

Proceeding Paper

Cumulative Cytotoxicity Assay of the Aqueous and Ethanolic Extracts of the Selected Medicinal Plants Using Crown Gall Tumor Disc Bioassay [†]

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Abstract: The present study was carried out to test an in vivo crown gall tumor disc bioassay using potato discs of the aqueous and ethanolic extracts of *Annona reticulata* with *Allium sativum*, *Allium fistulosum*, *Brassica oleraceae*, and then correlate the cytotoxicity results with the known pharmacological activities of the plants. Cytotoxicity was evaluated according to the crown gall tumor disc bioassay, a highly specific, quantitative method that requires only a short period of time to return results of crown gall tumor formation. The results shows the cumulative activity of the extracts of *Annona reticulata* extract with *Allium sativum*, *Allium fistulosum* and *Brassica oleraceae* extracts and shown they were potent against the bioassay as compared to their being used alone with combined extracts. The results indicated that their bioactive components have considerable pharmacological effects. Thus, the results supported the uses of these plant species in traditional medicine.

Keywords: crown gall tumor disc bioassay; *Annona reticulata*; *Allium sativum*; *Allium fistulosum* and *Brassica oleraceae*; potato disc bioassay; antitumour; cytotoxicity

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1. Introduction

Crown gall is a neoplastic disease of plants that occurs in more than 60 families of dicotyledons and many gymnosperms. The disease is characterized by the transformation of normal plant cells into autonomous tumor cells in a short period of time. Once initiated, the tumor possesses the capacity for autonomous growth independent of the normal control mechanism of the host [1]. The causative agents of this disease are specific strains of the Gram-negative bacterium *Agrobacterium tumefaciens* [2]. The relevance of the crown gall tumor system to the general cancer problem has been thoroughly reviewed [3,4]. The use of highly specific, quantitative bioassays that require only a short period of time to obtain results are available for studying crown gall tumor formation [5,6]. Using the potato disc bioassay we examined extracts and purified compounds of plant origin, some of which had shown antitumor activity in animals, for their effect on the initiation of crown gall tumors.

The first step in a drug discovery is the screening of biological and synthetic bioactive compounds [7]. The potato-disc assay is one of the methods that has proven to be useful in checking molecules' known and novel antitumor properties. This bioassay was based on *Agrobacterium tumefaciens* infection on a potato disc [8]. The tumorigenic mechanism

that it initiated in plant tissues was in many ways similar to that found in animals [9]. *A. tumefaciens*, a rod-shaped and virulent Gram-negative soil-borne bacterium, is the causative agent of crown gall disease. It is a neoplastic disease that produces tissue masses that bulge from the stems and roots of woody and herbaceous plants. These tumor masses could be spongy or hard, with or without a deleterious effect on the plant. The tumor produced is histologically similar to those in animals or humans. Ti-plasmid produces cell proliferation and blocks apoptosis by tumor induction just as in animal or human cancer cells [10]. As a result, it was proposed that the crown gall tumor (potato disc) assay be used to prescreen for antitumor activity [11–13]. Although a lot of aseptic technique is required to carry out the procedure, it can be performed with minimal technical training.

A variable chemical that interferes with cell cycles and has different modes of action was sensitive for the antitumor potato disc assay [14]. Therefore, a simple test is performed that needs aseptic conditions and it permits the detection and isolation of many anti-tumor compounds from plant microbes or biomolecules that were confirmed by *in vivo* animal tumor inhibition [15].

2. Materials and Methods

The leaves of *A. reticulata*, *Allium sativum* (bulbs), *A. fistulosum* and *B. Oleraceae* were collected from the town of Karjat, Raigad District, Maharashtra, India, in December 2018. All plant materials were authenticated at “The Blatter Herbarium” at St. Xavier’s College, Mumbai.

For the experiment, the leaves of the plants were collected after being identified and authenticated. They were shade-dried and made into a coarse powder that was first defatted with petroleum ether and then subjected to cold maceration for 72 h using a 1:1 mixture of methanol and water as a solvent to prepare a hydro-alcoholic extract of *Annona reticulata* leaves (percentage yield 20.5% *w/w* with respect to dried powder). The extract was filtered and then concentrated by a rotary evaporator. For the preparation, the different fractions were used [16–18].

The sun-dried and powdered leaves (76 g) of *A. reticulata* were successively extracted in a Soxhlet extractor at an elevated temperature (40–60 °C) using 200 mL of distilled n-hexane followed by extraction of different solvent like petroleum ether, methanol, and chloroform. All extracts were collected and filtered independently through filter paper and transferred in petri dishes so that liquid solvents could evaporate to give dry extracts. These were weighed and stored in an air-tight container with the necessary identification markings and kept in a refrigerator for future investigation.

Ten grams of spring onion leaves or bulbs were soaked in 100 mL of methanol and water, respectively. The prepared samples were shaken using orbital shaker for 7 h followed by centrifugation for 15 min at 7000 rpm. The extracts were then filtered using a vacuum filtration assembly and assessed using a brine shrimp lethality bioassay.

Red cabbage leaves were shade-dried followed by drying in a hot air oven at 50 °C and then ground into a fine powder and stored in an air-tight container for analysis. Fresh red cabbage leaves were ground in the mixer to collect juice. The coarse powder and juice of the red cabbage were extracted using methanol and water. These extracts of red cabbage powder and juice were collected separately and filtered using Whatman filter paper. All extracts were concentrated and excessive solvents were evaporated in a vacuum.

2.1. Phytopathogenicity Test

Phytopathogenicity tests were done using potato disc bioassays [19]. The strain of *A. tumefaciens* used for the tumor induction was obtained from the National Collection of Industrial Microorganisms (Pune 2145, India).

2.2. Disc Bioassay Method

The Luria Bertani (LB) agar medium was used to prepare the culture of *A. tumefaciens* strains. A single colony was transferred into the LB broth medium and incubated at 30 °C for 24 h. Potatoes (*Solanum tuberosum* L.) were disinfested by scrubbing under running water with a brush and then immersed in 2% Clorox for 5 min. Potato discs, (5 mm × 8 mm) were made using a cork borer and immersed in 2% Clorox for 30 min. Each disc was rinsed thrice in autoclaved distilled water for 15 min. After washing and rinsing, the potato discs were removed from the distilled water and blotted on sterile paper towels to remove excess water. In all, 16 potato discs were kept in the petri plates containing an autoclaved agar medium (2%). Suspensions of *A. tumefaciens* on the LB broth medium were standardized. Each disc was overlain with 50 µL of bacterial suspension. Petri plates were sealed by parafilm and incubated at room temperature (25–30 °C). The experiment was repeated at least twice and used with 10 replications. After 21 days, the potato discs were stained with Lugol's solution (10% KI + 5% I₂) and the tumors were counted under a dissecting microscope [20]. Lugol's reagent stained the starch in the potato tissue dark-blue to dark-brown, but the tumors produced by *A. tumefaciens* did not take up the stain and appeared creamy to orange [21–23]

$$\text{Percentage inhibition} = 100 - (\text{number of tumor with sample} / \text{number of tumor with control}) \times 100$$

2.3. Statistical Analysis

Experiments were Performed in triplicates and data were analyzed by taking their mean.

3. Results and Discussion

Table 1 lists the effects of all of the samples tested on the initiation of crown gall tumors on potato discs. The samples are listed in the experimental order in which they were assayed. A definite correlation exists between tumor formation of these samples, and their ability to inhibit crown gall tumor formation on the potato discs. Data from a typical experiment are shown in (Table 2) as a percent inhibition. Each sample was assayed in triplicate experiments. The initial step in the formation of crown gall tumors involved the attachment of the bacterium to a tumor-binding site [24,25]. The amount of inhibition obtained with the active samples was consistent whether or not these extracts were added to the potato discs. These results eliminated any possible effects of these samples on bacterial attachment.

Table 1. Comparative activity of various plant materials against initiation of crown gall tumors for cytotoxicity.

Plant Extracts Concentrations	Mean Number of Tumors (Mean ± SE)		
	1 mg/mL	10 mg/mL	50 mg/mL
Control (Distilled water)	0.0	0.0	0.0
Control (DMSO)	18.66 ± 2.25	21.33 ± 2.28	23.33 ± 1.85
Standard (Colchicine)	9.66 ± 1.90	11.33 ± 0.78	14.33 ± 1.13
<i>Annona reticulate</i> (Alcoholic extracts)	9.00 ± 0.33	11.67 ± 0.87	14.67 ± 0.62
<i>Annona reticulate</i> (Aqueous extract)	7.33 ± 0.11	8.67 ± 0.40	9.33 ± 0.62
<i>Allium sativum</i> (Alcoholic)	8.33 ± 0.11	11.67 ± 0.59	15.00 ± 0.67
<i>Allium sativum</i> (Aqueous extract)	6.00 ± 0.58	7.33 ± 0.87	9.33 ± 0.29
<i>Allium fistulosum</i> (Alcoholic extracts)	9.00 ± 0.58	8.67 ± 0.95	11.00 ± 1.20
<i>Allium fistulosum</i> (Aqueous extract)	9.00 ± 1.20	10.67 ± 0.95	13.67 ± 0.87
<i>Brassica oleraceae</i> (Alcoholic extracts)	7.00 ± 0.33	12.33 ± 1.18	14.33 ± 0.11
<i>Brassica oleraceae</i> (Aqueous extract)	8.66 ± 0.62	1.00 ± 0.67	13.33 ± 0.73
<i>Annona reticulate</i> and <i>Allium sativum</i> (1:1) Alcoholic extracts	13.00 ± 1.20	16.00 ± 1.20	20.33 ± 1.74

<i>Annona reticulata</i> and <i>Allium sativum</i> (1:1) Aqueous extracts	6.66 ± 0.87	8.33 ± 0.59	10.33 ± 0.91
<i>Allium fistulosum</i> and <i>Annona reticulata</i> (Alcoholic extracts)	12.00 ± 0.58	14.67 ± 0.97	15.67 ± 1.24
<i>Allium fistulosum</i> and <i>Annona reticulata</i> (Aqueous extract)	9.00 ± 0.33	11.67 ± 0.80	12.67 ± 0.48
<i>Brassica oleraceae</i> and <i>Annona reticulata</i> (Alcoholic extracts)	5.66 ± 0.29	8.33 ± 0.87	9.33 ± 0.48
<i>Brassica oleraceae</i> and <i>Annona reticulata</i> (Aqueous extract)	5.33 ± 0.62	6.67 ± 0.91	7.67 ± 1.24

Table 2. Comparative % inhibition of crown gall tumors for cytotoxicity.

Plant Extracts Concentrations	% Inhibition of Tumors When Compared with Control		
	1 mg/mL	10 mg/mL	50 mg/mL
Standard (Colchicine)	51.78	53.13	61.44
<i>Annona reticulata</i> (Alcoholic extracts)	48.21	54.70	62.87
<i>Annona reticulata</i> (Aqueous extract)	39.28	40.63	41.43
<i>Allium sativum</i> (Alcoholic)	44.63	54.70	64.29
<i>Allium sativum</i> (Aqueous extract)	32.14	34.38	40.01
<i>Allium fistulosum</i> (Alcoholic extracts)	48.21	51.57	54.29
<i>Allium fistulosum</i> (Aqueous extract)	48.21	50.01	58.58
<i>Brassica oleraceae</i> (Alcoholic extracts)	37.49	57.82	61.44
<i>Brassica oleraceae</i> (Aqueous extract)	46.42	46.88	57.15
<i>Annona reticulata</i> and <i>Allium sativum</i> (1:1) Alcoholic extracts	69.63	75.01	87.16
<i>Annona reticulata</i> and <i>Allium sativum</i> (1:1) Aqueous extracts	35.71	39.07	44.29
<i>Allium fistulosum</i> and <i>Annona reticulata</i> (Alcoholic extracts)	64.27	68.76	67.15
<i>Allium fistulosum</i> and <i>Annona reticulata</i> (Aqueous extract)	48.21	54.70	54.29
<i>Brassica oleraceae</i> and <i>Annona reticulata</i> (Alcoholic extracts)	30.35	39.07	40.01
<i>Brassica oleraceae</i> and <i>Annona reticulata</i> (Aqueous extract)	28.57	31.25	32.86

Statistical analysis showed that the methanol extract inhibited tumor growth on the potato discs significantly in a concentration-dependent manner across the strains (Table 1). A highly significant difference was observed, and agrobacterium suggests their different activity (Table 1). Maximum tumor inhibition was observed at 50 mg/mL plant extract against the strain. No significant tumor inhibition was observed at 1 mg/mL concentration. The inhibition percentage was calculated to compare with the control. On the basis of tumor forming ability, it was observed that *A. tumefaciens* inhibited the tumour growth. Our study results showed that an alcoholic extract significantly inhibited tumor formation on potato discs, which indicates it could be used for its antitumor properties. Several workers conducted similar types of investigations and recommended a large number of plant extracts as a potential source of anticancer agents [26]. Crown gall is a neoplastic disease of plants caused by *Agrobacterium tumefaciens* [27]; which occurs in more than 60 families of dicotyledons and many gymnosperms. Due to a similar mechanism of tumor development for both cases, we concluded that our studied plant extract might be of use for drug development for tumor treatment in humans.

4. Conclusions

Annona reticulata had been reported to be a potential antitumor agent for a long time, and the present study confirmed the cumulative effect of its antitumor potential. Significant tumor inhibition by an alcoholic extract of *Annona reticulata* on potato discs at different concentrations may lead to the conclusion that it might be used as a potential source of an antitumor agent.

Institutional Review Board Statement: This study was approved by the Institutional Review Board (IRB) of, Konkan Gyanpeeth Rahul Dharkar College of Pharmacy and Research Institute, Karjat, and the protocols used in the study were approved by the Committee.

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Conflicts of Interest: Declared none.

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