CHAPTER 5

ANTI-TUMOUR PROMOTING ACTIVITY OF PALM OIL TOCOTRIENOLS

5.1 INTRODUCTION

5.1.1 Biological Activity of Vitamin E

The earliest studies leading to the discovery of vitamin E in the 1920's were related to its function in animal reproduction [165,404,615]. Since then, vitamin E has often been described as an antisterility vitamin [80]. To date, thousands of reports on vitamin E have accumulated in the literature, covering a wide range of biological studies. Among the various natural vitamin E components, α-tocopherol (α-T) is the most extensively studied in relation to its chemistry and biological activities. The other vitamin E compounds, although having a wide distribution in plants and vegetable oils, have not been given sufficient attention. Research interest in tocotrienols has been initiated recently since massive isolation of tocotrienols from palm oil was available. Despite initial concerns about tocotrienols, some encouraging experimental results have emerged to indicate that they are more effective than the tocopherols in certain aspects, being anticancer [322,348,452] and hypocholesterolaemic agents [498,499,528,529,623]. For instance, γ-tocotrienol (γ-T_3) has been found to be hypocholesterolaemic in rats fed atherogenic diets [663] and act more effectively than γ-tocopherol (γ-T) by suppressing the activity of β-hydroxy-β-methylglutaryl-CoA reductase (HMGCoA reductase) [499]. Similarly, α-tocotrienol (α-T_3) has also been shown to inhibit the biosynthesis of cholesterol through suppression of HMGCoA reductase activity [528].

5.1.2 Chemical Carcinogenesis

There is accumulating evidence to support the hypothesis that most of human cancers are developed *via* a multistep process which may extend over one or several decades. For instance, in some common human cancers of viral origin, such as Burkitt's disease or carcinoma of the uterine cervix, the viral infection is only the first step toward carcinogenesis. The multistage nature of chemically-induced carcinogenesis [42] is now generally recognized as involving initiation by carcinogens, promotion by tumour promoters and finally progression to cancer. The terms "initiation" and "promotion" were

first introduced by Friedwald and Rous after their experiments on skin cancer [181]. Initiation is a very rapid and irreversible process whereas tumour promotion is a long but reversible process. In the first stage of carcinogenesis, exposure to a sub-carcinogenic dose of an initiator irreversibly convert normal cells to potential tumour cells which are carrying deleted biological information [258]; the growth of the potential tumour cells is under controlled by the target tissue at this stage. Previous experiments have shown that a single application of sub-carcinogenic dose of carcinogens such as 7,12-dimethylbenz[a]-anthracene (DMBA) seldom provoke any skin cancer. But when a tumour-promoting substance such as croton oil was applied to the skin, a large number of cancerous growth were observed. In the second stage, further exposure of the initiated target tissues to any amplifying factor such as by repeated dose of a tumour promoter, promotion of the potential tumour cells into visible benign tumours will occur, but the growth of the tumour is still controlled by the target tissue in this early promotion stage. In the progression stage of carcinogenesis, the benign tumours grow invasively, this lethal phase may occur spontaneously or induced by one additional sub-carcinogenic dose of an initiator in the late promotion stage [537]. In practice, it is difficult to prevent the induction of carcinogenesis caused by environmental carcinogens because a large variety of chemicals are being used in food products, toiletries and even pharmaceuticals, without a thorough understanding of their carcinogenicity and long-term toxicity. In view of these, the inhibition of the tumour promotion stage is generally recognized as a crucial preventive step against chemical carcinogenesis and cancer.

5.1.3 The Role of Free Radicals in Carcinogenesis

There is compelling experimental results indicating that free radicals are involved in chemical carcinogenesis [644], particularly in the process of tumour promotion [329]. The evidence includes: (a) potential free-radical generating compounds such as organic peroxides have been related to tumour promotion, (b) tumour promoters which stimulate the production of reactive oxygen species from endogenous sources in a variety of cell

types, (c) reactive oxygen species that can mimic the biochemical action of tumour promoters, (d) tumour promoters modulate cellular antioxidant defense systems and (e) free radical scavengers and detoxifiers can inhibit tumour promotion. Active oxygen (e.g. dioxygen, O_2; superoxide, O_2^- and hydrogen peroxide, H_2O_2), oxy-radicals (the hydroperoxy radical $HO_2^\bullet$ and the hydroxyl radical $HO^\bullet$) and other free radicals appear to play an important role in the promotion phase of carcinogenesis, in which gene expression of initiated cells is modulated by affecting the genes that regulate cell differentiation and growth. For instance, high oxygen tensions could increase the transformation frequency of mouse embryo cells irradiated with fluorescent light [556]; O_2^- promotes radiation or chemically initiated transformation of mouse embryo fibroblasts. H_2O_2, peroxyacetic acid, benzoyl peroxide and other organic peroxides also promote the chemically initiated transformation of mouse epidermal cells [61,583,585,648,707]. Furthermore, hydrogen peroxide also exhibits an enhancing effect upon duodenal and upper jejunal carcinogenesis in rats [272]. Certain promoters act by generation of oxygen radicals and resultant lipid peroxidation [7,154,155,220,399]. Moreover, phorbol esters have been reported as capable of generating oxygen radicals, which can cause chromosome breaks [53] and increase gene copy number [65].

Various mechanisms have been suggested for different types of carcinogens [401,480,644], some carcinogens are known to involve specific radical intermediates in their conversion from a pre-carcinogen to the proximate carcinogen form. For instance, *N*-hydroxy-2-acetylaminofluorene is known to be converted to a nitroxyl radical in the course of being converted to a carcinogen [31,173], and this is a general mechanism used to explain the radical-mediated carcinogenesis. An alternative theory on the conversion to an electrophilic ultimate carcinogen is also well-known. For example, leukocytes could produce superoxide which may trigger mutation [675]. There are many carcinogens known to be easily autoxidized or metabolized to produce superoxide and are related to cancer development. Benzoyl peroxide is a compound used in many dermatological

products, its tumour promoting effect [585] also suggests a general mechanism which relates chemical carcinogenesis with peroxidic species.

Another body of evidence that provides strong support for the role of free radicals in carcinogenesis is that many antioxidants, either of synthetic or natural origin, are anti-carcinogens or possess cancer prevention activity [408,419,478]. For example, the anti-promotional activity of butylated hydroxy toluene and butylated hydroxyanisole in inhibiting the transformation of mouse skin cells promoted by phorbol-12-myristate-13-acetate [41,519,584] and benzoyl peroxide [583], has been attributed to their antioxidant capability.

5.1.4 Epstein-Barr Virus and Nasopharyngeal Carcinoma

Epstein-Barr virus (EBV) was the first virus found to be associated with human cancer [157] and was isolated from Burkitt's lymphoma cells in 1964. It is a member of the Herpes group, which can readily transform human lymphocytes into lymphoblastoids having infinite replicating capability [131]. Infection by this common and widespread virus in humans has long been identified as the initiating factor in Burkitt's lymphoma and nasopharyngeal carcinoma which is a very common cancer found in the southern region of China [139,141]. The presence of EBV in a patient is linked to early antigens (EA) which are some EBV-associated products appearing soon after cell infection. EBV EA was first detected by an indirect immunofluorescence technique in certain lymphoblastoid cell lines abortively infected by EBV [263].

In many parts of Asia, there is a fairly high incidence of nasopharyngeal carcinoma which may be related to dietary habits or environmental factors (e.g. exposure to phorbol esters and derivatives). For instance, the high incidence of nasopharyngeal carcinoma in the southern region of China has been circumstantially linked to the distribution of plants such as *Aleurites fordii* L. and *Croton tiglium* L. which are commonly grown for industrial purposes and also used as medicinal herbs [303]. Antibodies to the EA have also been found in the sera of patients with nasopharyngeal carcinoma [262] which has been strongly

associated with EBV infection for many years since the first observation by Old *et al.* [487,488].

5.1.5 Tumour Promoters

Tumour promoting compounds were once known to be non-carcinogenic by nature but when repeatedly applied to tumour potential cells will promote the carcinogenesis process and enhance the growth of preformed tumour cells. While many tumour promoters are synthetic compounds, an abundance of naturally occurring tumour promoters have also been reported in plants, some of the examples being listed in Table 5.1. Among these tumour-promoting substances, phorbol esters have been found to be very effective promoters; they are present in the Euphorbiaceae family, some of which are commonly used as folk remedies or herbal teas. Many plants in Malaysia belonging to the Euphorbiaceae and Thymelaeceae families have medicinal uses. Among these plants, extracts from *Croton tiglium*, *Euphorbia lathylis*, *Aleurites fordii* [302], *Jatropha curcas*, *Euphorbia antiquorum*, *Euphorbia milli*, *Euphorbia pekinensis*, *Euphorbia kansui*, and *Daphne odora*, have been found to exert EBV-activating effects [692]. Indeed, a strong circumstantial link between the high incidence of nasopharyngeal carcinoma and the distribution of Euphorbiaceae and Thymelaeceae plants has been established in the southern regions of China [270].

Using a two-stage carcinogenesis test, diterpene esters with structure related to 12-*O*-tetradecanoylphorbol-13-acetate (TPA), can be tested by inducing tumour promotion after an initiating dose of DMBA; the structures of such carcinogenic compounds are shown in Fig. 5.1. TPA is the best known promoter of mouse skin tumour [259] and is the active component in croton oil extracted from the seed of *Croton tiglium* L. [70,256,479] which is a plant belonging to the Euphorbiaceae [257,649]. Other TPA-type tumour promoters (need not structurally be TPA-type) are teleocidin [182], aplysiatoxin [185], 12-*O*-hexadecanoyl-16-hydroxyphorbol-13-acetate [271] and 12-*O*-hexadecanoylphorbol-13-acetate [362]. Teleocidin is a compound isolated from

Table 5.1 Classes of Various Tumour Promoters

TPA types

Teleocidin class

- Dihydroteleocidin B
- Teleocidin A (Lyngbyatoxin A)
- Teleocidin B
- Des-*O*-methylolivoretin C
- (-)-Indolactam-V
- *N*-Geranyl-indolactam-V

Aplysiatoxin class

- Debromoaplysiatoxin
- Aplysiatoxin
- Bromoaplysiatoxin
- Dibromoaplysiatoxin
- Oscillatoxin A
- Anhydrodebromoaplysiatoxin

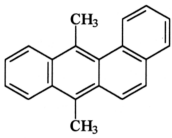
Phorbol ester class

- intramolecular 13,16-diester of 12-deoxy-16-hydroxyphorbol

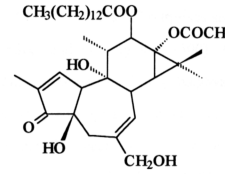
Non-TPA types

Okadaic acid class

- Okadaic acid
- Dinophysistoxin-1
- Calyculin A



7,12-dimethylbenz[a]anthracene
(or 9,10-dimethyl-1,2-benzanthracene)
(DMBA)



12-*O*-tetradecanoylphorbol-13-acetate
(TPA)

Fig. 5.1 Structures of DMBA (a carcinogen) and TPA (a tumour promoter).

Streptomyces mediocidicus [185] and it is also a potent tumour promoter in mouse skin carcinogenesis [611]. 12-*O*-hexadecanoyl-16-hydroxyphorbol-13-acetate is the active tumour promoter found in tung oil extracted from *Aleurites fordii* [271,482] or so-called tung oil tree, a plant commonly grown in Southern China for industrial purposes such as oil paints, varnishes, waterproof agents, anticorrosives and printing inks [300]; 12-*O*-hexadecanoylphorbol-13-acetate is isolated from *Sapium sebiferum* [481], lyngbyatoxin and debromoaplysiatoxin also induce EBV antigens [151]. Examples of tumour promoters effective in epidermal and non-epidermal tissues include saccharin, dichlorodiphenyl trichloroethane and others, all of which also actively induce EBV antigens [619]. Other skin tumour promoters of non-TPA type are okadaic acid, palytoxin, etc.

Mechanisms / pathways

It has been suggested that the mechanistic pathway of TPA-type tumour promoters is through a binding to the so-called phorbol ester receptors and somehow activate the protein kinase C which is then induce the synthesis of phosphoproteins in larger amounts. Okadaic acid is a non-TPA type promoter which inhibits dephosphorylation of phosphoserine and phosphothreonine through protein phosphatases 1 and 2A, therefore also causing an accumulation of the phosphoproteins [188]; this okadaic acid pathway is a general mechanism of tumour promotion in various organs. TPA is a producer of free radical [67] and its tumour-promoting activity is closely related with active oxygen radicals in some studies [328,643,707]. Many workers suggested that TPA exerts its biological activity *via* O_2^- while some reports indicated that HO$^\bullet$ may be an effective factor in the action of TPA [476].

5.1.6 Naturally Occurring Anti-tumour Promoters

Inhibition of carcinogenesis at the promotion stage has been suggested to be one of the potential measures for chemoprevention of cancers [666]. Common anti-tumour promoters are classified into the following groups: (a) retinoic acid and related compounds [652,669], (b) anti-inflammatory steroids (e.g. fluocinolone acetonide) [669], (c) some

protease inhibitors (e.g. leupeptins) [285], (d) polyamine synthesis inhibitors (e.g. difluoromethylornithine) [586], (e) prostaglandin synthesis inhibitors (e.g. indomethacin and naproxen) [651], (f) analogues of promoters such as phorbol esters with short chain fatty acid residues [559], and (g) others [138,469]. Many natural compounds have been demonstrated to be capable of inhibiting the tumour promoting stage of certain carcinogenic processes. Some dietary items, such as garlic [118,280] and onion [40], can have protective effects against human cancers and has been attributed to their anti-tumour promoting activity [40,470,472]. Furthermore, (-)-epigallocatechin gallate (in Japanese green tea leaves [701]), sarcophytol A and B (in a soft coral *Sarcophyton glaucum* [187]), laxogenin (in the tuber of *Allium bakeri* Rgl.), diallyl sulfide [662] and allixin (from garlic) [472], have been identified as the active components. (-)-Epigallocatechin gallate has been reported to have inhibitory effect on experimental mouse duodenal carcinogenesis induced by *N*-ethyl-*N'*-nitro-*N*-nitrosougua-nidine [191]. A decreased risk of gastric cancer was also noticed among patients with greater consumption of green tea [257]. Sarcophytol A has been found to inhibit the development of large bowel cancer in Fischer rats induced by *N*-methyl-*N*-nitrosourea [446]; a diet containing sarcophytol A also reduced the development of spontaneous mammary tumours in mice [189].

Naturally occurring compounds, especially food constituents such as ascorbic acid [420,421], tocopherols [457], retinoids [425,653], carotenoids [536,564], phenolics [381,438] including flavonoids [664] and coumarins [665], are found in vegetables and have the potential to reduce the risk of cancer development. Retinoic acid [494,568] and ascorbic acid are well known anti-cancer agents and inhibitors of tumour promotion, they have been shown to exhibit intervening effect on the activation of EBV from the genome-carrying human lymphoblastoid cells [299,693]. Flavonoids from medicinal plants also possess inhibitory effect on tumour promotion [184]. For instance, lophirone A [440] is a biflavonoid-related polyphenol from *Lophira alata* (Ochnaceae) [441], which is medicinally used as an analgesic in West Africa. Examples for other flavonoid type

inhibitor of tumour promotion are quercetin [233] and apigenin [670]. Ursolic acid and oleanolic acid are two types of triterpene carboxylic acids isolated from a Chinese medicinal plant with anti-inflammatory activity, *Glechoma hederacea*, which could effectively inhibit the tumour promotion in mouse skin carcinogenesis[632].

Mechanisms / pathways

It has been reported that sarcophytol A inhibits H_2O_2 formation by TPA-activated human neutrophils, and also inhibits oxidative DNA damage as determined by the formation of thymidine glycol and 5-hydroxymethyl-2'-deoxyuridine, this suggests that anti-tumour promoters may act by suppressing oxy-radicals formation [180]. (-)-Epigallocatechin gallate also inhibits the activation of protein kinase C by teleocidin [701]. It was thought that (-)-epigallocatechin gallate interacts with a protein kinase C complex consisting of phosphatidylserine, Ca^{2+} ion, a tumour promoter, and protein kinase C. It has been reported that (-)-epigallocatechin gallate and penta-*O*-galloyl-β-D-glucose inhibit H_2O_2 formation by TPA-activated human polymorphonuclear leukocytes, act by suppressing oxy-radical formation [705].

Bioassay and screening for potential anti-tumour promoters

EBV-activation bioassay [301] is a short-term *in vitro* assay widely used to investigate the activity of naturally occurring anti-tumour promoters; the inhibitory activity against tumour promoter-induced EBV-activation in Raji cells is measured. It is a relatively effective and rapid method for screening new anti-tumour promoters [484]. TPA [708], 5-iododeoxyuridine [610] or *n*-butyrate [390] are usually used as inducing agents for EBV EA expression in the bioassay of human lymphoblastoid cells.

5.1.7 Anti-cancer and Anti-tumour Properties of Vitamin E and Other Antioxidants

Many research work on cells and animals has demonstrated that many antioxidants are anticarcinogens [408,419,478], some of the antioxidants also exhibit beneficial effect as anti-tumour promoters [66,101,171,483], and the anti-promotional activity has been

related to their antioxidant capability. For instances, butylated hydroxytoluene and butylated hydroxyanisole inhibit the transformation of mouse skin cells promoted by phorbol-12-myristate-13-acetate [41,519,584] and benzoyl peroxide [583]. Tumour promotion is generally believed to be associated with the generation of free radical species [585,618]. Furthermore, the inhibitory effect of antioxidants such as butylated hydroxytoluene in the carcinogen-induced skin tumourogenesis has been related to their radical quenching properties [584]. Vitamins E, A and C, β-carotene and selenium are some of the common nutritional anticarcinogens having a protective effect in prevention of certain human cancers; the dietary intake or high blood levels of these antioxidants in combination have been related to lower risk of certain cancers [655].

Vitamin E acts as an excellent oxy-radical scavenger, it helps to terminate *in vivo* free radical-chain reactions. The function of vitamin E as an important *in vivo* antioxidant [126] protects unsaturated lipids from free radical peroxidation [193,563,689] which occurs especially in biomembranes [389,625], and thus prevents tissues from cellular damage associated with lipid peroxidation. Free radical chain reactions in lipid peroxidations of biological systems are generally believed to be one of the factors causative of heart disease [211], ageing process [379], cancer and a variety of pathological events [391,516]. Low vitamin E levels have been related to an increased risk of certain cancers in humans [414,657], and it was reported to possess anti-cancer properties in many cell culture and animal experiments [113,246,278]. Besides the well known antioxidant effects, it has been reported that the vitamin E possess a biomodulating properties and it was suggested this may account for the anti-tumour activity of vitamin E by inducing tumour cells to produce an anti-proliferative factor [346]; however, information in this aspect is still limited.

5.1.8 Anti-Cancer Constituents in Palm Oil

It is now realized that most of human cancers are caused by exogenous factors associated to lifestyle such as dietary factors, smoking and alcohol consumption. To lower

the risk of cancer, a change in lifestyle is necessary. In addition to a balanced diet, the roles of micronutrients have been given substantial attention in recent years with the view of countering the consistently high incidence of cancer particularly in the developed and industrialized countries. In concerns of dietary factors, a number of studies provide evidence that the nature of dietary fats has a close relationship with the cancer development. For example, the incidence of DMBA-induced mammary tumour in rats fed palm oil was lower than that in rats fed corn oil and soyabean oil [613]; minor amounts of carotenoids and tocotrienols present in the dietary palm oil were suggested to be responsible for the lower tumour incidence. Palm oil possesses some cancer prevention substances such as carotenoids and vitamin E [349,473]. However, direct evidence on the preventive properties of the tocotrienols is still insufficient. A Japanese study [471] has demonstrated that carotenoids isolated from palm oil showed anti-cancer activity. Earlier studies [322,348] indicated that tocotrienols have anti-tumour effects, high dose of α-T_3 applied by intraperitoneally injection has been shown to exert a stronger anti-tumour action than the tocopherols [322]; however, acetates of the tocopherols and tocotrienols did not show significant differences from the free chromanols in the anti-tumour study [322]. Tocotrienols possess a high protective activity against cardiotoxicity of the anti-tumour redox cycling drug, i.e. adriamycin, and suppress the microsomal lipid peroxidation induced by NADPH and adriamycin [348].

5.1.9 Objectives of the Present Research

Various natural tocopherol and tocotrienol components, and some of their derivatives were examined for their *in vitro* anti-tumour promoter activities. This was done by a short term *in vitro* assay based on the inhibition of EBV early antigen (EBV EA) expression in EBV genome-carrying human lymphoblastoid cells [301] by TPA. Preliminary experiments are also carried out on mouse *in vivo* two-stage skin carcinogenesis.

5.2 EXPERIMENTAL

5.2.1 Chemicals

Palm oil vitamin E concentrate containing tocotrienols and tocopherols were obtained from palm fatty acid distillate [218]. Briefly, the free fatty acids were crystallized and removed by filtration, the residual fatty acids were methylated and distilled off under vacuum. Individual vitamin E components in the residue were isolated by silica column chromatography using ethyl acetate and *n*-hexane as the eluant [219]. Dimers derived from the oxidation of γ-T_3 or γ-T by alkaline ferricyanide, were also purified by the same chromatographic method [219].

TPA and sodium *n*-butyrate purchased from Sigma Co. were used as the inducers for the EBV EA expression, both of them and the vitamin E compounds were dissolved in dimethylsulfoxide (Sigma) and further diluted with culture medium.

5.2.2 Cells and Culture Medium

The Raji cell line used in this study was EBV-genome-carrying human lymphoblastoid cells obtained from the National Cancer Institute, Bethesda. The cells were kept in RPMI 1640 medium supplemented with penicilin (100 I.U./mL), streptomycin (100 μg/mL), L-glutamine (120 μg/mL), fungizone (50 μg/mL), 10% heat-inactivated fetal calf serum, and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

5.2.3 Assay for EBV-activation Activity [692]

EBV-activation

A short-term *in vitro* assay was carried out to examine the anti-tumour promoting effect of vitamin E based on the activation of EBV EA expression in EBV genome-carrying human lymphoblastoid cells [301]. The rapidly growing Raji cells at a density of $1x10^6$ cells per mL culture medium were incubated with varying concentrations of vitamin E components and dimers of γ-T and γ-T_3; TPA (10 ng/mL) was added as the tumour promoter, 4 mM of sodium *n*-butyrate was added as synergist [298,324,390]. After

incubation for 72 hours in a humidified incubator at 37°C with 5% CO_2 in air, the cells were harvested by centrifugation at 1000 rpm for 5 minutes and washed in 4 mL phosphate buffer saline, pH 7.2 (PBS). The cells were resuspended in 0.3 mL of PBS and 50 μL aliquot were dispensed onto a Teflon-coated multi-well slide, dried under cold air and then fixed in cold acetone (-20°C) for 10 min.

Indirect Immunofluorescence Assay [475]

Nasopharyngeal carcinoma serum containing IgG antibody to EBV-EA, obtained from the patients with nasopharyngeal carcinoma at the Kuala Lumpur General Hospital, was used to detect the presence of EBV EA by means of indirect immunofluorescence assay. The conjugate used was anti-human IgG (γ-chain) fluorescein-conjugate (purchased from Behringwerke) prepared in rabbit. The fixed Raji cells were overlaid with 20 μL of diluted serum (EA titer 1:20), incubated in a humidity chamber at 37°C for 45 min., washed with PBS for 10 min, the slides were reincubated with 20 μL 1:15 dilution of the anti-human IgG conjugated to fluorescin-isothiocyanate at 37°C for 45 min. After washing with PBS, the slides were dried under cold air and mounted in glycerol-PBS (9:1), and the number of fluorescent cells were counted under a microscope equipped with epi-ultraviolet source, BG12 exciter filter, BG 38 suppressor filter and 530 barrier ocular filter. The inhibitory activity was evaluated by the following grades: (a) positive = 0% of EA induction (or complete inhibition of EBV EA expression), (b) weakly positive = less than 30% of EA induction (or partial inhibition of EBV EA expression), and (c) negative = 30% of the EA induction as in the control (or no inhibition of EBV-EA expression). Positive inhibition in the EBV early antigen expression induced by TPA would indicate that the vitamin E compounds can function as an anti-tumour promoter.

5.2.4 Two-stage Skin Carcinogenesis Experiment on Mouse [183,186]

Forty eight female mice aged 7-week old were used in this experiment. Four days before initiation by DMBA, the back of the mice were shaved by an electrical clipper

model 900 blade No. 40. The mice were divided into three groups:- two groups of the mice (skin) were treated with γ-T_3 or δ-T_3, no tocotrienol was applied for a control group. Tumourogenesis was initiated by a single topical application of 100 μg DMBA in 0.1 mL acetone (10 mg/10mL). One week after initiation, tocotrienol (0.5 mg/100 μL acetone) was applied at ½ hour prior to the application of a promoter, i.e. croton oil (1 mg/100 μL acetone). The treatment of vitamin E compounds and croton oil was done two times a week and applied on the same area of the mice two times weekly. The hair on topically treated area of the mice was shaved every two weeks throughout the experiment, and their body weight was recorded. The number of tumour with a size of 1 mm or more in diameter was counted every week. The percentage of tumour-bearing mice for each group was recorded weekly for evaluation of the anti-tumour promoting activity of vitamin E.

5.3 RESULTS AND DISCUSSION

5.3.1 *In Vitro* Anti-tumour Promoting Activity of Tocotrienols

The Raji cells treated with TPA and *n*-butyrate induced about 30% EBV early antigen in the absence of vitamin E compounds. The results on anti-tumour promoting effects of tocotrienols and tocopherols are shown in Table 5.2. Complete inhibition of the TPA induction was observed when pure γ-T_3 and δ-T_3 were applied at 20 μg/mL and above while α-T_3 showed inhibition at higher concentrations, i.e. 80 μg/mL and above. α-T and γ-T did not inhibit TPA induction at any concentration whereas δ-T inhibits the EBV EA expression only at 80-160 μg/mL.

Vitamin E, particularly α-T, has been widely regarded as an anticarcinogenic antioxidant and associated with lower risk of certain cancers in many cell culture and animal studies [655]. The anti-tumour promoter potential of pure tocotrienol components has now been studied in order to elicit their *in vitro* and *in vivo* anti-tumour effect. Tocopherols and tocotrienols are known to be excellent antioxidants in inhibiting lipid peroxidation, and their chain-breaking activity is associated with the ability of donating phenolic hydrogen and formation of phenoxyl free radicals [219]. Although tocopherols and the corresponding tocotrienols have similar chromanol structures and possess comparable antioxidant activity, only tocotrienols (especially the γ- and δ-homologues) are found to exhibit remarkable *in vitro* anti-tumour promoting activity in the present study. The actual mechanism by which the tocotrienols inhibit tumour promotion is not known, it may not just involve a direct action of their antioxidant property, but requires the unique structure which incorporates a less substituted chromanol moiety and a triunsaturated isoprenoid side chain as present in the natural tocotrienols (more compact than tocopherols). The present results indicate that the less methylated tocols (γ- and δ-vitamers) are substantially more active than the fully trimethylated chromanols (α-vitamers) and it appears that this is in the reverse order of antioxidant activity of the vitamin E components (i.e. $\alpha > \gamma > \delta$).

Table 5.2 Anti-tumour Promoting Activity of Vitamin E Components*

Vitamin E	Concentration (μg/mL)				
	10	20	40	80	160
α-T	-	-	-	-	-
α-T_3	-	-	-	+	+
γ-T	-	-	-	-	-
γ-T_3	-	weakly +	+	+	+
δ-T	-	-	-	weakly +	+
δ-T_3	-	+	+	+	+

* T = tocopherol, T_3 = tocotrienol (Structures are shown in page 3). Anti-tumour promoting effects are presented as: (i) positive (+) = complete inhibition of EBV EA expression, (ii) weakly positive (weakly +) = partial inhibition of EBV EA expression, (iii) negative (-) = no inhibition of EBV-EA expression.

In order to examine the relationship between the anti-tumour promoting activity and the antioxidant activity of the vitamin E components, further experiments were carried out using dimers of γ-T_3 which are also known to possess excellent antioxidant properties [219]. The results for the anti-tumour promoting experiment of dimers of γ-T and γ-T_3 are shown in Table 5.3. Although the γ-T_3 has shown an excellent anti-tumour promoting effect, all of its dimers (i.e. γ-T_3DED, (*R*)-5,5'-bi-γ-T_3 and (*S*)-5,5'-bi-γ-T_3) as well as those derived from γ-T (i.e. γ-TDED and (*R*)-5,5'-bi-γ-T), even though presented at high doses did not inhibit the EBV-activation. These dimers retain the unchanged triunsaturated side chains, but have a modified chromanol moiety which is more substituted and bulky arising from dimerization. Although γ-T_3 showed a strong inhibition on EBV EA expression, its bulky dimers including γ-tocotrienol diphenyl ether dimer (γ-T_3DED) and (*R*)- & (*S*)-5,5'-bi-γ-tocotrienyls (structures shown in page 26) do not possess the anti-tumour promoting activity despite the fact that γ-T_3DED produce phenoxyl radicals which are several times more stable than the phenoxyl radical generated from γ-T_3. Likewise, dimers of γ-T, i.e. γ-tocopherol diphenyl ether dimer (γ-TDED) and (*R*)-5,5'-bi-γ-tocopheryl (structures given in page 26) also did not show any anti-tumour promoting activity. Present results suggest that the anti-tumour promoting activity of vitamin E components is very much dependent on their structures, the tocotrienols having an unsaturated side chain are more active than tocopherols with saturated side chain. Among the tocotrienol homologues the mono-methyl tocol, i.e. δ-T_3, is more active than the dimethyl tocol, γ-T_3, followed by the trimethyl tocol, α-T_3. The generally accepted order for the antioxidant activity of tocopherols is $\alpha > \gamma > \delta$, whereas the anti-tumour promoting activity of tocotrienols appears to be in the reverse order $\delta > \gamma > \alpha$. The present study suggests that a potential health benefit may be derived from the widespread use of palm oil as a dietary fat particularly in the Asian region in view of the relatively high incidence of nasopharyngeal carcinoma caused by Epstein-Barr virus. Moreover, it may be one of the reasons that substantial dietary intake of tocotrienols present in fruits and vegetables could

Table 5.3 Anti-tumour Promoter Activity of γ-T, γ-T_3 and Their Dimers*

Vitamin E	Concentration (μg/mL)					
	10	20	40	80	160	320
γ-T	-	-	-	-	-	N.A.
γ-TDED	-	-	-	-	-	-
(*R*)-5,5'-bi-γ-T	-	-	-	-	-	-
γ-T_3	-	weak +	+	+	+	N.A.
γ-T_3DED	-	-	-	-	-	-
(*S*)-5,5'-bi-γ-T_3	-	-	-	-	-	-
(*R*)-5,5'-bi-γ-T_3	-	-	-	-	-	-

* T = tocopherol, T_3 = tocotrienol, TDED = tocopherol diphenyl ether dimer, 5,5'-bi-γ-T = 5,5'-bi-γ-tocopheryl dimer, T_3DED = tocotrienol diphenyl ether dimer, 5,5'-bi-γ-T_3 = 5,5'-bi-γ-tocotrienyl dimer (Structures are shown in page 26). Anti-tumour promoting effects are presented as: (i) positive (+) = complete inhibition of EBV EA expression, (ii) weakly positive (weakly +) = partial inhibition of EBV EA expression, (iii) negative (-) = no inhibition of EBV EA expression. N.A. = results not available.

be protective against cancer [246,322,348] and other degenerative diseases.

5.3.2 Two-stage Skin Carcinogenesis Experiments

The anti-tumour promoting activity of tocotrienols was also studied by a two-stage skin carcinogenesis experiment. The DMBA-induced carcinogenesis on mouse skin was monitored by examining the percentage of mice carrying tumours, the results are presented in Fig 5.2. Comparing with the mice treated with γ-T_3 and δ-T_3, the number of mice carrying skin tumours were slightly higher when the mice were not treated with tocotrienol. However, the latency for tumour appearance in mice treated with γ-T_3 and δ-T_3 (both 5 weeks) was apparently shorter than that for control mice (7 weeks).

Presently, these preliminary *in vivo* results (Fig. 5.2) indicate that γ-T_3 is not an anti-tumour promoter which is inconsistent with the *in vitro* results. Treatment with tocotrienols prior to the application of tumour promoter appeared to shorten the latency period before the onset of the chemically-induced skin carcinogenesis. Previously, vitamin E (α-T) was reported to be a tumour promoter in DMBA-initiated mouse skin carcinogenesis, and it was suggested that the tumour promotional process may be triggered by reduced levels of cellular oxidant [422]. One of the difficulties in animal experiments is the difficulty in dosage under a variety of conditions in oxygen concentrations. Vitamin E is normally antioxidant but can be prooxidant under highly oxidative conditions. Different experimental conditions in the application of tocotrienols may be the explanation of the discrepancy between the cell culture experiment and the mouse skin carcinogenesis experiments. Also, the former involved chemically-mediated expression of antigens by genome-carrying cells, whereas the latter is a carcinogenesis chemically-induced on normal cells. Moreover, the optimal concentrations for an anticarcinogen to effectively prevent cancer development is an important factor and must be considered in the animal experiment. Further research is therefore needed to investigate the controversial effects of tocopherols and tocotrienols in the skin tumour-promotional process.

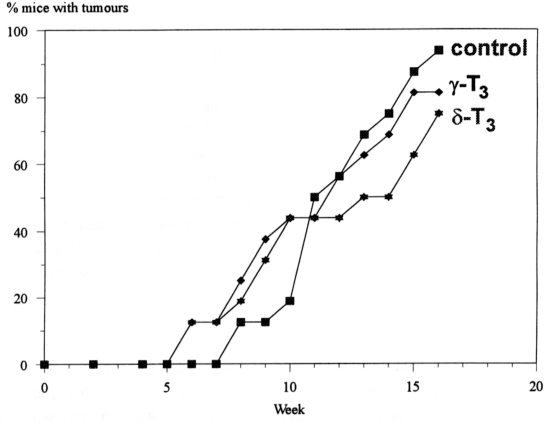


Fig. 5.2 **Effect of tocotrienols on the percentage of mice with skin tumours chemically-induced by DMBA and croton oil.**