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

Sandeep Waghulde [1,*], Nilesh Gorde [1], Tushar Baviskar [1], Praful Patil [1], Shweta Singh [1], Mohan K. Kale [1] and Vijay R. Patil [2]

[1] Konkan Gyanpeeth Rahul Dharkar College of Pharmacy and Research Institute, Karjat, Raigad, India
[2] Hon'ble Loksevak Madhukarrao Chaudhari College of Pharmacy, Faizpur, Jalgaon, India
* Correspondence: sandeepwaghulde@yahoo.com
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Abstract: The present study was conducted to test for in vivo crown gall tumor disc bioassay using potato discs of the aqueous and ethanolic extracts of Annona reticulata with Allium sativum, Allium fistolisum Brassica oleraceae and correlate cytotoxicity results with known pharmacological activities of the plants. Cytotoxicity was evaluated in terms of Crown Gall Tumor Disc Bioassay. The use of highly specific, quantitative bioassays which require only a short period of time to obtain results are available for studying crown gall tumor formation. Results showed that the extracts of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae were potent against the Crown Gall Tumor Disc Bioassay when compared alone with combined extracts. It indicated that bioactive components are present in these plants that could be accounted for its pharmacological effects. Thus, the results support the uses of these plant species in traditional medicine.

Keywords: Crown Gall Tumor Disc Bioassay; Annona reticulate; Allium sativum; Allium fistolisum and Brassica oleraceae; potato disc bioassay; antitumour; cytotoxicity

1. Introduction

Crown gall is a neoplastic disease of plants which 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 (Lippincott JA, BB Lippincott, 1975). The causative agents of this disease are specific strains of the gram negative bacterium *Agrobacterium tumefaciens* (Braun AC, 1972). The relevance of the crown gall tumor system to the general cancer problem has been thoroughly reviewed (Cloud W, 1974 and Anand VK, GT Heberlein 1977). The use of highly specific, quantitative bioassays which require only a short period of time to obtain results are available for studying crown gall tumor formation (Lippincott JA, GT Heberlein 1965 and Islam MS et al., 2013). Using the potato disc bioassay we examined extracts and purified compounds of plant origin, some of which had known antitumor activity in animals, for their effect on the initiation of crown gall tumors.

The preliminary step in drug discovery which allow the screening of biological and synthetic bioactive compounds (Islam MS, et al., 2009). Potato disc assay was shown to be useful for checking known and novel antitumor molecules' properties. This bioassay is based on *Agrobacterium tumefaciens* infection on potato disc (Srirama R, et al., 2007).The validity for the use of such assay is

that the tumorogenic mechanism initiated in plant tissues by *A. tumefaciens* is in many ways similar to that of animals (Kempf VAJ, et al., 2002). A. tumefaciens, is a Gram-negative soil borne bacterium, rod–shaped and virulent that is the causative agent of Crown Gall Disease. Crown Gall is a neoplastic disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. The tumor masses could be spongy or hard, with or without a deleterious effect on the plant. The produced tumor is histologically similar to animal or human ones. The process of tumor induction by Ti-plasmid is the result of cell proliferation and blocking of apoptosis like in animal or human cancer cells (David SG. 2004). As a consequence, it was proposed to adopt the crown gall tumor (potato disc) assay as a prescreen for antitumor activity (Jerry LM, Lingling LR. 1998., Galsky AG, Wilsey JP, Powell RG. 1980, Ferigni NR, et al., 1982). Although aseptic technique is required, the methodology is simple and can be performed with minimal technical training.

The antitumor potato disc assay was shown to be sensitive for variable chemicals that interfere with cell cycle and have different modes of action (Coker PS, Radecke J, Guy C, Camper ND. 2003 and Kahl G. 1982.). This simple test that needs aseptic conditions has allowed the detection and isolation of many anti tumor compounds from plant microbial or biomolecules that were confirmed by in vivo animal tumor inhibition (McLaughlin JL. 1991).

2. Materials and Methods

The leaves of A. reticulate, Allium sativum (bulbs), A. fistolisum and B. Oleraceae were collected from regions of Karjat Dist-Raigad, Maharashtra, India in December 2018. All Plant materials were authenticated at "The Blatter Herbarium" St. Xavier's College, Mumbai.

After identification and authentication of the plant, leaves of the plant were collected for the experimental process. The leaves were shade dried, made into coarse powder and the powdered material was initially defatted with petroleum ether and then subjected to cold maceration process for 72-h using 1:1 mixture of methanol and water as solvent to prepare hydro-alcoholic extract of Annona reticulata leave (percentage yield 20.5% *w*/*w* with respect to dried powder). The extract was filtered and concentrated by rotary evaporator. For the preparation of different fractions method was used (Zhishen,J.;Mengcheng,T.;Jianming,W.1999, Bondet,V.; Brand Williams,W.; Berset,C.1997 and Brand-Williams,W.; Cuvelier,M.E.; Berset,C.1999).

The sun dried and powdered leaves (76 g) of A. reticulata were successively extracted in a Soxhlet extractor at elevated temperature using 200 mL of distilled n-hexane (40–60) °C which was followed by petroleum ether, methanol, and chloroform. All extracts were filtered individually through filter paper and poured on petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container with necessary markings for identification and kept in a refrigerator for future investigations.

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

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

2.1. Phytopathogenicity Test

Phytopathogenicity tests were done using potato disc bioassays (Kahl G. 1982. and McLaughlin JL. 1991). The strain is obtained from NCIM, Pune 2145 of A. tumefaciens was used for the tumor induction.

2.2. Disc Bioassay Method

A. tumefaciens strains were cultured on Luria Bertani (LB) agar medium. A single colony was transferred into LB broth medium and incubated at 30 °C for 24 h. potatoes (Solanum tuberosum L.) and were disinfested by scrubbing under running water with a brush, then immersed in 2% Clorox for 5 min. Potato, (5 mm × 8 mm) were made with cork borer and immersed in 2% Clorox for 30 min. Each disc was rinsed thrice in autoclaved distilled water for 15 min. After rinsing, the discs were removed from the distilled water, blotted on sterile paper towels. Sixteen discs were placed on Petri plates containing autoclaved agar medium (2%). Suspensions of A. tumefaciens on LB broth medium were standardized. Each disc was overlaid with 50 μL of bacterial suspension. Petri plates were sealed by parafilm and incubated at room temperature (25–30 °C). Ten replications were used and experiment was repeated at least twice. After 21 days, potato discs were stained with Lugol's solution (10% KI + 5% I_2) and tumors were counted under dissecting microscope (Islam MS, 2010). Lugol's reagent stains the starch in the potato tissue a dark blue to dark brown colour, but the tumors produced by A. tumefaciens will not take up the stain, and appear creamy to orange (Chen FC et al., 1999, Aysan Y, et al., 2003 and Hussain A, Zia M, Mirza B. 2007).

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

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 the tumor formation of these samples, and their ability to inhibit crown gall tumor formation on potato discs. Data from a typical experiment are shown in Table 2 as % inhibition. Each sample was assayed in at triplicate experiments. The initial step in the formation of crown gall tumors involves the attachment of the bacterium to a tumor-binding site (Glogowski W, AG Galsky 1978 and Lippincott BB, JA Lippincott, 1969). The amount of inhibition obtained with the active samples is consistent whether these extracts are added to the potato discs. These results eliminate 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	**Mean Number of Tumors (Mean SE ±)**		
Concentrations	**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
Annona reticulate (Alcoholic extracts)	9.00 ± 0.33	11.67 ± 0.87	14.67 ± 0.62
Annona reticulate (Aqueous extract)	7.33 ± 0.11	8.67 ± 0.40	9.33 ± 0.62
Allium sativum (Alcoholic)	8.33 ± 0.11	11.67 ± 0.59	15.00 ± 0.67
Allium sativum (Aqueous extract)	6.00 ± 0.58	7.33 ± 0.87	9.33 ± 0.29
Allium fistolisum (Alcoholic extracts)	9.00 ± 0.58	8.67 ± 0.95	11.00 ± 1.20
Allium fistolisum (Aqueous extract)	9.00 ± 1.20	10.67 ± 0.95	13.67 ± 0.87
Brassica oleraceae (Alcoholic extracts)	7.00 ± 0.33	12.33 ± 1.18	14.33 ± 0.11

Brassica oleraceae (Aqueous extract)	8.66 ± 0.62	1.00 ± 0.67	13.33 ± 0.73
Annona reticulate and Allium sativum (1:1) Alcoholic extracts	13.00 ± 1.20	16.00 ± 1.20	20.33 ± 1.74
Annona reticulate and Allium sativum (1:1) Aqueous extracts	6.66 ± 0.87	8.33 ± 0.59	10.33 ± 0.91
Allium fistolisum and Annona reticulate (Alcoholic extracts)	12.00 ± 0.58	14.67 ± 0.97	15.67 ± 1.24
Allium fistolisum and Annona reticulate (Aqueous extract)	9.00 ± 0.33	11.67 ± 0.80	12.67 ± 0.48
Brassica oleraceae and Annona reticulate (Alcoholic extracts)	5.66 ± 0.29	8.33 ± 0.87	9.33 ± 0.48
Brassica oleraceae and Annona reticulata (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	**% Inhibition of Tumors When Compared with Control**		
Concentrations	**1 mg/mL**	**10 mg/mL**	**50 mg/mL**
Standard (Colchicine)	51.78	53.13	61.44
Annona reticulate (Alcoholic extracts)	48.21	54.70	62.87
Annona reticulate (Aqueous extract)	39.28	40.63	41.43
Allium sativum (Alcoholic)	44.63	54.70	64.29
Allium sativum (Aqueous extract)	32.14	34.38	40.01
Allium fistolisum (Alcoholic extracts)	48.21	51.57	54.29
Allium fistolisum (Aqueous extract)	48.21	50.01	58.58
Brassica oleraceae (Alcoholic extracts)	37.49	57.82	61.44
Brassica oleraceae (Aqueous extract)	46.42	46.88	57.15
Annona reticulate and Allium sativum (1:1) Alcoholic extracts	69.63	75.01	87.16
Annona reticulate and Allium sativum (1:1) Aqueous extracts	35.71	39.07	44.29
Allium fistolisum and Annona reticulate (Alcoholic extracts)	64.27	68.76	67.15
Allium fistolisum and Annona reticulate (Aqueous extract)	48.21	54.70	54.29
Brassica oleraceae and Annona reticulate (Alcoholic extracts)	30.35	39.07	40.01
Brassica oleraceae and Annona reticulata (Aqueous extract)	28.57	31.25	32.86

Statistical analysis showed that the methanol extract inhibit tumor growth on potato disc significantly in a concentration dependent manner across the strains (Table 1). Highly significant difference was observed 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. Inhibition percentage was calculated to compare with the control. On the basis of tumor forming ability, it was observed that A. tumefaciens. Our study results showed that alcoholic extract significantly inhibited tumor formation on potato discs which indicates it could be a potential source of antitumor properties. Several workers conducted similar type of investigation and recommend large number of plant extracts as a potential source of anticancer agent (Turker and Camper, 2002; Inayatullah et al., 2007; Hussain et al., 2007). Crown gall is a neoplastic disease of plants caused by Agrobacterium tumefaciens (Kahl and Schell, 1982; Lippincott and Lippincott, 1975) which occurs in more than 60 families of dicotyledons and many gymnosperms (Galsky and Wilsey, 1980). Due to similar mechanism of tumor development for both cases concluded that our studied plant extract might be of use for drug development for tumor treatment in human.

4. Conclusions

Annona reticulate is reported as a potential source of antitumor agent since long time. The present study confirms the cumulative effect of their antitumor potential. Significant tumor inhibition by the alcoholic extract of Annona reticulate on potato disc at different concentrations may lead to conclude that it might be used as a potential source of antitumor agent.

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