Tumour-promoting diterpene esters of the plant family Euphorbiaceae

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The plants of the family Euphorbiaceae produce highly skin inflammatory, caustic, irritant latex and seed oil. This is mainly due to the presence of various long chain esters of saturated fatty acids, attached at C-12 and C-13 positions of the diterpene phorbol and at C-3 position of ingenol moieties. These diterpene esters are strong tumour promoters in Berenblum's experiment and are well established as carcinogens. The use of croton oil for fish killing in small ponds and consumption of bitter honey, contaminated with ingenol esters, play positive role in exacerbating the overall process of carcinogenesis, among the population exposed to these tumour promoters. This observation is well sustained from epidemiological studies.

THE earliest, and probably the most significant biological effect of croton oil, which is obtained from the seeds of *Croton tiglium* L., belonging to the family Euphorbiaceae and growing as leafy shrub in India, Ceylon, Philippines, Mauritius and China, was demonstrated by Berenblum in 1941. The weekly application to the interscapular skin of mice of acetone solutions of benzpyrene and this oil or benzpyrene and croton resin, caused significant augmentation of carcinogenesis, in comparison to that observed with benzpyrene solution alone[1]. Although such cocarcinogenic activity had previously been shown with oleic acid[2], these experiments showed that croton oil was the most potent naturally occurring cocarcinogen known.

An important step towards understanding the actions of croton oil was the observation by Mottram, that benzpyrene need only be applied once in a sub-manifestational dose to prepare the skin so that subsequent multiple applications of croton oil can elicit tumours[3]. Based on these observations Berenblum and Shubik[4] proposed two-stage theory of chemical carcinogenesis. Further refinement of this experiment lead it to be known as Berenblum's experiment[5].

The initial sub-threshold dose of the carcinogen such as benzpyrene induced an irreversible initiating process, resulting in the formation of latent tumour cells. Subsequent repeated applications of croton oil or *Euphorbia* lattices, caused an epicarcinogenic or promoting process, which enabled such latent tumour cells to develop into malignant cells.

The term cocarcinogenecity is a general one which refers to all forms of augmentation of tumour induction. The phenomenon can arise in many ways, including additive, synergistic, preparative or incomplete carcinogenic action[6]. Tumour promoters are, thus, only one type of cocarcinogen and have been classified as incomplete carcinogens since they can complete the process begun by the initiator[7–9].

It has been demonstrated experimentally that the information, 'potential tumour cells' generated by the initiator, persist in the target tissue for a very long period, i.e. for at least 2/3 to 3/4 of the average life span of mice[10]. Therefore it is reasonable to assume that the growth of 'potential tumour cells' remains under the control of the surrounding cells. If, however, after initiation the target tissue is treated repeatedly with promoter, for example, croton oil or its most active constituent: 12-tetradecanoyl-phorbol-13-acetate (TPA)[7]; the growth of the potential tumour cell is prompted to yield papillomas. These papillomas become malignant even after some time due to the inherent tendency. Therefore, in addition to the stages of tumour initiation and promotion, a third stage called tumour progression, was also postulated for mouse skin[11–14] (Figure 1).

TPA (Figure 2 *b*) and other 12,13-diesters of diterpene phorbol represented only 50% of the entire phorbol content of croton oil and the rest of the phorbol was present in the hydrophobic portion as unidentified form of phorbol-12,13,20-triester. This was demonstrated for the first time with the isolation of a new 'cryptic irritant and cocarcinogen' from the seed oil of *Croton sparciflorus*. This new substance was found to be

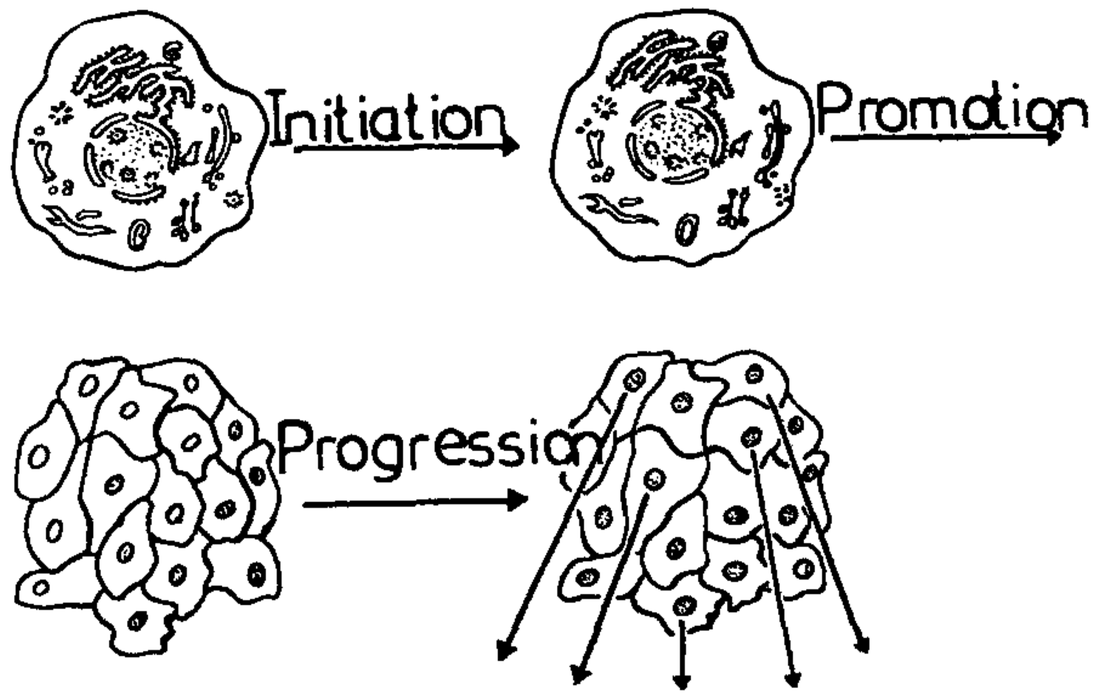
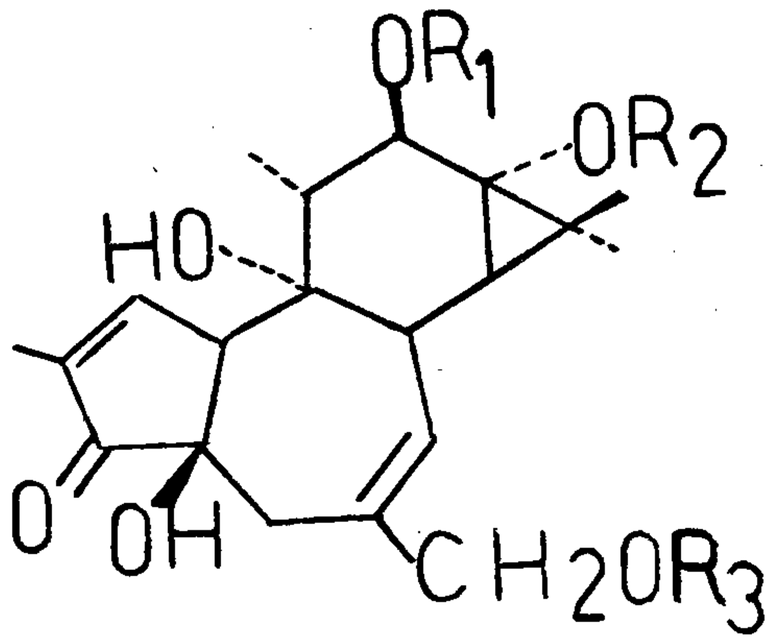


Figure 1. The three phases of tumorigenesis.

12-dodecanoyl-13-acetyl-phorbol-20-linolinate[15]. This ester of phorbol exhibited very little irritant activity as compared to the other phorbol 12,13-diesters. However, mild hydrolytic treatment of this, caused the linolinic acid attached to position 20 of phorbol moiety (Figure 2 *c*) to be released selectively to yield corresponding highly active and irritant 12-dodecanoyl-phorbol-13-acetate, which is a strong tumour promoter on mouse back skin experiment. It is likely that such activation can probably take place in tissues exposed to 12,13,20-triesters[15].

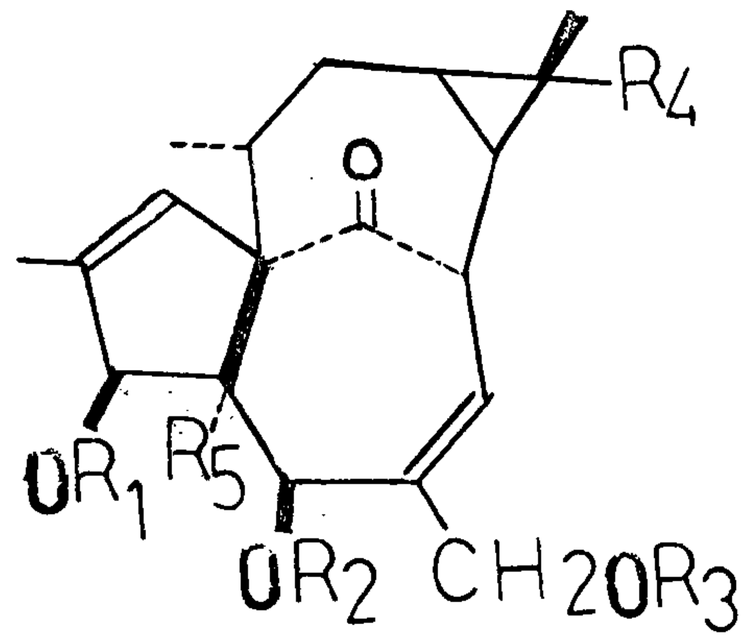


a) $R_1 = R_2 = R_3 = H$ = Phorbol

b) $R_1 = CO{-}(CH_2)_{12}{-}CH_3$, $R_2 = COCH_3$, $R_3 = H$ = TPA

c) $R_1 = CO{-}(CH_2)_{10}{-}CH_3$, $R_2 = COCH_3$, R_3 = Linolinate

Figure 2. Structure of phorbol and its esters.



$R_1 = R_2 = R_3 = R_4 = H$, $R_5 = OH$ = Ingenol

a) $R_1 = R_2 = R_3 = CH_3$, $R_4 = H$, $R_5 = OH$

b) $R_2 = R_3 = R_4 = H$, $R_5 = OH$, R_1 = Hexadecanoate

c) $R_1 = R_2 = R_3 = H$, $R_4 = CH_2OH$, $R_5 = OH$

d) $R_2 = R_3 = R_4 = H$, $R_5 = OH$, R_1 – Palmitate

e) $R_2 = R_3 = R_4 = H$, $R_5 = OH$, R_1 = Dodecanoate

f) $R_2 = R_3 = R_4 = H$, $R_5 = OH$, R_1 = Decanoate

g) $R_2 = R_3 = R_4 = H$, $R_5 = OH$, R_1 = 2-methyldecanoate

h) $R_1 = R_2 = R_3 = R_4 = R_5 = H$

Figure 3. Ingenol and its esters isolated from *Euphorbia* species growing in Azarbaijan province of Iran.

From the skin irritant and purgative seed oil of *Euphorbia lathyris* L. and from its highly skin inflammatory latex, esters of another diterpene, which is reminiscent of diterpene phorbol, called ingenol, have been isolated. Out of these esters ingenol-3-hexadecanoate had been found to be skin irritant and tumour promoting in Berenblum's experiment[16]. Further, from the skin-inflammatory and tumour promoting latex of *Euphorbia lactea*, which causes severe dermatitis and inflammation to skin, to human and animals alike, hydroxylated derivative of ingenol, called 16-hydroxy ingenol, had been isolated[17] (Figure 3 *b*, *c*). In a standard experiment on the preshaved and preinitiated back skin of NMRI mouse, 0.1 µm of 7,12-dimethyl benzanthracene in acetone produced squamous cell carcinoma[17] after 28 weeks.

The caustic, skin irritant and tumour-promoting latex of *Euphorbia serrata* L., which grows profusely around Tabriz, capital of Azarbaijan province of Iran, and the latex of *Euphorbia seguieriana* Neck. obtained from the same region, were found to contain parent diterpene ingenol[18,19]. However, in tumour-promoting experiments on preshaved back skin of NMRI mouse initiated with dimethyl benzanthracene in acetone as usual, the latex preparation of *Euphorbia serrata* produced squamous cell papilloma within a short span of 16 weeks, due to the presence of ingenol-3-palmitate in the latex sample[20] (Figure 3 *d*), whereas the latex preparation of *Euphorbia seguieriana* Neck. produced squamous cell papilloma within 14 weeks, on the back skin of NMRI mice.

The plant of *Euphorbia esula* L. is known to cause sheep mortality and produces inflammation with the loss of hairs from the feet of the horses. The latex causes blistering with severe irritation if allowed to remain on the skin and it can lead to partial blindness if dropped into the eyes[21]. From this latex highly skin inflammatory unsaturated Δ-2,4,6,8,10-pentene-tetradecanoic acid, esterified at C-3 position of diterpene ingenol has been isolated. However this ingenol ester is nontumour promoting[22] in mouse back skin experiments. From this latex another ester of ingenol, though less irritant yet tumour promoting on mice skin, was found to be ingenol-3-dodecanoate (Figure 3 *e*). This produced squamous cell papilloma in a short duration of 20 weeks[22]. *Euphorbia striatella* Boiss, which not only contained highly inflammatory latex but has various medicinal properties, was found to contain highly skin irritant, but nontumour promoting ingenol-Δ-2,4,6-decatrinoate[23]. In tumour-promoting experiments on back skin of NMRI mice, the latex promoted the formation of

squamous cell papilloma; due to the presence of ingenol-3-decanoate, within 22 weeks[23] (Figure 3*f*). *Euphorbia virgata*, which grows profusely in the Saidabad area of Tabriz, have been found to contain highly skin irritant latex, from which non skin irritant esters of diterpene ingenol, such as ingenol-3-phenyl acetate, and ingenol-3-benzoate were isolated. However, the irritant activity of the latex was represented by ingenol-3-tigliate and ingenol-3-(2)-phenyl decanoate. Out of these two ingenols 3-(2)-methyl decanoate (Figure 3 *g*) was found to promote the process of tumour formation on mouse back skin[24]. From another plant, viz. *Euphorbia megalantha* Boiss, the latex of which is moderately skin inflammatory; ingenol and a new diterpene derivative of ingenol called 4-deoxy ingenol, has been isolated[25]. The structure of this ingenol derivative is shown in Figure 3 *h*.

In continuation of our studies on skin irritant and tumour-promoting diterpene esters from the plants of *Euphorbiaceae* family, growing in Azarbaijan province of Iran[26], out of the 19 species, with curious exception of *Chrozophora tinctoria* Jass., all species were found to contain no cryptic irritant, but short chain esters of diterpene ingenol only[27]. Long chain esters were identified as usual and for the short chain esters of ingenol, gas chromotographic techniques were found highly suitable[28], after these were purified by column and thick layer chromatography in the standard way[29,30].

It has also been shown that ingenol-3,5,20-triacetate (Figure 3 *a*) which is totally ineffective as tumour promoter and weakly skin irritant produces in initiation promotion experiment lung adenoma in NMRI mice instead[31]. The seed oil of *Croton tiglium* L. is used by natives in India, for fish killing in small ponds, which contain mostly mud dwelling, air breathing, hardy fish *Heteropneustes fossilis*, along with other fish species[32]. The other plants of *Euphorbiaceae* are also employed by the tribals of Southern Rajasthan for stupefying the fish in ponds[33]. These facts prompted us to take up the freshwater fish, *Heteropneustes fossilis*, as a model to study the process of liver carcinogenesis in this species.

Further, the seed oil of the plants of the family *Euphorbiaceae* growing around Faizabad was screened for its toxic effect on *H. fossilis*, and among all oils tested, the oil of *Croton tiglium* was found to be highly toxic to this fish species[34]. Interestingly, the acetone preparations of some latex sample of the *Euphorbia* species growing in abundance around Ayodhya–Faizabad, showed strong anti-termite effects[35].

It has been demonstrated that administration of 0.1 μM of 7,12-dimethyl benzanthracene in tricaprylin, pro-os to this fish, only once, followed by oral administration of croton oil in tricaprylin repeatedly, triggered the formation of marked basophilia in the liver[36]. The formation of basophilia along with other changes in the liver were also noted when initiation has been done as recorded above and promotion was performed by pricking the liver of this fish repeatedly at fixed intervals[37]. Various cellular changes such as hyperplasia, thickening of cell wall, loss of cytoplasm, vaccuolization, nuclear displacement, and very high degree of mitosis coupled with marked basophilia, which was indicative of preneoplasia leading to hepatocellular carcinoma formation[38], were noted, when these fish were exposed under suitable conditions to 7,12-dimethyl benzanthracene (in high doses)[39], 2-acetyl aminofluorene, urethane and methyl carbamate-1-napthol[40]. The Godrej hair dye, permanent black, liquid preparation, produces carcinogenic symptoms in the liver of this fish[41], whereas edible dyes such as Sun Set Yellow and Brilliant blue, induce degenerative changes in the liver of *Heteropneustes fossilis*[42]. Interestingly, some synthetic strained ring compounds, having marked pharmaceutical applications, were also found to induce marked basophila in the liver of *H. fossilis*[43], as did HCH (ref. 44).

It is now well established that tumour promoters make up a group of compounds largely differing from one another in chemical structure that, though by themselves non-carcinogenic, enhance the development of malignant transformation in carcinogen-initiated parenchymal and stromal cells of various organs[45]. Tumour promoters also evoke a pleiotropic-hyperplastic response and several phenotypically neoplastic features in normal, i.e. non-initiated cells[46]. The complex operative biological mechanism of such agents and the relation of their manifold biological effects to the actual promotion of neoplasia are the current subjects of intense investigations. Several early metabolic events, like the induction of a pro-oxidant state and of enzymatic changes at the plasmalemal level, the stimulation of metabolism of phospholipids and of arachidonic acid, the biosynthesis of polyamines etc. seem to play a relevant role in the enhancement of the responses triggered by tumour promoters[47].

The transmembrane Ca^{2+} fluxes and events requiring the activation of Ca^{2+} binding sites, of calmodulin modulated enzymes and of ubiquitous Ca^{2+} activated phospholipid dependent protein kinase-C seemingly play a key role in the control of normal and abnormal cell proliferation and also of differentiation and functioning[48–52]. It is a well-established fact that TPA and tumour promoters of marine origin such as teleocidine, lyngbyatoxin-A and aplysiatoxin induce tumour promotion through the activation of protein kinase-C. In this process the amounts of phosphoproteins increased and then expression of early response genes such as *C-fos* and *C-jun* was initiated[53].

Thus, the early event of tumour promotion is the increase of phosphoproteins and these act as signals for gene expression resulting ultimately in induction of clonal growth of the initiated cells[54,55], and are possibly

involved in the inhibition of apoptosis in tumour promoting phase[56].

Although induction of Epstein-Barr virus by these tumour promoters of phorbol and ingenol type is well established[57–59], procedure for detection, but the development of basophilia in the liver of *H. fossilis*, can also be used for detecting the presence of these tumour promoters in polluted waters[60,61] on one side and on the other it indicates the unsuitability of such fish for human consumption, as has been the case with bitter honey, collected from Saidabad area of Azarbaijan. In Saidabad area, the plants of *E. siguieriana* grow profusely and honey bees collect the nectar from the flowers. This honey tastes bitter, as it is contaminated with the tumour promoters of ingenol type[62]. The consumption of such honey appears to be the main cause for increased risk of oesophagus cancer, in hilly regions of Azarbaijan province of Iran, a fact well sustained from the epidemiological studies[62,63].

It has also been shown that the high incidence of oesophageal cancer on the Caribbean island of Cura-cao is mainly due to the consumption of Welensali-tea, made from *Croton flavens* leaves from which phorbol derivatives exhibiting tumour promoting activity in the oesophagus have been isolated[64]. These tumour promoters contaminate indirectly milk and meat when the livestock food get mixed with the plants of Euphorbiaceae[65].

Likewise, environmental carcinogens other than those emanating from the Euphorbiaceae family, include algal microcystins, which are known to contaminate the ditch water and enhance the chances of liver cancer in certain parts of People's Republic of China[66,67], indicating the role these tumour promoters play in excerbating the total carcinogenic load of the environment.

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RESEARCH ARTICLES

Submarine terrace limestones from the continental slope off Saurashtra–Bombay: Evidence of Late Quaternary neotectonic activity

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Bathymetric and shallow seismic data from the continental slope off Saurashtra–Bombay indicate wide submarine terraces at 130, 145 and 170 m and reefal structures at 320–360 m water depths. 10 cm thick slabs of limestones are recovered from the 130 m depth terrace. Some of these limestones consist of thin micrite layer on the top and a sandy layer below and others are similar to calcarenites. They contain >95% aragonite and minor high- and low-magnesium calcite. Acicular aragonite cements occur as isopachous crusts. Dissolution and clotting of aragonite needles and drusy calcite in the interstices indicate cementation of the limestones at intertidal conditions. The age of the limestones is 11,900 years BP. These imply that the 130 m depth terrace was at intertidal depths at about 12,000 years BP. The eustatic sea-level, however, was at −90 m at 12,000 years BP. This disparity suggests neotectonic activity and subsidence by about 40 m on the Saurashtra–Bombay region some time after 12,000 years BP.

SUBMARINE terraces are important geomorphic features on the continental margins and may record former sea-levels[1]. Submarine terraces have been reported[2–4] on the western continental shelf of India between 35 and 115 m and also between 115 and 170 m water depths on the continental slope off Saurashtra–Bombay[5]. Saurashtra was tectonically unstable during the Pleistocene[6–9]. The eustatic sea-level[10] low during the Last Glacial Maxima (LGM) was only −120 m. The deeper terraces may therefore have implications to neotectonism and Quaternary sea-level changes. We report here the investigations on the limestones from a 130 m depth submarine terrace off Saurashtra–Bombay and provide evidence of Late Quaternary neotectonic activity, hitherto unknown from the western offshore.

Materials and methods

During the cruise 150 of the *R. V. Gaveshani*, bathymetric and shallow seismic data were collected from the continental shelf and slope off Saurashtra–Bombay. Sediment and limestone samples were collected with a Peterson grab (Figure 1). Mineralogy of the representative samples was determined by X-ray diffraction. Freshly broken surfaces of the limestone fragments were examined under a scanning electron microscope (JEOL T 20). Radiochemistry of the limestones was carried out at the Hydrogeology and Isotope Geochemistry Laboratory, University of Paris, Orsay. Polished and thin sections of the limestones were studied under petrographic microscope.