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RESEARCH ARTICLE

Inhibition of crown-gall tumorigenesis with plant extracts

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Abstract

Measurement of the inhibition of crown-gall tumors on potato discs is an antitumor bioassay method for detection of anti-tumor compounds from higher plants. In this study, after surface sterilization, tuber discs were co-cultivated with *Agrobacterium tumefaciens* B_6S_3 for two days. The discs were then inoculated on MS media with the crude extracts obtained from different parts of various plants – *Allium sativum* L. (Liliaceae), *Salvia verbenaca* L., *Rosmarinus officinalis* L., *Ocimum basiculum* L., *Lavandula stoechas* L. (Lamiaceae), *Althaea cannabina* L. (Malvaceae), *Petroselinum sativum* Mill. (Apiaceae), *Pelargonium radicula* L. (Geraniaceaea), *Juglans regia* L. (Juglandaceae), *Platanus orientalis* L. (Platanaceae), *Laurus nobilis* L. (Lauraceae), *Ranunculus ficaria* L. (Ranunculaceae), and *Abies equi-trojani* Aschers. et Sint. Ex Boiss (Pinaceae). Tumors appeared after 10-15 days, and tumor inhibition was detected only in six plant extracts of 13 (*A. sativum, R. officinalis, P. orientalis, L. nobilis, R. ficaria*, and *A. equi-trojani*) when compared to control material. Constitutive tumor inhibition activities were higher in these plant extracts (63.5%, 56.1%, 61.7%, 54.6%, 69.7%, and 57.9% at 0 min; 58.8%, 54.6%, 58.8%, 48.4%, 62.6%, and 51.6% for 15 min after bacterial inoculation).

Keywords: *Agrobacterium tumefaciens; crown-gall; plant extracts; potato discs; tumor inhibition*

Introduction

Crown-gall is a neoplastic disease of plants which occurs in more than 60 families of dicotyledons, also many gymnosperms, and induced by specific strains of *Agrobacterium tumefaciens*. The bacteria contain Ti (tumor inducing) plasmids which carry genetic information (T-DNA) that transforms normal wounded plant cells into autonomous tumor cells (Braun & Winzler, 1972). The relevance of the crown-gall tumor system to the general cancer problem has been thoroughly reviewed (Binns & Thomashow, 1988). Tumor induction on potato discs was first described by Anand and Heberlein (1977). Inhibition of development of crown-gall tumors on discs of potato tubers showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (P388, *in vivo,* murine leukemia) antitumor assay (Ferrigni et al., 1982). The modified assay has been initially performed in a series of natural anti-tumor compounds, on plant extracts suspected to have 3PS activity (Galsky et al., 1981). This modified procedure for crown-gall tumors on potato discs could routinely be employed as a comparatively rapid, inexpensive and statistically reliable prescreen for detection of compounds from higher plants. In this research we investigated the antitumor effects of various plant crude extracts (Table 1) on potato crown-gall tumor development.

Materials and methods

Agrobacterium tumafaciens B_6S_3 (wild type) strain was obtained from Zagreb University, Croatia. The bacteria were grown at 28°C on solidified LB media suspended in liquid LB media and adjusted to a density of $OD_{600} = 0.2$.

Preparation of plant extracts

The leaves of *Allium sativum* L. (Liliaceae), *Salvia verbenaca* L., *Rosmarinus officinalis* L., *Ocimum basiculum* L., *Lavandula stoechas* L. (Lamiaceae), *Althaea*

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Table 1. Effects of various plant exracts on induction of crown-gall on potato.

Addition to potato discs[a]	Mean no. of tumors/discs[b]
Control (5×10^9 cells/mL)	55.4 ± 3.9[c]
Allium sativum	22.8 ± 2.3 (58.9)[d]
Salvia verbenaca	45.5 ± 1.5 (-)
Petroselinum sativum	52.2 ± 1.8 (-)
Ocimum basiculum	44.2 ± 2.4 (-)
Pelargonium radicula	46.7 ± 3.4 (-)
Lavandula stoeas	50.5 ± 3.1 (-)
Rosmarinus officinalis	22.7 ± 5.3 (59)[d]
Juglans regia	45.5 ± 1.9 (-)
Platanus orientalis	21.5 ± 1.3 (61.2)[d]
Laurus nobilis	24.5 ± 0.9 (55.8)[d]
Nerium oleander	44.9 ± 1.8 (-)
Ranunculus ficaria	15.5 ± 2.2 (72)[d]
Abies. equi-trojani	23.3 ± 4.2 (57.9)[d]

[a]Inoculum (0.5 mL) containing 2.5×10^9 cells of *A. tumafaciens* B_6S_3, and 0.05 mg of the experimental material were added to the potato discs.
[b]Fifty discs were used for the determination.
[c]Standard error.
[d]Percentage inhibition (p <0.001).

cannabina L. (Malvaceae), *Petroselinum sativum* Mill. (Apiaceae), *Pelargonium radicula* L. (Geraniaceaea), *Juglans regia* L. (Juglandaceae), *Platanus orientalis* L. (Platanaceae), *Laurus nobilis* L. (Lauraceae), *Ranunculus ficaria* L. (Ranunculaceae) and cones of *Abies equi-trojani* Aschers. et Sint. Ex Boiss (Pinaceae) were shade-dried and ground to a coarse powder. Powdered plant materials (25 g each) were individually extracted with water/alcohol (200 mL) and then filtered according to Galsky et al. (1981). Filtrates were concentrated with drying under vacuum and used for activity studies. The crude extract (4 mg) of each plant was dissolved in 1 mL DMSO. Inoculums were prepared using 0.5 mL sample (4 mg extract/mL DMSO) with 1.5 mL sterile distilled water.

Transformation and inoculums

Tuber discs were co-cultivated with the bacterial suspension (OD_{600} = 0.2) then blotted dry between sterile Whatman filter papers and then transferred on solidified MS media (Murashige & Skoog, 1962) for 2 days in dark conditions. Then the discs were washed for 4 h in distilled water containing 1 μM of cefotaxime (Cx) and one drop (0.05 mL) of inoculum was added per disc at various times (0 and 15 min after bacterial inoculation) for testing the possibility any of the active test materials have in any way preventing bacterial attachment. The discs (each of the Petri dishes contained 6 discs) were transferred on MS media (hormone free) containing 1 μM of Cx. After 10-15 days tumors appeared on the discs. Culture was kept at 25°C in a controlled environmental chamber in dark conditions and relative humidity was 70%. Experiments were repeated three times. Inhibitions of crown-gall tumors were calculated according to McLaughlin (1991).

Table 2. Activity of six plant samples on crown-gall tumor inhibition when added to potato discs at various times after bacterial inoculation.

	Mean no. tumors/disc[b]	
	Time elasped prior to addition of plant extract to potato discs previously inoculated with *A. tumefaciens* B_6S_3	
Addition to potato discs[a]	0	15 min
Control (5×10^9 cells/mL)	33.7 ± 3.9[c]	
Allium sativum	12.3 ± 2.3 (63.5)[d]	13.9 ± 1.9 (58.8)
Rosmarinus officinalis	14.8 ± 5.3 (56.1)	15.3 ± 2.6 (54.6)
Platanus orientalis	12.9 ± 1.3 (61.7)	13.9 ± 3.3 (58.8)
Laurus nobilis	15.3 ± 0.9 (54.6)	17.4 ± 5.6 (48.4)
Ranunculus ficaria	10.2 ± 2.2 (69.7)	12.6 ± 8.9 (62.6)
A. equi-trojani (Aschers et Sinten)	14.2 ± 4.2 (57.9)	16.3 ± 0.9 (51.6)

[a]Inoculum (0.5 mL) containing 2.5×10^9 cells of *A. tumafaciens* B_6S_3, and 0.05 mg of the experimental material were added to the potato discs.
[b]Fifty discs were used for the determination.
[c]Standard error.
[d]Percentage inhibition (p <0.001).

$$\text{Inhibition \%} = 100 - \frac{\text{Average number of tumors per disc of sample}}{\text{Average number of tumors per disc of control}}$$

Results and discussion

Crude extracts from 13 plants were subjected to potato disc cytotoxicity assay (Table 1) and tumors appeared after 10-15 days after application. Tumor inhibition was detected in six plant extracts when compared to control material (Table 2).

Allium sativum (garlic) has strong antioxidant properties and its role in preventing age-related diseases like cardiovascular diseases, cancer, arthritis, cataract formation, etc., has been investigated for the past 10-15 years (Rahman, 2003; Çelik & Aslantürk, 2007). In our study, we determined that *A. sativum* has a gradual inhibition (63.5% for 0 min and 58.8% for 15 min after inoculation) on tumor inhibition.

Oils of *Rosmarinus officinalis* (thyme, rosemary, lavender, and cedarwood) rubbed onto the scalp helped with alopecia

for 44% of patients versus 15% of controls, double blind study of 86 patients (Hay et al., 1998). Also, tumor inhibition was determined (56.1% for 0 min and 54.6% for 15 min) in *R. officinalis* extract in the present study.

The leaves of *Platanus orientalis* are astringent and vulnerary. The fresh leaves are bruised and applied to the eyes in the treatment of ophthalmia. Tumor inhibition of *P. orientalis* extract was observed on the potato discs (61.7% for 0 min and 58.8% for 15 min).

The leaves and berries of *Lauris nobilis* (bay laurel) contain the essential oils eugenol, cineol and geraniol, which account for the distinctive spicy aroma. Infusion is reputed to soothe the stomach and relieve flatulence. Oil pressed from the berries was once a popular liniment for arthritis and sore muscles and still is used in perfumes, candles, and soaps. As shown in Table 2, tumor inhibition of *L. nobilis* is 54.6% for 0 min and 48.4% for 15 min after inoculation.

Ranunculus ficaria has been used for thousands of years in the treatment of hemorrhoids and ulcers. It is not recommended for internal use because it contains several toxic components. The whole plant, including the root, is astringent (Bown, 1995). It is widely used as a remedy for piles and is considered almost a specific. It is also applied externally to perineal damage after childbirth (Bown, 1995). According to the knowledge about *R. ficaria,* we found significant inhibition effects of the root extract, 69.7% for 0 min and 62.6% for 15 min after inoculation on crown-gall development.

Limited information is available on the usage of herbs such as *Abies equi-trojani* and its cones. But, it is known that the cones are used for the treatment of bronchial asthma in Anatolia (middle and east of Anatolia), the Black sea region, and southwest of Anatolia (Ida mountain/Canakkale region). Based on this information we decided to test cones of *A. equi-trojani*, and crown-gall tumor inhibition was also determined in extracts from the cones (57.9% for 0 min and 51.6% for 15 min after inoculation).

The initial step in the formation of crown gall tumors involves the attachment of the bacterium to a tumor-binding site (Glogowski & Galsky, 1978; Lippincott & Lippincott, 1969). This attachment on the potato disc system is complete within 15 min following inoculation (Glogowski & Galsky, 1978). To test the possibility that any of the active test materials were in any way preventing bacterial attachment, they were added at various times after the inoculation of the bacteria to the potato discs (Table 2). The amount of inhibition obtained with the active samples is consistent, whether these extracts are added to the potato discs simultaneously with the bacteria, or 15 min following bacterial inoculation. These results eliminate any possible effects of these samples on bacterial viability when added on potato discs.

To rule out the possibility that these results are unique to tumor induction by *A. tumefaciens* strain B_6S_3, several of the experimental compounds were assayed for their activity on tumor initiation by TT-107, another virulent strain of *A. tumefaciens.* The results were similar to those found when B_6S_3 was used as the initiating strain. Similar to our work, extracts from different plants have been used for both crown-gall tumor bioassay and in human cancer cell lines (Hui et al. 1989; Arican et al., 2000; Arican & Ozalpan, 2007). However, Ibrahim et al. (1995) studied extracted material from marine algae, and they confirm the method is useful for screening of the bio-active material from various materials.

The results of this study show the ability of the antitumor activity of these samples to inhibit crown-gall tumor inhibition on potato discs. Although further studies are necessary, it appears that the crown-gall tumor bioassay could be used as an aid in the screening of potential antitumor compounds.

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