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CROWN GALL TUMOR TEST FOR ANTICANCER ACTIVITY OF PROTEIN EXTRACTS OF *VISCUM ALBUM* L.

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The crown gall tumor test system on potato tuber discs was used for the study of antiumor activity of mistletoe protein extracts. By estimating the number and weight of tumors we found that crude mistletoe protein extract inhibits tumor induction and growth. The refined protein fraction was even more efficient. The mistletoe proteins also showed stimulative effects on rhizogenesis.

Introduction

Mistletoe has been used as a drug against different diseases in popular medicine for a long time, but the systematic research into anti-neoplastic activity of mistletoe extracts started only recently. Biological and anti-tumor activity of many protein fractions from mistletoe extract has been thouroughly investigated by Vester et al. (1968 a, b), who determined optimal conditions for the isolation of easily denatured active components — basic proteins similar to histones.

Lectin from *Viscum album* L. inhibits protein synthesis in a lysate of rabbit reticulocytes (Stirpe et al. 1980), while Helixor^{R}, representing the whole mistletoe extract, reduces the growth of permanent human and mouse cell lines, to a much greater extent than the growth of cells with limited capacity for propagation (Ulrich and Mechelke 1980). In medical treatment of unoperable colo-rectal tumors Helixor^{R} can also be applied with success (Denck and Karrer 1980).

Abreviations: Rubisco = ribulose-1,5-bis-phosphate carboxylase/oxygenase

Konopa et al. (1980) separated five cytotoxic components from the basic polypeptide fraction of *Viscum album* L. Cytotoxic viscotoxins bind to DNA and, according to Woynarowski and Konopa (1980), the interaction between the viscotoxin IVb and the DNA molecule has some features in common with the interaction of various histones with DNA.

In vitro culture of crown gall tumor tissue of Boston ivy *(Parthenocissus tricuspidata)* has been recently proposed as a convenient system for testing new anticancer drugs (Vervoitte 1984).

The aim of the present work was to assess the potential of another crown gall tumor test system — potato tuber discs (Anand and Heberlein 1977) — in the study of biological and antitumor activity of mistletoe protein extracts.

Material and Methods

Preparation of mistletoe protein extracts. Protein extracts of ***Viscum album*** were prepared by the method described by Vester et al. (1968a), and modified slightly in our laboratory. Young stems and leaves were frozen in liquid nitrogen and homogenized into fine powder. Soluble proteins were extracted with cold 0,15 mol · l^{-1} NaCl solution at a ratio of 3 ml of saline per 1 g of mistletoe powder. The suspension was centrifuged at 10 000 g for 20 minutes at 4° C, the supernatant was used as the crude extract or fraction /00/. The refined fractions /12/ and /16/ were further prepared as outlined in Table 1.

Crown gall tumor test system on potato tuber discs. Potato tuber discs were inoculated according to Anand and Heberlein (1977), either with pure culture of *Agrobacterium tumefaciens* ($B_{6/804}$) or with mixtures

Table 1. Flow diagram of major steps in the extraction of mistletoe proteins

Leaves and young stems	
↓	freezing in liquid nitrogen grinding in precooled porcelain mortar
Fine powder	
↓	extraction in 0.15 mol · l^{-1} NaCl
Dense paste	
↓	centrifugation (10,000 g, 20 min)
Supernatant =	crude extract or *fraction /00/*
↓	salting out with 1.0 mol · l^{-1} $(NH_4)_2SO_4$ centrifugation (10,000 g, 30 min)
Supernatant	
↓	salting out with 1.9 mol · l^{-1} $(NH_4)_2SO_4$ centrifugation (10,000 g, 30 min)
Pellet	
↓	dissolved and dialysed against distilled water
Retentate	
↓	centrifugation (20,000 g, 30 min)
Pellet	
↓	salting in with 0.1 mol · l^{-1} $(NH_4)_2SO_4$
Faction /12/	
↓	desorption of pigments on DEAE-Sephadex
Eluate = *Fraction /16/*	

of bacteria and various mistletoe extracts. The titer of bacteria in the inoculum was 10^8 cells per ml. The protein concentration in the extracts was estimated photometrically according to Bradford (1976). The mistletoe protein extract ranging in concentrations from 0.5 — 1.0 mg per ml, does not effect the growth of bacteria. The whole procedure was performed under axenic conditions.

Gel electrophoresis. The electrophoretic investigations were done on polyacrylamide gels at pH 8.9 according to the method of Davis (1964) and Ornstein (1964). The gels were stained in 0.01% Coomassie brilliant blue solution and destained by diffusion. Densitometric recordings were carried out in a Joyce Loebl, Chromoscan MK II Densitometer using fine resolution attachment and a Coomassie brilliant blue complementary filter.

Results

The protein extracts were prepared from mistletoe plants collected from three different host trees: apple, locust and willow during winter months in the surroundings of Zagreb.

The inhibitory effects of mistletoe protein extract, applied simultaneously with *Agrobacterium tumefaciens* ($B_{6/804}$) to potato tuber discs is presented in Table 2.

The crude protein extract mildly inhibits tumor induction (Table 2). The inhibition of tumor induction expressed in the number of tumors per tuber disc, seems to correlate with tumor growth, expressed by the total tumor mass.

The inhibitory effect depends on the total soluble protein content (Table 3) as well as on the mistletoe host tree.

It has also been observed that mistletoe protein extract stimulates rhizogenesis in the tumor tissue (Fig. 2.6). The frequency of root differentiation increased from 9% in the control group to 20% observed in the group where the mistletoe protein fraction /16/ was added to potato tuber discs, supressing the number and the mass of tumors.

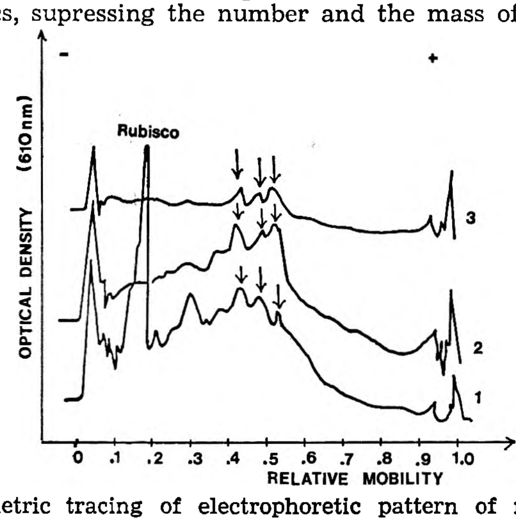

Fig 1. Densitometric tracing of electrophoretic pattern of mistletoe protein extracts
1 — crude extract (fraction /00/, 2 — fraction /12/, 3 — fraction /16/.

Table 2. Effect of mistletoe crude extract — fraction /00/ on the induction and growth of crown gall tumors on potato tuber discs.

Inoculum	Number of tumors per disc	Weight of total tumor mass per disc in g
B_6	35.25 ± 7.1[d]	0.318 ± 0.16[d]
B_6 + /00/[a]	27.20 ± 6.3	0.280 ± 0.16
B_6 + /00/[b]	25.30 ± 5.9	0.263 ± 0.18
B_6 + /00/[c]	22.70 ± 5.6	0.220 ± 1.21

Protein content of the extracts: 1 mg · ml^{-1}
Mistletoe host trees: a — apple, b — locust, c — willow. d — Standard error of the mean

Table 3. Dependence of the mistletoe crude extract /00/ inhibitory effect on its protein content.

Inoculum	Protein concentration (mg · ml^{-1})	Number of tumors induced per disc	Weight of total tumor mass per disc in g
B_6		38.9 ± 10.01[a]	0.483 ± 0.083[a]
B_6 + /00/	1.8	27.9 ± 8.93	0.462 ± 0.080
B_6 + /00/	1.2	21.3 ± 6.97	0.420 ± 0.069
B_6 + /00/	0.8	16.3 ± 7.08	0.301 ± 0.070
B_6 + /16/	0.8	13.7 ± 5.64	0.287 ± 0.047

a — Standard error of the mean for 50 discs in each class

Table 4. Inhibitory effect of the mistletoe crude extract in comparison with the refined fractions /12/ and /16/ and the commercial cytostatic Adriblastin[R] /A/.

Inoculum	Number of tumors per disc	Weight of total tumor mass per disc in g
B_6	35.40 ± 9.56[a]	0.462 ± 0.083[a]
B_6 + /00/	19.02 ± 5.30	0.338 ± 0.065
B_6 + /12/	14.02 ± 4.17	0.234 ± 0.063
B_6 + /16/	12.30 ± 4.40	0.145 ± 0.059
B_6 + A	5.04 ± 3.73	0.009 ± 0.005

Protein content of the extracts: 0.8 mg · ml^{-1}
a — Standard error of the mean for 50 discs in each class.

The effects of the refined fraction /12/ and especially fraction /16/ on tumor induction are more pronounced than the one of the crude extract /00/ (Table 4, Fig. 2.1 — 2.4). However, the complete inhibition was not yet obtained.

Electrophoretic analysis of the protein composition and heterogeneity showed that it is the chloroplast enzyme Rubisco (Rm 0.13), which is most abundantly present in the crude extract, (Fig. 1). The refined

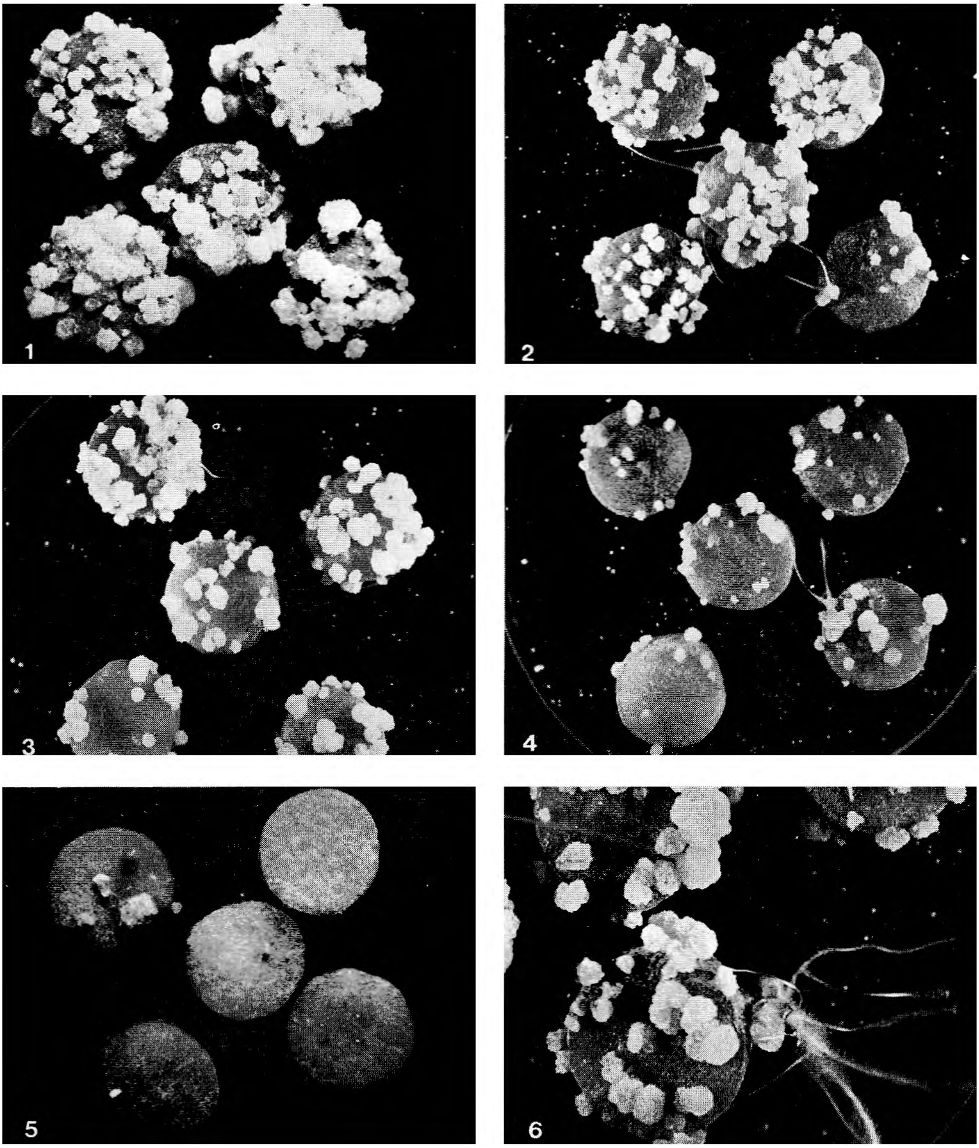

Fig. 2. **1** Crown gall tumors on potato tuber discs induced with bacterium *A. tumefaciens* B_6 **2** Mild inhibitory effect of crude extract /00/ on tumor induction and growth. **3** Stronger inhibitory effect of fraction /12/ obtained by salting out Rubisco from crude extract. **4** Clear inhibitory effect of fraction /16/ obtained by desorption of pigments from fraction /12/. **5** Strong inhibitory effect of anticancerous Adriblastin^{R}. **6** Well developed roots from tumor tissue treated with crude mistletoe extract.

fractions /12/ and /16/ are free of this enzyme. The three bands in the middle of the gel, moving with relative mobilities of 0.43, 0.48, 0.51 are quantitatively dominant in the fraction /12/, as well as in the most effective fraction /16/.

Discussion

The crown gall tumor test system of the Boston ivy *(Parthenocissus tricuspidata)* has recently been described as a very convenient *in vitro* plant tissue culture system for testing new anticancer drugs (Vervoitte 1984). Our experiments performed on another plant, *Solanum tuberosum,* and under different culture conditions, indicate that the crown gall tumor tissue on potato tuber discs is also an extremely suitable antitumor activity screening system.

We have tested the antitumor activity of mistletoe protein extracts and compared their inhibitory effects to those of the commercially available anticancer drug Adriblastin[R]. On the basis of our results we can conclude that the efficiency of the extract is dependent on mistletoe host-tree species, the most efficient being the crude extract prepared from the plant collected from the willow tree. These results are in good agreement with those of Vester et al (1968 a).

Further purification of the crude extract and preparation of refined fractions /12/ and /16/ yielded the increased antitumor activity. Both of these most efficient fractions contain three common protein components of intermediate electrophoretic mobility. We assume that among them is the protein fraction with maximal biological activity.

Mistletoe protein extract seems to stimulate rhizogenesis in the tumor tissue, which indicates an incomplete tumor transformation (Schilperoort and Bomhoff 1975, Beiderbeck 1977).

The high auxin/cytokinin ratio is known to induce rhizogenesis. The question remains open whether in our case the active mistletoe proteins effect rhizogenesis by inactivating genes for synthesis of cytokinins similarly to the case where certain mutations in the Ti-plasmid determine the morphogenesis of the tumors (shooty or rooty tumors), or by auxin synthesis stimulation.

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References

Anand, V. K., Heberlein, G. T., 1977: Crown gall tumorigenesis in potato tuber tissue. Amer. J. Bot. *64,* 153—158.

Beiderbeck, R., 1977: Pflanzentumoren. Ulmer Verlag, Stuttgart, 124—127.

Bradford, M. M., 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proetin-dye binding. Anal. Biochem. *72,* 248—254.

Davis, B. J., 1964: Disc electrophoresis II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. *121*, 404—427.

Denck, H., Karrer, K., 1980: Kolo-rektale Tumoren. Schriftenreihe Krebsgeschehen *23*, 65—76.

Konopa, J., Woynarowski, J. M., Sewandowska-Gumieniak, M., 1980: Isolation of viscotoxins. Hoppe-Seyler's Z. Physiol. Chem. *361*, 1525—1533.

Ornstein, L., 1964: Disc electrophoresis. I Background and theory. Ann. N. Y. Acad. Sci. *121*, 321—349.

Schilperoort, R. A., Bomhoff, G. H., 1975: Crown gall: A model for tumor research. In: Genetic manipulations with plant material. (Ed. Ledoux L.) New York, 141—162.

Stirpe, R., Legg, L., Onyon J., Ziska, P., Franz, H., 1980: Inhibiton of protein synthesis by a toxic lectin from *Viscum album* L. (mistletoe). Biochem. J. *190*, 843—845.

Ulrich, W., Mechelke, F., 1980: Reaktion der *In-vitro*-Kulturen vom menschlichen Fibroblasten HeLa-Zellen und von murinen L. Zellen bei Applikakation eines Präparates aus *Viscum album* L. Arzneim. Forsch/Drug Res. *30*, 2—11.

Vervoitte, V., 1984: Applicability of *in vitro* plant tissue cultures in discovery of new anticancer drugs. Application to the study of alstonine. Bio-Science *3*, 14—16.

Vester, F., Seel, A., Stoll, J., Müller M., 1968a: Zur Kenntnis der Inhaltsstoffe von *Viscum album*. III Isolierung und Reinigung cancerostatischer Proteinfraktionen. Hoppe-Seyler's Z. Physiol. Chem. *349*, 125—147.

Vester, F., Bohne, L., El-Rouly, M., 1968b: Zur Kenntnis der Inhaltsstoffe von *Viscum album*, IV Biologisches Verhalten einzelner Proteinfraktionen. Hoppe-Seyler's Z. Physiol. Chem. *349*, 495—511.

Woynarowski, J. M., Konopa, J., 1980: Interaction between DNA and viscotoxins. Hoppe-Seyler's Z. Physiol. Chem. *361*, 1535—1545.

SAŽETAK

CROWN-GALL TUMOR KAO TEST ZA ODREĐIVANJE ANTITUMORSKE AKTIVNOSTI PROTEINA IMELE (*VISCUM ALBUM* L.)

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Crown-gall tumori na tkivu gomolja krumpira korišteni su kao test-sistem za ispitivanje antitumorske aktivnosti proteina u ekstraktu imele. Određivanje broja i mase tumora pokazalo je, sa statističkom sigurnošću, da već sirovi ekstrakt inhibira tumorsku indukciju i rast. Inhibitorno djelovanje pročišćenih proteinskih frakcija još je izrazitije. Proteinski ekstrakt stimulirao je rizogenezu u tumorskom tkivu.

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