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# Tumor Promotion by Euphorbia Latices\*

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## SUMMARY

Numerous papillomas and two malignant tumors were elicited in 101 strain mice treated once weekly with applications of acetone extracts of latices of ten species of Euphorbia after a single dose of DMBA in acetone. Of the species tested *E. tirucalli* was the most potent. Three papillomas were seen in 40 mice treated with DMBA only and three among 100 mice treated with latices only. These results indicate the presence of potent tumor-promoting agents in the latices. With one exception, there was good correlation between degree of epidermal hyperplasia and tumor-promoting effect.

In the course of studies on the two-stage mechanism of skin tumor production in mice and rabbits, many tumor-initiating agents have been discovered. Several of the carcinogenic polycyclic hydrocarbons (e.g., 3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene), applied in subcarcinogenic doses, have been shown to initiate tumor formation. In addition, substances which are not carcinogenic for the skin, e.g. urethan and triethylene melamine, have been shown to act in this way (21, 24).

On the other hand, the choice of tumor-promoting agent has always been much more limited. Until recently croton oil was the only promoting agent of sufficient potency for reliable use in studying the two-stage mechanism in mouse skin. Many attempts to isolate the active principle of croton oil have failed (1, 2, 5, 6, 9, 26), although highly potent fractions of it have been prepared (1). Lijinsky has recently obtained crystalline products (12), but these have not yet been tested for biological activity.

Since 1953, several pure substances have been found to possess promoting activity for mouse skin; among these are iodoacetic acid (10), chloracetophenone (10), several surface-active agents (25), and many phenolic compounds including phenol itself (3). Of these pure chemical substances phenol is the most active promoter; nevertheless, it is considerably less potent than croton oil or its resin (cf. [3] and [1]). It follows that the identifica-

tion of the active principle of croton oil is an objective of considerable importance, and it is hoped that the experiments described here, in addition to any intrinsic interest they may have, will lead indirectly toward that goal.

About 18 months ago a colleague, Dr. J. S. Fawcett, of the Department of Experimental Biochemistry, London Hospital, suggested that we should test the latex of *Euphorbia ingens* for tumor-promoting activity. He had come across this material during the war, while searching for alternatives to natural rubber, which was in short supply. The latex was so irritating to human skin that it was obviously not a suitable substitute. Because of this irritant effect, and because *E. ingens* belongs to the same family as *Croton tiglium*, from which croton oil is obtained, Fawcett thought it might be interesting to test it for tumor-promoting activity.

In due course, a sample of the latex was obtained and tested. The present paper describes experiments with the latex of *E. ingens*, and also with latices from nine other species of Euphorbia.

## MATERIALS AND METHODS

*Mice.*—Male and female mice of the "101" inbred strain were used for all experiments. They were housed in groups of ten in zinc or galvanized iron cages. White wood shavings and sawdust were used as bedding. The mice were fed on cubed Diet 41B (obtained from Messrs. E. Dixon and Sons Limited, Ware, Herts.) and water ad libitum.

All mice when 6–8 weeks old were vaccinated on the tail with sheep lymph against ectromelia. Only those showing a positive reaction were used.

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*Technic of application to the skin.*—The hair of the entire dorsal area from the root of the tail to the nape of the neck was removed with electric clippers from all mice before the beginning of the experiment and thereafter at 2- to 3-week intervals.

All test substances were applied to the skin from calibrated pipettes, care being taken to ensure that the solution spread evenly over the entire clipped area.

*Recording of tumors.*—All mice were examined at fortnightly intervals for tumors of the skin and other organs. Sick mice were killed and, together with those found dead, examined post mortem.

*Histology.*—Specimens of skin, benign and malignant skin tumors, and other tissues were examined histologically. Either Zenker's fluid or formol saline was used as a fixative, and the sections were stained with hematoxylin and eosin.

*Chemicals.*—9,10-Dimethyl-1,2-benzanthracene (DMBA) was obtained from L. Light and Co., and acetone (Analar grade) from British Drug Houses Ltd.

The *Euphorbia* latices were obtained from three sources.<sup>1</sup> The collection of latex from the succulent species presented little difficulty, since it flowed freely from the incised stems. Collection from the nonsucculent, *E. wulfenii*, was far more tedious: only the cut tip of the stem could be used, and each stem provided no more than 3 small drops of latex. Rubber gloves were worn to protect the hands during collection.

*Preparation and storage of extracts.*—The samples sent from South Africa were preserved by the addition of a few drops of toluene immediately after collection. Shortly after receipt, 5 per cent acetone extracts were prepared from the semi-solid latices by grinding in a pestle and mortar, adding acetone, shaking, and finally filtering. The resulting extracts were stored in amber-glass bottles at 4° C. The latex of *E. ingens*, and all those obtained from The Royal Botanic Gardens, were liquids. Five per cent extracts of these were prepared in the same way, except that grinding was unnecessary. More dilute solutions were made up from the stock solutions as required.

Samples of the extracts left in the light at room temperature changed from colorless to red-brown

in a few weeks, whereas none of the stock solutions showed any visible deterioration after 30 weeks at 4° C. in the dark.

## RESULTS

*Experiment I: Euphorbia ingens.*—Preliminary tests were carried out to see whether the latex was irritating to the skin of mice. Concentrations in acetone above 10 per cent caused complete necrosis within 24 hours. A 10 per cent acetone extract caused patchy necrosis of the skin, which took up to 3 days to become manifest to the naked eye. Damage to the skin was most marked when the skin was in the resting phase of the hair cycle. Histologically, there was marked epidermal hyperplasia in those areas which had not undergone necrosis. Five per cent and 1 per cent acetone extracts caused less necrosis and more hyperplasia. A 0.1 per cent extract caused hyperplasia without necrosis.

After treatment with a tumor-initiating agent, mice tend to develop skin tumors at the margins of healing wounds, irrespective of how the wounds are caused (13, 15, 16, 19, 23). Therefore, in testing chemical substances for tumor promotion it is desirable to avoid excessive damage to the epidermis. With this in mind, caution was exercised in testing the latex of *Euphorbia ingens* for tumor promotion: treatment was begun with a 0.1 per cent solution.

*Test of the latex of E. ingens for tumor-promoting activity and carcinogenicity.*—Three groups of mice were used in the main experiment. Each group consisted of ten males and ten females. Groups I and III received a single application of 0.2 ml. 0.15 per cent DMBA in acetone (300 µg.) to the clipped dorsal skin. Mice of Group II were clipped but not treated. Three weeks later mice of Groups I and II began to receive once-weekly applications of an acetone extract of *E. ingens* latex. The concentration of the latex was held at 0.1 per cent for 3 weeks, and then, since there was no macroscopic damage, increased to 0.2 per cent. After a further 3 weeks at this level, it was increased to 0.5 per cent for 7 weeks. During this period the first papillomas appeared. The concentration was increased to 1 per cent during the 14th week of secondary treatment, and to 2 per cent for the 15th and 16th weeks. However, the 2 per cent solution caused patchy necrosis, and so the concentration was reduced again to 1 per cent and held there from the 17th to 32d weeks. Except for periodic clipping, mice of Group III received no treatment after the initial single application of DMBA.

Papillomas began to appear on the backs of mice of Group I during the 10th week of treatment with the extract. The incidence of papillomas

<sup>1</sup> Dr. P. R. Enslin, National Chemical Research Laboratories, Private Bay 191, Pretoria, South Africa, kindly supplied samples of *Euphorbia ingens*. Dr. R. A. Dyer of the Department of Agricultural Technical Services, Pretoria, South Africa, sent the samples of latex from *E. tirucalli*, *E. grandidens*, and *E. triangularis*. The remaining latices were obtained from the Royal Botanic Gardens, Kew, Surrey, through the courtesy and cooperation of the Director, Dr. G. Taylor, and of Dr. F. N. Howes.

steadily increased, and by the 26th week twelve of the twenty survivors bore a total of 54 papillomas (see Table 1).

In contrast, no tumors were seen on the backs of nineteen survivors from the group treated with DMBA only (Group III); and only one arose among the seventeen survivors treated with the latex extract only (Group II).

During subsequent observation four small papillomas arose on the skin outside the treated area in Group III. The occurrence of such ectopic tumors in greater numbers following treatment with DMBA only, as compared with DMBA plus a promoting agent, has been observed previously (17).

No malignant skin tumors were seen in this experiment. However, malignant tumors rarely occur before the 36th week in this kind of experiment (17), and survival beyond the 36th week was very poor. The main cause of death was the renal dis-

of 0.3 ml. of 5 per cent latex with an interval of 1 week between applications, and the remaining four were given two similarly spaced applications of 1 per cent latex. Ulceration and crusting of the skin occurred, and specimens of skin were removed from the treated areas of all mice and examined histologically. Eventually all the skin lesions healed, their place being taken by persistent hairless scars. After an interval of 6 weeks all mice began an 18-week course of 0.3 ml. 0.1 per cent croton oil in acetone. Such a course of croton oil treatment regularly elicits numerous papillomas in mice previously initiated with DMBA, urethan, or other initiators. In the present experiment, however, not a single tumor arose during the whole course.

It was concluded that treatment with the latex of *E. ingens* did not initiate tumor formation under the conditions described.

TABLE 1  
TUMOR-PROMOTION BY LATEX OF *E. ingens*

Group	Primary DMBA treatment (μg.)	Secondary treatment* with <i>E. ingens</i> (per cent)	Survivors at 26 wk.†	No. mice with papillomas	Total no. papillomas
I	300	0.1-2.0	20	12	54
II	None	0.1-2.0	17	1	1
III	300	None	19	0	0

\* Once weekly applications of 0.3 ml. each. For details of concentrations, see text.

† Groups consisted of twenty mice at the beginning of the experiment.

ease, papillonephritis, described by Gorer (8) and further studied by Dunn (7). The occurrence of this disease in our colony of 101 strain mice has already been recorded (22); it is apparently unrelated to treatment. The disease is a serious handicap in long-term experiments, and we are currently endeavoring to eradicate it. One mouse of Group I died of lymphatic leukemia 10 months after the beginning of treatment. It is not thought that the *Euphorbia* latex had anything to do with its causation, since lymphatic leukemia is not infrequently seen in mice treated with DMBA only.

The results of these tests indicated clearly that the latex of *Euphorbia ingens* contains a potent tumor-promoting substance.

*Test of latex of E. ingens for tumor-initiating activity.*—Sixteen male mice were used in this test. These mice had been used in the preliminary tests of the latex for irritant effect on the skin. Four had received a single drop of crude latex and four a single application of 0.3 ml. 10 per cent latex in acetone. A further four received two applications

*Experiment II: Euphorbia tirucalli and eight other species.*—As in the case of *E. ingens*, preliminary tests were carried out to assess the irritant properties of the nine latices. All were tested at a concentration of 1 per cent, four mice being used in each test. Two applications of 0.25 ml. each were given with an interval of 1 week between them. Three days after the second application a specimen of dorsal skin was taken from each mouse for microscopic examination. Table 2 lists the nine latices alphabetically and, in the third column, shows the number of cell layers in the epidermis expressed as an average for the four mice in each test. As will be seen from the table, all the latices produced epidermal hyperplasia, some very marked, some only slight. In addition, ulcers, scabs, and, later, scars were seen in mice treated with *E. canariensis*, *E. candelabrum*, *E. obovifolia*, and *E. tirucalli*. Despite these apparent differences in response it was decided to test all the latices at the same concentration. It would have been very laborious to adjust the concentrations to

give equal degrees of hyperplasia, and in any case one of our basic interests was to see whether there is a correlation between the level of epidermal hyperplasia produced and tumor-promoting power.

One hundred and twenty male and 120 female mice were used in the main experiment. Mice of each sex were allotted at random into twelve batches of ten, and one batch of each sex was used for each test group.

Groups IV to XII and Group XV received a single application of 150  $\mu$ g. DMBA in 0.1 ml. acetone to the clipped dorsal skin on the first day of the experiment. Groups XIII and XIV were clipped at this time but not treated. Three weeks

later Groups IV–XIV, inclusive, began to receive once-weekly applications of 0.25 ml. of the extracts of the latexes as shown in Table 3. Groups XIII and XIV, which had not been previously treated with DMBA, were tests for carcinogenicity of the latexes of *E. cooperi* and *E. triangularis*. Group XV was a control group which received treatment with DMBA only.

As in experiment I, treatment with the latexes was begun cautiously, to avoid, as far as possible, necrosis and ulceration of the skin. A concentration of 0.2 per cent in acetone was used for the first and second applications, 0.5 per cent for the third and fourth, 0.75 per cent for the fifth and sixth,

TABLE 2  
HYPERPLASTIC RESPONSE OF MOUSE EPIDERMIS TO VARIOUS EUPHORBIA LATEXES

GROUP	SUBSTANCE	DEGREE OF EPIDERMAL HYPERPLASIA: No. OF CELL LAYERS			ASSESSMENT OF HYPERPLASTIC RESPONSE IN ARBITRARY UNITS
		3 days after 2d applica- tion	3 days after 18th applica- tion	3 days after 2d applica- tion with solution stored for 18 weeks	
IV	<i>E. abyssinica</i>	3	2-3	3	+
V	<i>E. canariensis</i>	4	6	2-3	+++
VI	<i>E. candelabrum</i>	3	4	3	++
VII	<i>E. cooperi</i>	3	3	3	+
VIII	<i>E. grandidens</i>	3	4	4-5	+++
IX	<i>E. obovalifolia</i>	4-5	7	6	++++
X	<i>E. tirucalli</i>	5	5	5-6	+++++
XI	<i>E. triangularis</i>	1-2	2	3	+
XII	<i>E. wulfenii</i>	4	2	3-4	++

TABLE 3  
TUMOR-PROMOTING EFFECT OF VARIOUS EUPHORBIA LATEXES AND ITS RELATION TO  
HYPERPLASTIC RESPONSE

Group	Primary treatment with DMBA ( $\mu$ g.)	Secondary treatment	Hyperplastic response*	Survivors at 26 wk.	No. mice with papil- lomas	Total no. papillomas
IV	150	<i>E. abyssinica</i>	+	19	10	28
V	"	<i>E. canariensis</i>	+++	17	10	103
VI	"	<i>E. candelabrum</i>	++	19	15	68
VII	"	<i>E. cooperi</i>	+	18	3	4
VIII	"	<i>E. grandidens</i>	+++	16	13	163
IX	"	<i>E. obovalifolia</i>	+++++	20	7	40
X	"	<i>E. tirucalli</i>	+++++	15	15	358
XI	"	<i>E. triangularis</i>	+	20	1	1
XII	"	<i>E. wulfenii</i>	++	18	11	69
XIII	None	<i>E. cooperi</i>	+	19	0	0
XIV	"	<i>E. triangularis</i>	+	19	0	0
XV	150	None		20	0	0
XVII†	None	<i>E. tirucalli</i>	+++++	20	2	2
XVIII†	"	<i>E. grandidens</i>	+++	17	0	0

\* See Table 2.

† Not contemporaneous with Groups IV–XV (see text).

and thereafter a 1 per cent solution. During the latter part of the experiment considerable thickening of the skin and some superficial ulceration and crusting occurred in the groups treated with the latices of *E. canariensis* (Group V), *E. obovalifolia* (Group IX), and *E. tirucalli* (Group X). It is interesting, however, that these macroscopic signs of irritation were mild compared with those seen in mice treated with the same concentration (i.e., 1 per cent) but without previous applications of lower concentrations (*vide supra*). These superficial lesions healed without scar formation, and it is not thought likely that they played any specific tumor-promoting role.

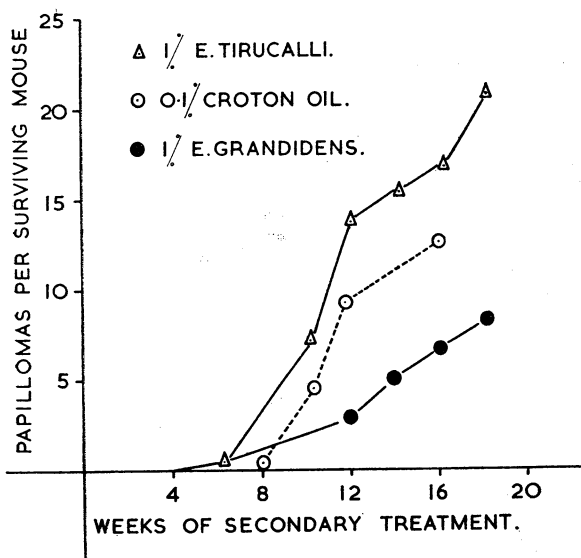


CHART 1.—Development of papillomas in mice treated with an initiating dose of DMBA followed by (a) *Euphorbia tirucalli* latex, (b) *Euphorbia grandidens* latex, and (c) croton oil.

Papillomas began to appear between the 6th and 16th weeks in all the test groups (i.e., groups treated with DMBA and latex), and the incidence of tumors tended to increase steadily as treatment was continued. However, the rate of increase differed markedly in the different groups. The incidence of papillomas after 26 weeks of treatment ranged from only one on twenty survivors in the case of *E. triangularis* (Group XI) to 358 on fifteen survivors in the case of *E. tirucalli*. Table 3 shows the incidence of tumor-bearing mice and of papillomas in all groups at 26 weeks, and Figure 1 shows a mouse from Group X bearing multiple papillomas. It is unfortunate that the two latices chosen for test for carcinogenicity (Groups XIII and XIV) were the two with the least promoting activity. The latices of *E. tirucalli* and *E. grandidens* were tested for carcinogenicity in a subse-

quent test (see below). A mouse in Group XV (DMBA only) developed two papillomas on the treated area, and one outside it, after the 46th week.

Chart 1 shows the rate of accumulation of papillomas in response to treatment with the latices of *E. grandidens* and *E. tirucalli*. For comparison, papilloma development in a comparable group of 101 mice from a parallel experiment begun 3 weeks later, and treated with 150  $\mu$ g. DMBA followed by once-weekly applications of 0.1 per cent croton oil, is also shown. These results indicate that 1 per cent acetone extracts of these two latices have promoting activity of approximately the same order as a 0.1 per cent solution of croton oil.

Treatment was stopped after 26 weeks in the case of Groups X and XII, after 30 weeks in Group V, and after 37 weeks in Groups IV, VI, VII, VIII, IX, XI, XIII, and XIV. Thereafter mice were inspected regularly for the development of malignant tumors.

A squamous-cell carcinoma infiltrating the panniculus muscle arose in Group IX (*E. obovalifolia*) during the 50th week (Fig. 3). Two similar tumors arose in Group VI (*E. candelabrum*) during the 37th and 44th weeks; both showed infiltration of dermal collagen, but had not reached the muscle layer (Fig. 2).

As in the experiment with *E. ingens* survival of mice into the period when malignant tumors might have been expected was extremely poor in all groups because of a high incidence of papillo-nephritis. In the 37th week when the first malignant tumor was seen, only 54 out of 120 mice were living in the six groups which bore the most benign tumors. This number was reduced to 33 by the 44th week when the second malignancy arose, and 2 weeks later was only seventeen. Clearly this poor survival of mice rendered the present experiment of little value as a study of the power of *Euphorbia* latices to promote malignant tumor formation.

*Tests for carcinogenic action of latices from E. tirucalli and E. grandidens.*—Two groups (Groups XVI and XVII), each of ten male and ten female mice, were treated once weekly with 0.25 ml. of a 1 per cent acetone extract of *E. tirucalli* and *E. grandidens*, respectively. The experiment is in its 40th week, and treatment is being continued.

One papilloma arose on each of two mice in Group XVI during the 17th and 28th weeks, respectively. The induction of benign tumors by treatments with potent promoting agents alone presents special difficulties in interpretation—e.g. croton oil (4, 18). This result is therefore equivocal.

*The relation between production of hyperplasia and tumor promotion.*—On the whole, the tumor-

promoting effect seemed to be directly related to the degree of hyperplasia seen in specimens of epidermis taken 3 days after a second application of latex solution. Only in the case of *E. obovalifolia* (Group IX) was there a marked discrepancy—the tumor yield being relatively small whereas the hyperplastic response was great.

To investigate this discrepancy further, histological studies were undertaken. Two possibilities were explored. First, it was possible that the stock solutions of latex extracts had deteriorated during storage while the experiments were in progress. Tests were therefore made on previously untreated mice, with 1 per cent extracts which had been stored at 4° C. in the dark for 18 weeks. There was a close correspondence between the hyperplasia produced in these tests and that produced by the fresh extracts (see Table 2, columns 3 and 5). Secondly, we knew from macroscopic observations that during a long course of weekly applications mice steadily become inured to the effects of chemical irritants, and we considered the possibility that the development of tolerance to the irritant properties of latices might occur more readily in some cases (e.g., *E. obovalifolia*) than others. Therefore, during the 18th week of treatment biopsies of treated skin were taken from two mice in each group. Table 2, column 4, indicates that these specimens showed sometimes more, sometimes less, hyperplasia than did mice which had received only two applications of either the fresh or stored latices. In the case of *E. obovalifolia* there was still considerable hyperplasia after the 18th application, and the relatively low tumor yield was therefore not due to the development of tolerance to the hyperplastic effect of the latex.

There is at present, therefore, no satisfactory explanation for the discrepancy between the hyperplastic and tumor-promoting effects of this particular latex.

## DISCUSSION

Acetone extracts of ten *Euphorbia* latices elicited skin tumors in mice pretreated with a subcarcinogenic dose of DMBA, whereas mice treated with the same dose of DMBA only developed no tumors. A small group of mice treated with *E. ingens* initially, and then repeatedly with croton oil, developed no tumors.

Only three papillomas were seen in 100 mice treated with latices only for up to 40 weeks.

A wide range of promoting activity was observed. The tumor-promoting effect of a 1 per cent acetone extract of the latex of *E. tirucalli* was of the same order as that of a 0.1 per cent solution of croton oil; other latices were less effective.

Only three malignant tumors were seen in the experiments reported. However, survival into the period when malignant tumors might have been expected (17, 20, 22) was extremely poor. If this fact is taken into consideration, it appears probable that the capacity of these latices to promote malignant tumor formation is similar to that of croton oil and other promoting agents. Further experiments to establish this point with the use of a different strain of mice are planned.

At present our knowledge of the chemical constitution of the *Euphorbia* latices is limited. Prof. F. L. Warren and his associates have reported the isolation of Euphol, taraxasterol, euphorbol, and tirucallol (11, 14). However, none of these substances is likely to be the active ingredient since, according to Warren,<sup>1</sup> none of them is an irritant for the human skin.

Two important questions arise as result of these findings: is the active principle the same for all the latices, and, if so, is it the same as that of croton oil? At the present time neither of these questions can be answered. The chemistry of croton oil, although studied by several workers (1, 2, 5, 6, 9, 12, 26) is still only at the stage of the biological testing of different fractions: pure substances have not yet been identified or tested. Boehm (2) considered that the active constituent of the dried juice of *Euphorbia resinifera* is a gum resin similar to croton resin.

Future coordinated chemical and biological studies on the *Euphorbia* latices may perhaps result in the discovery of a powerful promoting substance in pure form. At the same time light may be thrown on the nature of the active principle of croton oil.

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Fig. 1.—Papillomas on a mouse of Group X treated for 26 weeks with 1 per cent *Euphorbia tirucalli* latex after a single application of DMBA.

Fig. 2.—Malignant tumor on a mouse of Group VI treated for 37 weeks with 1 per cent *Euphorbia candelabrum* latex after a single application of DMBA.

Fig. 3.—Squamous carcinoma from a mouse of Group IX,

treated once with DMBA and then once weekly for 37 weeks with 1 per cent *Euphorbia obovalifolia* latex. It first appeared malignant 13 weeks after the end of treatment.

The panniculus muscle (right) has been penetrated and destroyed by tumor tissue. Collections of pigment cells such as those seen in the dermal collagen layer (top left) commonly occur in 101 strain mice treated with DMBA.



