Effect of Tocotrienols on the Growth of a Human Breast Cancer Cell Line in Culture[1]

Kalanithi Nesaretnam[a,*] Najla Guthrie[b], Ann F. Chambers[c], and Kenneth K. Carroll[b]

[a]Palm Oil Research Institute of Malaysia, Kuala Lumpur 50720, Malaysia, [b]University of Western Ontario, London, Ontario N6A 5Cl and [c]London Regional Cancer Research Centre, London, Ontario, N6A 4L6 Canada

ABSTRACT: The tocotrienol-rich fraction (TRF) of palm oil consists of tocotrienols and some α-tocopherol (α-T). Tocotrienols are a form of vitamin E having an unsaturated side-chain, rather than the saturated side-chain of the more common tocopherols. Because palm oil has been shown not to promote chemically-induced mammary carcinogenesis, we tested effects of TRF and α-T on the proliferation, growth, and plating efficiency (PE) of MDA-MB-435 estrogen-receptor-negative human breast cancer cells. TRF inhibited the proliferation of these cells with a concentration required to inhibit cell proliferation by 50% of 180 μg/mL, whereas α-T had no effect at concentrations up to 1000 μg/mL as measured by incorporation of [^{3}H]thymidine. The effects of TRF and α-T also were tested in longer-term growth experiments, using concentrations of 180 and 500 μg/mL. We found that TRF inhibited the growth of these cells by 50%, whereas α-T did not. Their effect on the ability of these cells to form colonies also was studied, and it was found that TRF inhibited PE, whereas α-T had no effect. These results suggest that the inhibition is due to the presence of tocotrienols in TRF rather than α-T.

Lipids 30, 1139–1143 (1995).

Earlier studies have shown that palm oil does not promote chemically-induced mammary tumors in rats like other dietary fats and oils (1–3). Furthermore, the growth of cells derived from a spontaneously arising mouse mammary tumor, when injected into animals pre-fed with various dietary lipids, was halved when 20% (of energy) palm oil was fed compared with 20% safflower oil, and was reduced to one-sixth in animals fed 5% palm oil (4). It has been suggested that this inhibition might be due to the fact that palm oil is not rich in linoleic acid, which has been shown to promote cancer in *in vivo* and *in vitro* studies (5–8). A second explanation may be found in the anticarcinogenic potential of palm oil's minor components, mainly its vitamin E tocotrienols, as recent studies have shown that palm oil stripped of the vitamin E fraction enhances tumorigenesis. It also was demonstrated that addition of the tocotrienol-rich fraction (TRF) isolated from palm oil to corn oil caused a delay in the appearance of tumors when compared with the corn oil control group (9). Tocotrienols also caused a delay in the onset of subcutaneous lymphoma by 2–4 wk in HRS/J hairless mice, a strain genetically susceptible to subcutaneous lymphoma (10). The life span of mice inoculated with transplanted tumor cells was increased with tocotrienols (11). An inhibition of tumor cell growth of Hela and p388 cells by tocotrienols in culture also has been reported (12). The two forms of vitamin E—tocopherol and tocotrienol—were tested for chemopreventive activity in two chemically-induced rat mammary tumor models. It was found that only the tocotrienol group had a statistically significant increase in tumor latency when mammary tumors were induced with 7,12-dimethylbenz(a)antracene (DMBA), and neither analogue modified latency when nitrosometylurea was used (13).

Tocotrienols account for about 70% of the vitamin E present in palm oil and have an unsaturated side-chain, whereas tocopherols have a saturated side-chain. Although tocotrienols are generally believed to be much less biologically active than tocopherols, they have been shown to have greater free radical scavenging properties as cell membrane constituents (14,15). It has been proposed that vitamin E act as scavenger of nitrite compounds, thus preventing the formation of cancer-promoting nitrosamines (16). Epidemiological data suggest that vitamin E and other antioxidants may decrease cancer incidence. Several studies have revealed that the subjects with the highest serum concentrations of vitamin E have a lower risk of certain cancers (17–20). In addition to this role as a free radical scavenger, high intakes of vitamin E also may enhance the body's immune response (21).

Our interest in tocotrienols was stimulated by observations that dietary palm oil only promotes chemically-induced carcinogenesis in rats when stripped of the TRF. In this study, we tested the effect of TRF isolated from palm oil in comparison with α-tocopherol (α-T) on the proliferation and growth of MDA-MB-435 human breast cancer cells.

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

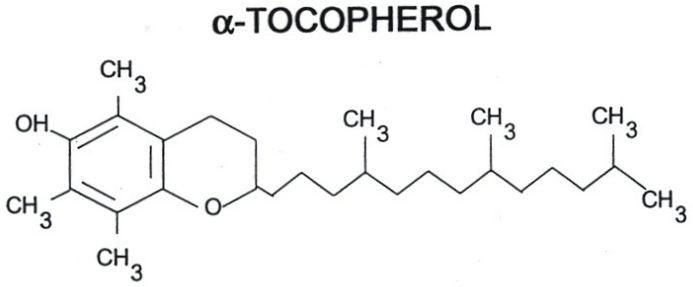
*To whom correspondence should be addressed at Palm Oil Research Institute of Malaysia, P.O. Box 10260, Kuala Lumpur 50720, Malaysia.

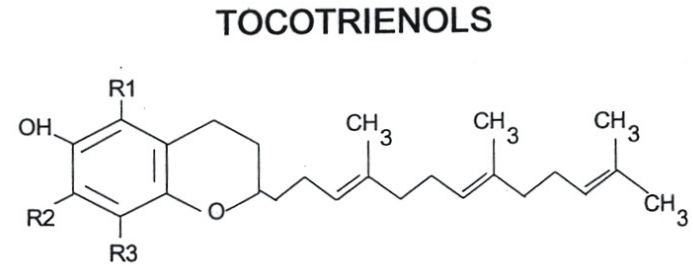
Abbreviations: α-T, α-tocopherol; DMBA, 7,12-dimethylbenz(a)anthracene; MTT, 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PE, plating efficiency; TRF, tocotrienol-rich fraction.

MATERIALS AND METHODS

Materials. Tissue culture plastic ware, growth media, and serum for cell culturing were purchased from Gibco Laboratories (Burlington, Ontario, Canada). [^{3}H]Thymidine (6.7 Ci/mmol) was purchased from ICN (Irvine, CA). Scintiverse was purchased from Fisher Scientific (Nepean, Ontario, Canada). The TRF was obtained from the Palm Oil Research Institute of Malaysia (Kuala Lumpur, Malaysia). α-T was obtained from Sigma Chemical Co. (St. Louis, MO). The TRF and α-T were dissolved in dimethyl sulfoxide and diluted with culture medium. All other chemicals were purchased from Sigma Chemical Co.

Isolation of TRF. The extraction of TRF from palm oil is described by Sundram and Gapor (22) and was performed as follows: Palm fatty acid distillate was used as the starting material, and this was converted into methyl esters by esterification. The methyl esters were then removed by distillation, leaving behind a vitamin E concentrate. This was then concentrated by crystallization and passed through an ion exchange column, resulting in vitamin E that was 60–70% pure. Further purification of this preparation was achieved by washing and then drying the concentrate and passing it through a second molecular distillation stage. The final purity of the vitamin E preparation was 95–99% and its composition was: α-T, 32%; α-tocotrienol, 25%; γ-tocotrienol, 29%; and δ-tocotrienol, 14%. The structural formulae of the compounds are depicted in Figure 1.





	R1	R2	R3
α-tocotrienol	CH_3	CH_3	CH_3
γ-tocotrienol	H	CH_3	CH_3
δ-tocotrienol	H	H	CH_3

FIG. 1. Structures of α-tocopherol and tocotrienols.

Cell culture experiments. The human breast cancer cell line MDA-MB-435 was obtained from Dr. Janet Price (M.D. Anderson Hospital, Houston, TX) (23) and was cultured routinely in minimum essential medium supplemented with 10% fetal bovine serum in a 95% air/5% CO_2 incubator at 37°C. Stock cultures were seeded at a density of 2×10^5 and allowed to grow to 80–90% confluence in T-75 flasks. Culture media was changed every two to three days.

The proliferation assay was performed in a 96-well, flat-bottom plate at a plating density of 2×10^4 cells/well in a total volume of 200 μL of medium. The cells were incubated at 37°C for 48 h, with or without the test compounds. Fifty μL of the TRF was added at the following final concentrations: 1 mg/mL, 500 μg/mL, 250 μg/mL, 200 μg/mL, 180 μg/mL, 160 μg/mL, 140 μg/mL, 120 μg/mL, 60 μg/mL, and 30 μg/mL. Tocopherol controls at the same concentrations were carried out simultaneously. [^{3}H]Thymidine (0.5 μCi/well) was then added, and after 4 h the cells were trypsinized and harvested onto a glass fiber filter paper on a semi-automatic 12-well cell harvester (Skatron, Sterling, VA) for evaluation of thymidine incorporation into DNA. Radioactivity on the paper was counted using Scintiverse for 10 min in a liquid scintillation counter.

Plating efficiency (PE) was determined by seeding a known number of cells in 60- or 100-mm tissue culture dishes with or without a given concentration of either TRF or α-T and counting the colonies 14 d later. PE was calculated as the number of colonies divided by the number of cells seeded. Relative PE was calculated as experimental PE divided by control PE.

For growth experiments, the cells were plated at 1×10^4 cells/60 mm dish in 7 mL of medium. TRF was added at concentrations of 125 and 500 μg/mL. The same concentrations of α-T were used to make allowance for any effects due to tocopherols in the TRF. Dishes were incubated for 2, 4, 6, 8, and 10 d, after which the cells were trypsinized and counted with a hemocytometer.

Viability of cells was measured by the 3,(4,5-dimethylthiazole-2-yl)2,5diphenyltetrazolium bromide (MTT) assay as described by Hansen *et al.* (24). In this assay, MTT is converted to a blue formazan product by dehydrogenases that are active in living cells. The intensity of the blue color developed is a measure of cell viability. MDA-MB-435 cells (8×10^4/well) were seeded with various concentrations of TRF and α-T in a 96-well plate in a total volume of 200 μL of medium. MTT (25 μL of 5 mg/mL) was added to each well. After 3 h, 100 μL of extraction buffer consisting of 20% SDS dissolved in a 50% dimethyl formamide/50% water solution at pH 4.0 was added. The blue color formed was measured at 590 nm.

RESULTS

To investigate the effects of TRF and α-T on cell proliferation, we examined the incorporation of [^{3}H]thymidine into MDA-MB-435 cells in the presence of various concentrations of these substances after 48 h. At a concentration of 180 μg/mL

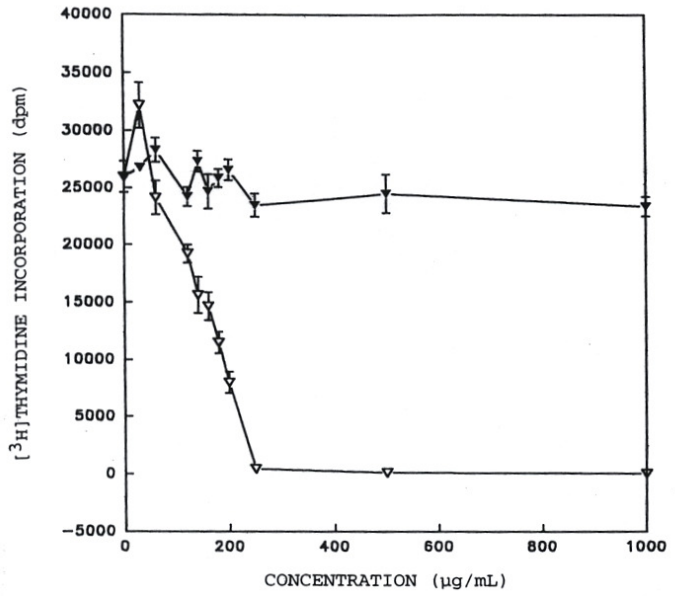


FIG. 2. Effects of α-tocopherol (α-T) (▼) and tocotrienol-rich fraction (TRF) (▽) on the proliferation of MDA-MB-435 cells. The cells were incubated with various concentrations of TRF or α-T (1 mg/mL or 30 µg/mL, respectively) for 48 h. [^{3}H]thymidine (0.5 µCi/well) was then added and the cells were harvested after 4 h to evaluate the incorporation of thymidine into DNA. Points are the average of mean values from three experiments ± SE.

TRF showed a 50% inhibitory effect (Fig. 2). At concentrations above 225 µg/mL, TRF completely inhibited the incorporation of [^{3}H]thymidine by MDA-MB-435 cells. However, there appeared to be a slight stimulation of thymidine incorporation by the cells at the lowest concentration (30 µg/mL) of TRF. In contrast, α-T did not show any inhibition of cell proliferation, even at the highest concentration of 1000 µg/mL.

Figure 3 shows the influence of TRF and α-T on the growth of the MDA-MB-435 cell line. Figure 3A shows the effect of TRF and α-T at a concentration 180 µg/mL on the cells. As shown earlier in the proliferation assay, TRF inhibited the growth of the cells to 50% of control values, and α-T did not. At a higher concentration of 500 µg/mL (Fig. 3B), TRF was shown to be extremely effective in producing complete growth suppression, whereas α-T had no effect.

The sensitivity of the human breast cancer cells to various concentrations of TRF or α-T was determined (Fig. 4). At 1000 µg/mL concentration of α-T, the PE of MDA-MB-435 cells was nearly identical to the control PE of the cells. At the same concentration of TRF, the PE of MDA-MB-435 cells was reduced by approximately three orders of magnitude.

The MTT assay uses the principle whereby cell growth or cell kill is indicated by the conversion or lack of conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically. As can be seen from Figure 5, the MDA-MB-435 cells incubated with various concentrations of α-T grew normally (no change in o.d. reading) and were comparable to the control cells, where there was no addition of α-T or TRF. The effect of various concentrations of TRF on the cells showed,

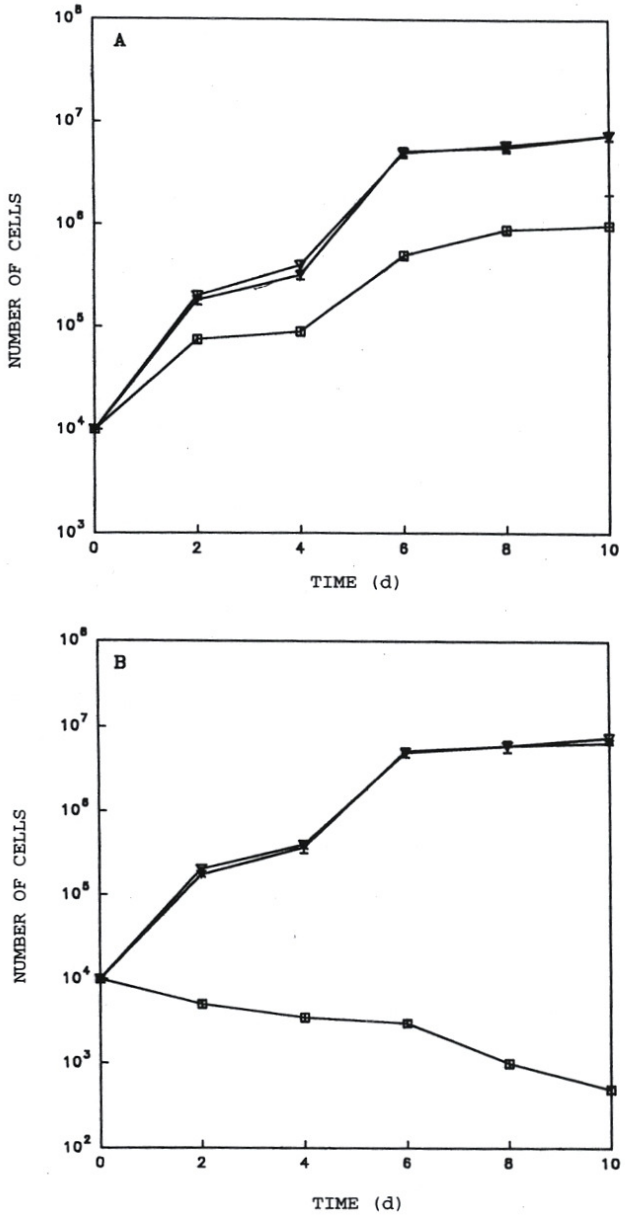


FIG. 3. Comparison of the inhibitory effects of α-tocopherol (α-T) (▼) and tocotrienol-rich fraction (TRF) (□) on MDA-MB-435 cell growth. TRF and α-T were added at concentrations of 180 (A) and 500 µg/mL (B). Neither TRF nor α-T was added to control cells (▽). Points are the average of mean values from three experiments ± SE.

however, that the cells were viable only up to 200 µg/mL; thereafter the o.d. curve dropped rapidly, indicating a substantial loss of cells.

DISCUSSION

As many dietary antioxidants have been shown to prevent cancer in animal studies, the potential activity of vitamin E (tocopherols and tocotrienols) is of interest. Although tocotrienols are known to possess a lower biological "vitamin E activity" than tocopherols (14,25), recent findings suggest that α-tocotrienol is a better antioxidant than α-T (15). In light of the fact that palm oil does not promote chemically-

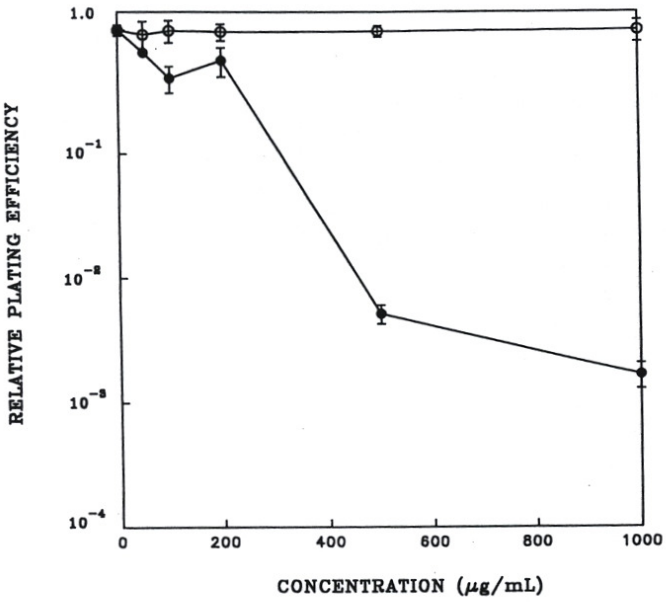


FIG. 4. Effect of tocotrienol-rich fraction (TRF) (●) and α-tocopherol (α-T) (○) on the plating efficiency (PE) of MDA-MB-435 cells relative to control PE without either compound. Cells were grown in 50- or 100-mm tissue culture dishes with or without a given concentration of either TRF or α-T. The colonies were counted 14 d later. Points are mean values ± SD.

induced mammary carcinogenesis when fed at a high level to rats in a semipurified diet, and this failure to promote appears to be due to the TRF of palm oil, we tested the effects of tocotrienols and α-T on human breast cancer cells in culture.

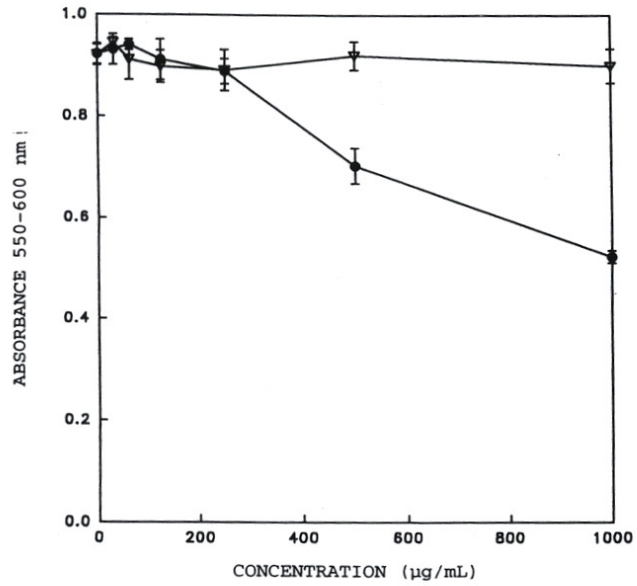


FIG. 5. Effect of tocotrienol-rich fraction (TRF) (●) and α-tocopherol (α-T) (▽) on the viability of MDA-MB-435 cells. MDA-MB-435 cells were incubated with various concentrations of TRF and α-T. 3,(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (25 μL) was added, and after 3 h, 100 μL of SDS extraction buffer was added and o.d. measurements made at 570 nm. Points are the average of mean values from three experiments ± SE.

We found that TRF markedly inhibited the proliferation of MDA-MB-435 human breast cancer cells in culture, having a concentration required to inhibit cell proliferation by 50% of 180 μg/mL, whereas α-T had no effect at concentrations up to 1000 μg/mL. We also found that in testing the compounds on the growth of these cells over a period of ten days, TRF inhibited the growth by 50% at 180 μg/mL (Fig. 3A) and completely inhibited growth at 500 μg/mL (Fig. 3B). Also, these cells were viable in the presence of TRF at concentrations up to 200 μg/mL, and they were viable at all concentrations used for α-T (Fig. 5). The ability of the cells to form colonies was not altered with α-T, but TRF inhibited the PE (Fig. 4). Because TRF in palm oil is made up of 30% α-T and because in our study α-T appeared to have no effect at the same concentration, this inhibition is likely due to the presence of tocotrienols.

Our present results confirm the findings of Komiyama and Yamaoka (12), who showed growth inhibition of human and mouse tumor cells (H69, HeLa, and P388) when the cells were exposed to tocotrienols for 72 h *in vitro*. Tocopherols, on the other hand, showed no significant effect. Also, vitamin E (α-T) supplementation alone has been ineffective in inhibiting the development of mammary carcinomas in rats treated with carcinogenic chemicals (26–28). In addition, initiation of colon tumors induced by the carcinogen, dimethylhydrazine, and mammary tumors induced by DMBA, were increased by feeding diets deficient in vitamin E tocopherols (29,30). Horvath and Ip (31), however, presented evidence that α-T, although ineffective by itself, was able to potentiate the ability of selenium to inhibit the development of mammary carcinomas induced by DMBA in female rats fed a high-fat diet. Tocotrienols, on the other hand, have been shown to inhibit the increase in plasma γ-glutamyltranspeptidase activity, a recognized marker of early neoplasia in rats treated with the hepatocarcinogen, 2-acetylaminofluorene (32). Survival of mice receiving transplanted tumor cells was also increased if they were fed tocotrienol in a dose-responsive way (11).

The exact reasons for the higher sensitivity of the breast cancer cells to tocotrienols are unknown, but the following possibilities can be suggested: (i) Tocotrienols may more easily cross the cell membrane, and (ii) they may undergo slow degradation within the cell. These observations indicate that tocotrienols merit further investigation as possible agents for breast cancer prevention and/or treatment.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Palm Oil Research Institute of Malaysia and in part by a grant to A.F.C. from the National Cancer Institute of Canada.

REFERENCES

1. Sylvester, P.W., Russell, M., Ip, M.M., and Ip, C. (1986) Comparative Effects of Different Animal and Vegetable Fats Before and During Carcinogenesis Administration on Mammary Tu-

morigenesis, Sexual Maturation and Endocrine Function in Rats, *Cancer Res. 46*, 757–762.
2. Sundram, K., Khor, H.T., Ong, A.S.H., and Pathmanathan, R. (1989), Effect of Dietary Palm Oils in Mammary Carcinogenesis in Female Rats Induced by 7,12-Dimethylbenz(α)anthracene, *Cancer Res. 49*, 1447–1451.
3. Kritchevsky, D., Weber, M.M., and Klurfeld, D.M. (1992) Influence of Different Fats (soybean oil, palm olein or hydrogenated oil) on Chemically-Induced Mammary Tumors in Rats, *Nutr. Res. 12*, S175–Sl79.
4. Hubbard, N.E., and Erickson, K.L. (1987) Influence of Dietary Fats on Cell Populations of Line Mouse Mammary Tumors; A Morphometric and Ultrastructural Study, *Cancer Lett. 35*, 281–294.
5. Hopkins, G.J., and Carroll K.K. (1979) Relationship Between Amount and Type of Dietary Fat in Promotion of Mammary Carcinogenesis Produced by 7,12-Dimethylbenz(α)anthracene, *J. Natl. Cancer Inst. 62,* 1009–1012.
6. Clinton, S.K., Mulloy, A.L., and Visek, W.J. (1984) Effects of Dietary Saturation on Prolactin Secretion, Carcinogen Metabolism and Mammary Carcinogenesis in Rats, *J. Nutr. 114,* 1630–1639.
7. Gammal, E.B., Carroll, K.K., and Plunkett. E.R. (1967) Effects of Dietary Fat on Mammary Carcinogenesis by 7,12-Dimethylbenz-α-anthracene in Rats, *Cancer Res. 27*, 1737–1742.
8. Rose, D.P., and Connolly, J.M. (1989) Stimulation of Growth of Human Breast Cancer Cell Lines in Culture by Linoleic Acid, *Biochem. Biophys. Res. Comm. 164*, 277–283.
9. Nesaretnam, K., Khor, H.T., Ganeson, J., Chong, Y.H., Sundram, K., and Gapor, A. (1992) The Effect of Vitamin E Tocotrienols from Palm Oil on Chemically Induced Mammary Carcinogenesis in Female Rats, *Nutr. Res. 12*, 879–892.
10. Tan, B. (1992) Antitumor Effects of Palm Carotenes and Tocotrienols in HRS/J Hairless Female Mice, *Nutr. Res. 12*, S163–Sl73.
11. Kato, A., Yamaoka, M., Tanaka, A., Komiyama, K., and Umezawa, I. (1985) Physiological Effect of Tocotrienols, *Yakugaku Zasshi 34*, 375–376.
12. Komiyama, K., and Yamaoka, M. (1989) Studies on the Biological Activity of Tocotrienols, *Chem. Pharm. Bull. 37*, 1369–1371.
13. Gould, M.N., Haag, J.D., Kennan, W.S., Tanner, M.A., and Elson, C.E. (1991) A Comparison of Tocopherol and Tocotrienol for the Chemoprevention of Chemically Induced Rat Mammary Tumors, *Am. J Clin. Nutr. 53,* 1068s–1070s.
14. Yamaoka, M., and Carrillo, M.J.H. (1990) Effect of Tocopherols and Tocotrienols on the Physicochemical Property of the Liposomal Membrane in Relation to Their Antioxidant Activity, *Chem. Phys. Lipids 55*, 295–300.
15. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) Free Radical Recycling and Intermembrane Mobility in the Antioxidant Properties of α-Tocopherol and α-Tocotrienol, *Free Radic. Biol. Med. 10*, 263–275.
16. Lathia, D., Braasch, A., and Theissen, U. (1988) Inhibitor Effects of Vitamin C and E on *In Vitro* Formation of *N*-Nitrosamine Under Physiological Conditions, *Front. Gastrointest. Res. 14*, 151–156.
17. Salomen, J.T., Salomen, R., and Lappetalainen, R. (1985) Risk of Cancer in Relation to Serum Concentrations of Selenium and Vitamin A and E, Match Case Control Analysis of Prospective Data, *Br. Med. J. 290,* 417–420.
18. Willet, W.C. (1986) Selenium, Vitamin E, Fiber and the Incidence of Human Cancer an Epidemiologic Perspective, *Adv. Exp. Med. Biol. 206,* 27–34.
19. Knekt, P., Aromaa, A., Maatela, J., Aaran, R.K., Nikkari, T., Hakama, M., Hakulinen, T., Peto, R., Saxen, E., and Teppo, L. (1988) Serum Vitamin E and Risk of Cancer Among Finnish Men During a Ten Year Follow-Up, *Am. J. Epidemiol. 127*, 28–41.
20. Knekt, P. (1988) Serum Vitamin E Level and Risk of Female Cancer, *Int. J. Epidemiol. 17*, 281–286.
21. Packer, L. (1991) Protective Role of Vitamin E in Biological System, *Am. J. Clin. Nutr. 53*, 1050S–1055S.
22. Sundram, K., and Gapor, A. (1992) A Vitamin E from Palm Oil Its Extraction and Nutritional Properties, *Lipid Technolog.,* November/December, 137–141.
23. Price, J.E., Polyzos, A., Zhang, R.D., and Daniels, L.M. (1990) Tumorigenicity and Metastasis of Human Breast Carcinoma Cell Lines in Nude Mice, *Cancer Res. 50*, 717–721.
24. Hansen, M.B., Nielsen, S.E., and Burg, K. (1989) Re-Examination and Further Development of a Precise and Rapid Dye Method for Measuring Cell Growth/Cell Kill, *J. Immunol. Methods 119,* 203–210.
25. Leth, T., and Sondergaard, H. (1977) Biological Activity of Vitamin E Compounds and Natural Materials by the Resorption-Gestation Test and Chemical Determination of the Vitamin E Activity in Foods and Feeds, *J Nutr. 107*, 2236–2243.
26. Wattenberg, L.W. (1972) Inhibition of Carcinogenic and Toxic Effects of Polycyclic Hydrocarbons by Phenolic Antioxidants and Ethoxyquin, *J. Natl. Cancer Inst. 48*, 1425–1430.
27. Dayton, S., Hashimoto, S., and Wollman, J. (1977) Effect of High-Oleic and High Linoleic Safflower Oils on Mammary Tumors Induced in Rats by 7,12-Dimethylbenzanthracene, *J. Nutr. 107*, 1353–1360.
28. Welsch, C.W. (1992) Relationship Between Dietary Fat and Experimental Mammary Tumorigenesis: A Review and Critique, *Cancer Res. 52*, 2040s–2048s.
29. Sumiyoshi, H. (1985) Effects of Vitamin E Deficiency on 1,2 Dimethylhydrazine Induced Intestinal Carcinogenesis in Rats, *Hiroshima J. Med. Sci. 34*, 363–369.
30. Ip, C. (1982) Dietary Vitamin E Intake and Mammary Carcinogenesis in Rats, *Carcinogenesis 3*, 1453–1456.
31. Horvath, P.M. and Ip, C. (1983) Synergistic Effect of Vitamin E and Selenium in the Chemoprevention of Mammary Carcinogenesis in Rats, *Cancer Res. 43,* 5335–5341.
32. Ngah, W.Z.W., Jarien, Z., San, M.M., Marzuki, A., Top, G.M., Shamaan, N.A., and Kadir, K.A. (1991) Effects of Tocotrienols on Hepatocarcinogenesis Induced by 2 Acetylaminofluorene in Rats, *Am. J. Clin. Nutr. 53*, 1076s–1081s.

[Received December 22, 1993, and in final revised form June 12, 1995; Revision accepted August 28, 1995]