75 $\mu$ M and IC<sub>20</sub> value of 300 $\mu$ M. On the other hand, both compounds have no effect on the cell proliferation of Chang cells at all concentrations used.

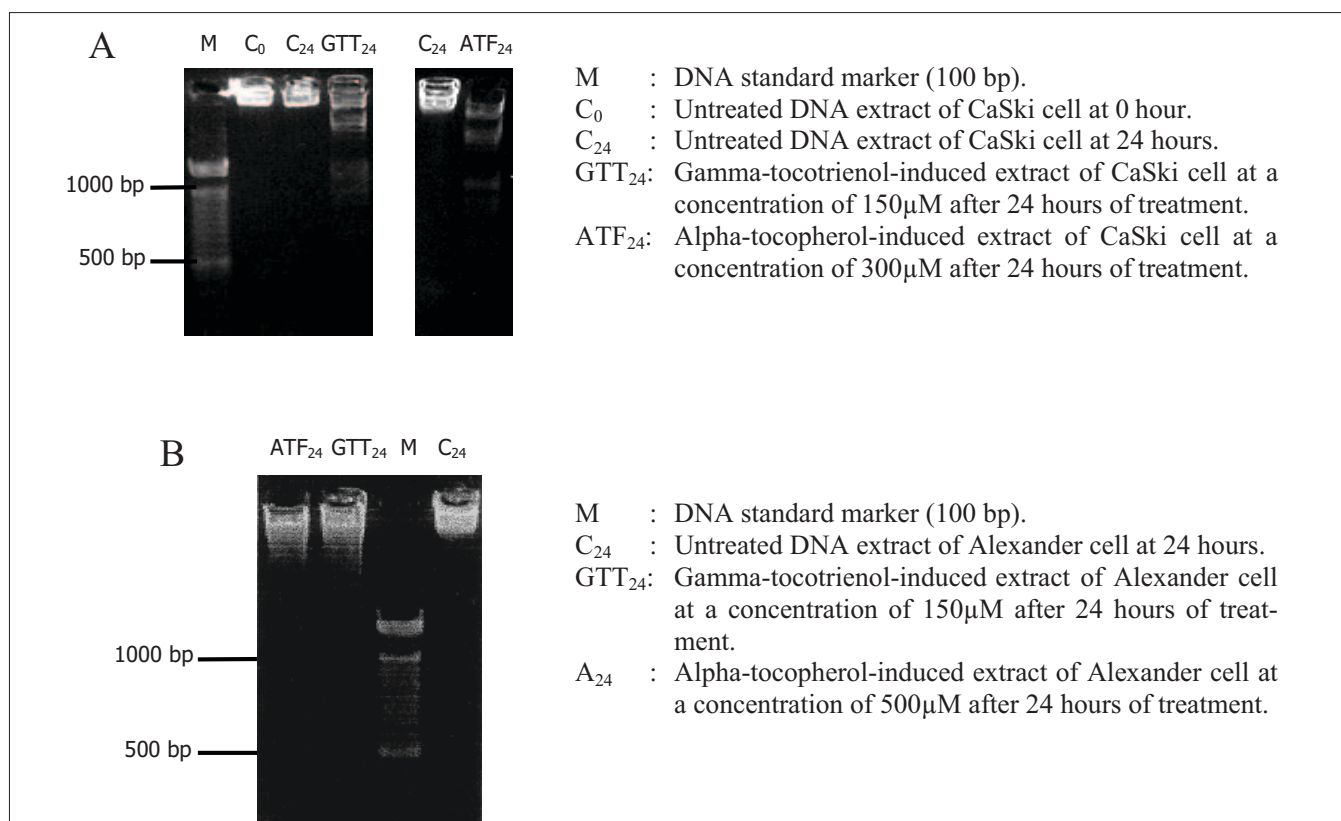
#### **Effects of $\gamma$ -tocotrienol and $\alpha$ -tocopherol on cell growth**

Gamma-tocotrienol reduced the growth of CaSki and Alexander cells by 50.0% (*P* is less than 0.05, *n* is equal to four) at IC<sub>50</sub> values of a concentration of 75 $\mu$ M and 66 $\mu$ M, respectively. Alpha-tocopherol shows a lesser growth inhibition of CaSki cell at IC<sub>40</sub> value of a concentration of 75 $\mu$ M (*p* is less than 0.05, *n* is equal to four) and Alexander cell at IC<sub>20</sub> value of a concentration of 300 $\mu$ M (*P* is less than 0.05, *n* is equal to four), respectively as compared to untreated cultures.

#### **Induction of apoptosis by $\gamma$ -tocotrienol and $\alpha$ -tocopherol by DNA fragmentation**

To further analyze the possible antiproliferation mechanism induced by Vitamin E on CaSki and Alexander cells, we analyzed the apoptotic properties of both compounds by measuring the cellular DNA fragmentation activity. The activity was investigated by determining the BrdU-labeled DNA fragments released into the cytoplasm during apoptosis.

Treatment with  $\gamma$ -tocotrienol at a concentration of 10 $\mu$ M has no effect on CaSki cell, though it enhanced the apoptotic activity of Alexander cell by 2.3-fold (*P* is less than 0.01, *n* is equal to four) as compared to control Figure 1. However at a concentration of 50 $\mu$ M, it has no effect on both cells. Interestingly, at a concentration of 100 $\mu$ M,  $\gamma$ -tocotrienol enhanced the apoptotic activity of both cells by 5.8-fold (*P* is less than 0.01, *n* is equal to four) and 3.9-fold (*P* is less than 0.01, *n* is equal to four) with the maximum activity of 6.8-fold (*P* less than 0.01, *n* is equal to four) and 5.2-fold (*P* less than 0.01, *n* is equal to four) at a concentration of 150 $\mu$ M, respectively. At higher concentrations of 200 $\mu$ M to 500 $\mu$ M, the apoptotic activity induced by  $\gamma$ -tocotrienol was slightly reduced to 5.6-fold to 3.7-fold and 2.7-fold, respectively, but the effect was still significant at *P* less than



**Figure 1:** DNA laddering fragmentation induced by  $\gamma$ -tocotrienol (GTT) and  $\alpha$ -tocopherol (ATF) in CaSki (A) and Alexander (B) cells

0.01 as compared to untreated cultures. Alpha-tocopherol enhanced the apoptotic activity of both CaSki and Alexander cells by 2.7-fold ( $P$  less than 0.01,  $n$  is equal to four) and 2.1-fold ( $P$  less than 0.01,  $n$  is equal to four) beginning at a concentration of 300μM and 500μM, respectively.

The apoptotic effect of  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol was confirmed by the DNA fragmentation patterns extracted from CaSki (A) and Alexander (B) cells.

Further evidence of apoptosis was obtained by the formation of DNA laddering fragments detected by electrophoresis.

#### Cell uptake

To ensure that both  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol were incorporated into all three cells, each concentration of treatment was analyzed using high performance liquid chromatography.

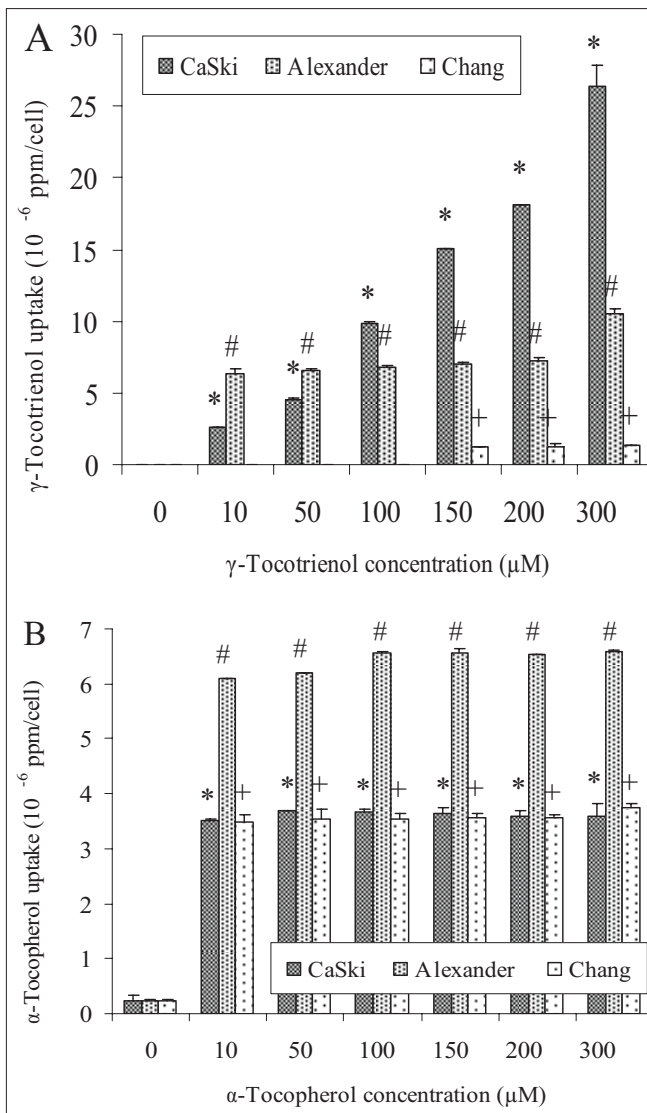
Gamma-tocotrienol was taken-up by CaSki and Alexander cells at all concentration used (Figure 2A). However, the extent of cellular uptake varied between the two cells. The increment uptake of  $\gamma$ -tocotrienol by CaSki cell correlated with all the concentrations used ( $P$  less than 0.01,  $n$  is equal to four), while Alexander cell shows a plateau uptake ( $P$  less

than 0.01,  $n$  is equal to four). In contrast, smaller amount of -tocotrienol ( $P$  less than 0.01,  $n$  is equal to four) was only detected at concentrations from 150μM to 300μM of treatments. Gamma-tocotrienol was taken-up most efficiently by CaSki as compared to Alexander and least of all Chang cell with a ratio of 12.4:5.8:1 (at a concentration of 150μM) and 19.7:7.8:1 (at a concentration of 300μM).

On the other hand, all three cells show plateau increment of  $\alpha$ -tocopherol uptake (Figure 2B) at all concentrations used ( $P$  less than 0.010.05,  $n$  is equal to four). However,  $\alpha$ -tocopherol was most efficiently taken up by Alexander, followed by both CaSki and Chang cells ( $P$  less than 0.010.01,  $n$  is equal to four) with a ratio of 1.7:1:1.

#### Discussion

Treatment of cancer cells with high dose of 13-*cis*-retinoic acid,  $\alpha$ -tocopheryl succinate, or  $\beta$ -carotene clearly alters the expression of specific genes, levels of proteins, and translocation of certain proteins from one cellular compartment to another, causing differentiation, proliferation inhibition, and apoptosis, depending on the type and form of antioxidant, treatment schedule, and type of tumor cell [15]. Alterations in gene expressions and



**Figure 2:** Gamma-tocotrienol (A) and  $\alpha$ -tocopherol (B) uptake by CaSki, Alexander and Chang cells. Gamma-tocotrienol was taken-up most efficiently by CaSki as compared to Alexander and least of all Chang cell ( $P < 0.01$ ,  $n = 4$ ) at all concentrations used, while  $\alpha$ -tocopherol was most efficiently taken up by Alexander, followed by both CaSki and Chang cells ( $P < 0.01$ ,  $n = 4$ ). \*significant as compared to untreated CaSki extract at  $P < 0.01$ . #significant as compared to untreated Alexander extract at  $P < 0.01$ . +significant as compared to untreated Chang extract at  $P < 0.01$ .

protein levels are directly related to proliferation inhibition and apoptosis [16].

Our findings demonstrated that treatment with concentrations from 100  $\mu$ M to 300  $\mu$ M of  $\gamma$ -tocotrienol efficiently inhibits the proliferation of CaSki and Alexander cells by 93.5 to 97.8% and 59.7 to 69.1%, respectively as compared to  $\alpha$ -tocopherol at a lesser magnitude of 19.7 to 39.4%

beginning at a concentration of 50  $\mu$ M and 16.9% to 19.6% reduction of the later cell at concentrations of 200  $\mu$ M and above. Gamma-tocotrienol at  $IC_{50}$  values (a concentration of 75  $\mu$ M and 66  $\mu$ M, respectively) reduced the growth of both cells by 50% as compared to  $\alpha$ -tocopherol at  $IC_{40}$  and  $IC_{20}$  values (a concentration of 300  $\mu$ M) caused a 40 and 20% reduction, respectively.

Other *in vitro* studies reported that  $\alpha$ -,  $\beta$ -,  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol efficiently function as potent inhibitors of DNA synthesis in human breast cancer cells (non-estrogen-responsive, MDA-MB-435 and estrogen-responsive, MCF 7). The effectiveness of growth inhibition by tocotrienols is independent of estrogen sensitivity. Therefore, tocotrienols have great potential for possible natural aid in the prevention and management of breast cancer. Treatment with a concentration of 180  $\mu$ g/mL and 2  $\mu$ g/mL of palm oil  $\gamma$ -tocotrienol reduced the proliferation of MDA-MB-435 and MCF-7 by 50%, respectively as compared to palm oil  $\alpha$ -tocopherol at a concentration of 125  $\mu$ g/mL in the later cells [17]. Other study reported that treatment with a concentration of  $\alpha$ -tocopherol at concentrations of 25  $\mu$ M and 50  $\mu$ M, inhibits the growth of several human cancer cell lines, including prostate (androgen-resistant, PC-3 and androgen-sensitive, LNCaP) and lung (A549) cells. In contrast, at similar concentrations  $\alpha$ -tocopherol has no effect on normal prostate epithelial (PrEC) cells [8].

To further investigate the antiproliferation mechanism induced by  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol, we analyzed the apoptotic properties of both the compounds on CaSki and Alexander cells by measuring the apoptotic activity of the cellular DNA fragmentation. Gamma-tocotrienol at a concentration of 150  $\mu$ M on CaSki and Alexander cells enhanced the maximum DNA fragmentation activity by 6.8-fold and 5.2-fold, while  $\alpha$ -tocopherol exhibit lesser magnitude of 2.0-fold at higher concentrations of 300  $\mu$ M and 500  $\mu$ M, respectively. Gamma-tocotrienol is more potent as apoptotic inducer as compared to  $\alpha$ -tocopherol. Our finding is in accordance with other data reported that  $\gamma$ -tocotrienol, RRR- $\alpha$ -tocopheryl succinate possessed the ability to induce cancer cells to undergo apoptosis without affecting normal cells. However, RRR- $\alpha$ -tocopherol does not induce cancer cell to undergo apoptosis [18]. Cellular DNA laddering fragments of CaSki and Alexander cells induced with a concentration of 150  $\mu$ M of  $\gamma$ -tocotrienol were evident using electrophoresis. Treatment with -tocopherol at concentrations of 300  $\mu$ M and 500  $\mu$ M also produced similar patterns. Previous study using the cellular DNA laddering detection indicated that  $\alpha$ -tocopherol or combination with  $\alpha$ -tocopherol, induced apoptosis in androgen-sensitive (LNCaP) but not the androgen-resistant (PC-3) human prostate cancer cells through the induction of cytochrome

c release, activation of caspase-9 and -3, cleavage of poly-ADP-ribose polymerase and involvement of caspase-independent pathways [8]. Another study reported that  $\alpha$ -tocopheryl succinate induces apoptosis of human breast cancer cells via translocation of Bax from the cytosol to the mitochondria and releasing cytochrome c from the mitochondria to the cytosol [10].

The greater apoptotic effect on the CaSki and Alexander cells by  $\gamma$ -tocotrienol may be due to its shorter unsaturated isoprenoid tail that allows easier mobility, more uniform distribution in cell membranes and greater recycling activity as compared to  $\alpha$ -tocopherol [4]. This is in accordance with our findings of cellular uptake of both the compounds by both the cell types using HPLC method. Gamma-tocotrienol was taken-up most efficiently by CaSki cell with a ratio of 4:1 (at a concentration of 150 $\mu$ M) and 7:1 (at a concentration of 300 $\mu$ M) as compared to  $\alpha$ -tocopherol, followed by Alexander cell with a ratio of 2:1 at a concentration of 300 $\mu$ M. The differential magnitude in the incorporation of  $\gamma$ -tocotrienol by CaSki and Alexander cells may also be due to the presence of specific receptor(s) that specifically binds to the compound.

In summary, our results showed that palm oil vitamin E, especially  $\gamma$ -tocotrienol, effectively induces apoptosis of the cells of the cervical carcinoma and hepatoma (CaSki and Alexander cells). The apoptosis inducing ability of  $\gamma$ -tocotrienol makes this compound a promising candidate for further characterization of its antitumor profile *in vivo* as a possible natural therapeutic agent in cancer management.

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