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Author(s)	YAMAMOTO, Kohtarō; KIMURA, Yanagi
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**Cytological Effects of Chemicals on Tumors, XXV.
Further Studies on the Effect of *Diphylleia grayi*
Extracts on Tumor Cells^{1),2)}**

By

Kohtaro Yamamoto and Yanagi Kimura

Zoological Institute, Hokkaido University

(With 2 Text-figures and 2 Tables)

Diphylleia grayi is a kind of the Podophyllaceae plants indigenous to Japan. The species of the Podophyllaceae are few in number with a distribution within extremely limited areas. Some plants of this family have been proved to contain colchicine-like antimitotic substances; podophyllin, podophyllotoxin, alpha- and beta-peltatin, quercetin, and sikkimotoxin, well established oncolytic agents, were all isolated from American and Indian species of this family (Hartwell 1947, Hartwell and Detty 1948, 1950, Leiter *et al.* 1950, Chatterjee 1952, Kelly and Hartwell 1954). Very recently, one of the authors (Kimura 1963b) found that the crude extract from *D. grayi* had powerful antitumor action on some transplantable animal tumors. According to her results, the action of the extract was similar to, but more powerful than that of podophyllin and colchicine, producing a marked regression of tumors.

The present work was undertaken with a view to examine a possible antitumor action of the extract on cells in tissue culture, as well as *in vivo* effects of four fractions from the plant on tumor cells.

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Materials and Methods

1) HeLa cells grown in tissue culture were selected as material for the study of *in vitro* effects of the saline extract of *D. grayi*. The tissue culture technique and the method for preparing the plant extracts used in the present experiments were essentially the same as

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those described previously (Kimura 1963a, b). Two to three days after the onset of subculture of HeLa cells, the extract was applied to the nutrient medium at concentrations of 0.5, 1, 2, 10 and 20mg/ml for 48 to 72 hours. Cells were fixed and stained after the method of Jacobson and Webb (1952) at appropriate intervals following the application of the extract.

2) The MTK-sarcoma III cells transplanted in rats of Wistar and Fisher strains were used for this study. The saline-extract and other fractions were prepared by the method illustrated in Figure 1. On the 3rd day of tumor transfer, fractions were injected into peritoneal cavities of rats at concentrations as listed in Table 2. Ascites tumor cells were drawn out at appropriate intervals following the treatment and then squashed with acetic dahlia for cytological observations.

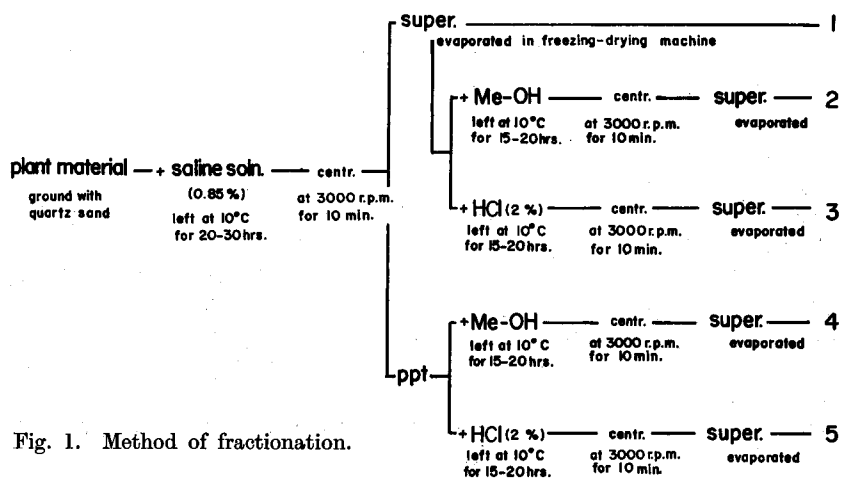


Fig. 1. Method of fractionation.

Results of Observations

1) Effects of the saline-extract of *D. grayi* on HeLa cells: As was observed previously in *in vivo* experiments, the number of dividing cells in extract-treated cultures increased strikingly as compared to that of untreated cultures. Table 1 shows the effect of the extract on HeLa cells. The increase in percentage of dividing cells is remarkable, with the increase in the number of metaphases in extract-treated cultures. The metaphases showed typical c-mitotic figures; that is, contraction, stickiness and scattering of the chromosomes. In general, almost all dividing cells were arrested at metaphase within 24 hours of treatment. Figure 2 shows the accumulation of mitotic cells subsequent to the addition of the extract to 2 day-cultures during 96 hours of treatment. It was observed that the number of metaphasic cells showed an increase until 48 hours following the treatment, and then a gradual decrease. The following chro-

mosome abnormalities were found in the later stages of the treatment: the chromosomes of affected cells formed irregular masses after condensation. In some instances, these masses stuck together, to form pycnotic aggregation of

Table 1. Effect of saline extract from *D. grayi* on HeLa cells, in 24 hour-treatment

Concentration mg/ml	Mitotic index	Percentage of dividing cells		
		Prophase	Metaphase	Ana-telophase
Control	3.2 (65/2041)	30.8	58.5	10.7
0.5	7.2 (149/2067)	22.8	57.0	20.2
1.0	23.2 (467/2011)	4.5	95.5	0
2.0	25.2 (539/2060)	2.8	97.2	0
10.0	27.8 (576/2073)	4.0	96.0	0
20.0	41.7 (847/2032)	2.4	97.6	0

chromatin. Such destructive actions of the extract were much severer upon HeLa cells than upon transplantable ascites tumors, as studied previously by one of the authors (Kimura 1963b). It was also found that the higher the dosage, the greater

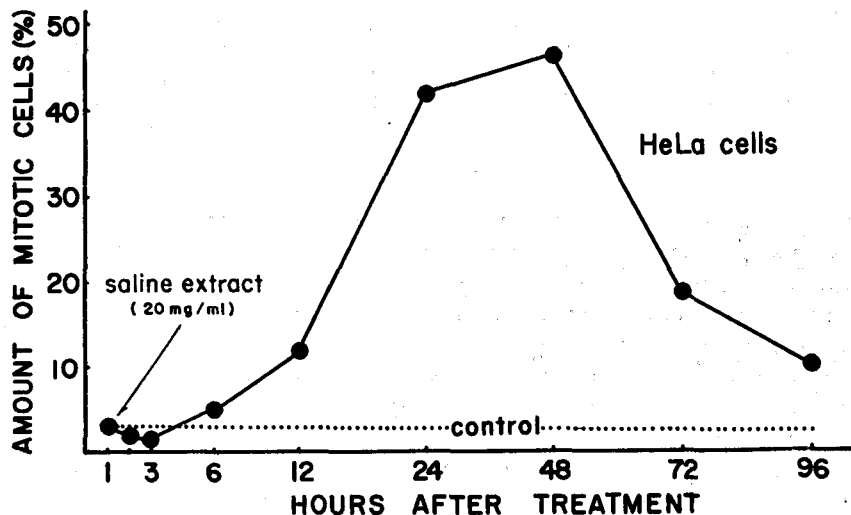


Fig. 2. Curve showing mitotic index of HeLa cells following exposure to saline extract from *D. grayi*.

the damage produced. However, the response pattern of HeLa cells remained unchanged at different doses of the extract. There was no evidence of abnormal chromosome configurations, such as chromosome translocation, fragmentation or bridges at the concentrations tested with this cell line.

2) Effects of some fractions of *D. grayi* on the MTK-sarcoma III: In the MTK-sarcoma III cells, cytological changes produced by the saline extract and four kinds of fractions (Fig. 1) were virtually identical, but the effect as an antimitotic agent was greater in the saline extracts than in the other fractions. Within 9 hours of treatment, most of the mitotic cells under the influence of these fractions were arrested at metaphase. High occurrence (33%) of metaphasic cells was seen in materials treated with fraction 2. Fractions 3, 4 and 5 were less effective in a decreasing order, showing 30, 26 and 19 percent at the peak of mitotic incidence, respectively, while 50 percent of the cells were at metaphase when treated with the saline extract (Table 2). The minimal effective dose, in a single injection was in the range of 4 to 7 mg per 100g in the four fractions, while 3 mg per 100g in the saline extract.

Table 2. Effects of alcohol and acid fractions on MTK-sarcoma III

	Minimum tolerated dose (mg/100g)	Mitotic index	% of metaphase cells	Life span
Control	—	3.5	57.4	8.9 (8-9)
Fraction 1 (Saline extract)	3	50.1	98.4	14.1 (9-24)
Fraction 2	4	33.3	97.7	9.3 (8-18)
" 3	4	30.2	98.3	10.1 (7-13)
" 4	5	26.4	96.0	9.7 (4-14)
" 5	7	19.4	94.1	8.3 (6-10)

Discussion

Among agents having colchicine-like effects, remarkable are podophyllin, an extract of the powdered roots of *Podophyllum peltatum*, and its allies (King and Sullivan 1946, Belkin 1948, Ormsbee *et al.* 1947, Greenspan *et al.* 1950, Leiter *et al.* 1950, MacCardle 1951, Makino and Cornman 1953, Makino and Tanaka 1953, Tanaka *et al.* 1955, Biesele 1958).

Recent work on chemical plant taxonomy by Hegnauer (1962, 1963) stimulated a search for podophyllin-like antitumor substances in the other species of the Podophyllaceae.

In Japan only two species of the family are found; the one is *Diphylleia grayi* and the other, *Glauucidium palmatum*. Saline extracts from these two species were tested against transplantable animal tumors *in vivo* in the previous study (Kimura 1963b). The results showed that extracts from *D. grayi* contained highly active antimitotic substances, while those from *G. palmatum* did not. Roots, stems, leaves, and berries of *D. grayi* all contained active substances, but extracts from the berries were the highest in toxicity. The leaves, from which extracts are easily made, contained the highest proportion of the substances, least in toxicity. In the present experiments, saline extract was prepared from leaves of *D. grayi*.

It was found in the present study that the abnormalities induced in HeLa cells by the extracts were morphologically identical with those observed previously with transplantable ascites tumors: they were represented by blockage of mitosis at metaphase, shortening and scattering of metaphase chromosomes and subsequent disintegration of tumor cells. Metaphase arrest was the most prominent feature of all abnormalities, and in that respect the extract was found to act more severely on HeLa cells than on ascites tumor cells.

A preliminary trial was made in the present study to prepare more purified substances with potential chemotherapeutic effectiveness from *D. grayi*. Firstly, methanol and acid fractions of the saline extract were prepared, and the efficiencies were compared with the original saline extract at the cytological level. It was found that although single injection of these fractions and the saline extract produced similar morphological damages to tumor cells, the fractions were much less effective than the extract. Secondly, methanol and acid fractions were prepared from the residue of saline extraction: they were found still less effective than the fractions of the saline extract.

Further trials to prepare more effective and purified fractions are now in progress.

Summary

The present study deals with some cytological effects of saline extract from *D. grayi* on HeLa cells, and with the efficiency of methanol and acid soluble fractions of the plant on the MTK-sarcoma III.

The saline extract was found to have powerful antimitotic effects on HeLa cells.

Methanol and acid soluble fractions prepared were found to contain antimitotic substances, though less effective than the saline extract.

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