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In vitro evaluation of *Croton bonplandianum* Baill. as potential antitumor properties using *Agrobacterium tumefaciens*

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Antitumor properties of twigs extract of *Croton bonplandianum* Baill. were proven using potato disc and radish seed bioassays. Tumor formation ability of *Agrobacterium* was distinctly inhibited on potato disc in presence of methanol extract. Significant variation on tumor formation ability was observed among the studied strains of *Agrobacterium* when treated with different concentrations (10, 100 and 1,000 ppm) of plant extracts. Tumor inhibition was increased with the increasing of concentrations of plant extract. Decreasing the root length and percentage of seed germination during radish seed bioassays further confirmed the antitumor properties of *Croton bonplandianum*. In conclusion it may be recommended that the bioactive compound of this plant can play important role in developing antitumor drugs for human beings, as there is a similarity between human and plant tumor formation mechanism.

Key words: Antitumor properties, *Croton bonplandianum*, potato disc bioassay, *Agrobacterium*

Introduction

Cancer is now serious health problems in human beings both in developed and developing countries. Some conventional systems such as surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods are being used for Cancer treatment. Most of the agents have been revealed as mutagenic and/or carcinogenic, and are highly toxic, not only for cancer but also for normal cells (Fatber, 1968). Due to the toxic and adverse side effects of synthetic medicines being observed round the glove, herbal medicine has made a comeback to improve the fulfillment of our present and future health needs (Harun-ur-Rashid *et al.*, 2002). Several plant-derived compounds have been approved as anti-cancer drugs i.e. vinblastine,

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vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan, just to name a few (Syrovets and Laumonnier, 2009).

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). The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (McLaughlin, 1991; Agrios, 1997). The potato disc assay demonstrates the inhibition of tumor formation on potato discs; materials (e.g. plant extracts) that inhibit these plant tumors have a high predictability of showing activity against the P388 (3PS) leukemia in mice (Ferrigni *et al*., 1982). Development of a simple antitumor prescreen using a convenient and inexpensive plant tumor assay systems can offer numerous advantages as alternatives to extensive animal testing in the search for new anticancer drugs (Turker and Camper, 2002).

Croton bonplandianum Baill. is native to the southern Bolivia, Paraguay, Southwestern Brazil, and Northern Argentina of South America (Chakrabarty and Balakrishnan, 1992). Croton is rich in secondary metabolites including alkaloids and terpenoids (Rizk, 1987), the latter including irritant co-carcinogenic phorbol esters (Phillipson, 1995). Diterpene resins found in many species of Croton have been used experimentally in studies of tumor initiation and conceivably prove to be useful in cancer therapy (Sharma, 2009). Considering its tremendous important in antitumor potential, the present study was undertaken to evaluate their antitumor and phytotoxicity by utilization of potato disc and radish seed bioassay.

Materials and methods

Plant material

Croton bonplandianum Baill. was collected from different places of Rajshahi University Campus. Taxonomic identity of this plant was confirmed by Mr. A. H. M. Mahbubur Rahman, Assistant Professor, Department of Botany, Rajshahi University, Rajshahi-6205, Bangladesh. Twigs were used for extraction.

Preparation of extracts

Extraction procedure was carried out according to Ahmad and Beg (2001) with some modification. Twigs were rinsed well with tap and distilled water (DW) and kept under shade still drying. Dried material coarsely powdered

using mortar and pestle followed by oven dry and further reduced to powder using an electric blender (Nokia, Osaka-Japan) and stored in air tight glass container. Powdered (50 g) were then dissolved in methanol by allowing to sediment at room temperature (27-30 °C) for 7 days with occasional shaking. For filtrating the material, Teton cloth and Whatman No.1 filter paper was used, respectively. The filtrates were then transferred in to glass beaker and dried into semisolid material using water bath (4 holes analogue, Thermostatic water bath, China). Particular concentration (10 ppm, 100 ppm and 1,000 ppm) of the plant extract was prepared using methanol for antitumor activity test, and 250 mg/ml were prepared for antibacterial assay.

Antitumor potato disc assay

Antitumor test of plant extracts was performed according to standard potato disc bioassay (Turker and Camper, 2002; Hussain *et al*., 2007). Three *A. tumefaciens* strains named AtTa0112, AtAc0114 and AtSl0105 (isolated and identified in our laboratory) were cultured on Luria-Bertani (LB) agar and then transferred into LB broth and incubated 48 hours. Six to seven loops of broth cultures were transferred in to test tube containing 10 ml phosphate buffered saline (PBS; pH 7.2). The following proportion was used for antitumor activity test: 600 µl test extract + 150 µl Double Distilled Water (DDW) + 750 µl *A. tumefaciens* in PBS. Camptothecin was used as positive control replacing test extracts.

Red skinned potatoes (*Solanum tuberosum* L.) were collected from local market and thoroughly washed with tap and DW. For surface sterilization, 0.1% $Hgcl_2$ solution was used. Potatoes were cut into 8 mm diameter in size cylindrical pieces using cork borer and transferred in to DDW containing conical flask. After washing, the cylindrical segments were cut into 5 mm × 8 mm disc and placed on to agar (15 g/l) plates (10 discs per plate). 50 µl of appropriate inoculums were placed on the surface of each potato disc. The plates were sealed with parafilm and incubated at room temperature at 27-30 ^{0}C for 21 days. After 21 days discs were stained with Lugol's solutions (10 % KI, 5 % I_2) and tumors were counted under a stereo microscope. The experiment was carried out in sterilized condition and repeated in three times. Percent inhibition of tumors was calculated (McLaughlin, 1991; McLaughlin *et al*., 1993; McLaughlin and Rogers, 1998). More than 20 % tumor inhibition is considered significant (Ferrigni, 1982). Data were analyzed using ANOVA.

Antibacterial assay

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity of studied plant extract (Bauer *et al*., 1966; Barry, 1980). Methanol was used as negative control and erythromycin (30 µg/ml), carbenicillin (100 µg/ml) and chloramphenicol (30 µg/ml) were used as positive control. Discs (Whatman No.1 filter paper) were impregnated with 10 µl of the extract and antibiotics followed by air dried, and then placed on seeded LB agar plates. 20 µl standard bacterial cultures (48 hours incubated) were used for spreading LB agar plates. Plates were then incubated at 28-30 °C for 24 hours. Antibacterial activity was evaluated by measurement of diameter zones of inhibition (mm) against studied *A. tumefaciens* strains. Each assay was carried out in triplicates.

Radish seed phytotoxicity assay

Radish seed phytotoxicity assay was conducted according to Turker and Camper (2002). Two different determinations: 1) root length and 2) percent seed germination were performed. For root length determination, two different concentrations (1,000 ppm and 10,000 ppm) of the extract were used. Five ml of each concentration of the extract was applied on filter paper in Petri plates. After evaporation of methanol, 5 ml DW and sterilized (using 0.1% $HgCl_2$) 20 radish seeds were placed in each Petri plate. Petri plates were sealed with parafilm and incubated at 23 ± 2 °C. DDW was used as a negative control. Root length was measured after 1, 3 and 5 days of interval. This experiment was repeated three times.

For seed germination, two different concentrations (1,000 ppm and 7,500) of the extract were used. This part of the experiment is similar to that of earlier experiment except for the extract concentrations and number of seeds. Here, 100 radish seeds were placed on each Petri plate. Germinated seeds were counted after every day upto 5 days. Experiment was repeated three times.

Results

Antitumor potato disc assay

Tumor producing three *A. tumefaciens* strains shows significantly different response from each others across all the concentrations of the plant extracts used, similarly, extract concentration also differ significantly on tumor inhibitions.

Maximum and minimum tumor inhibition was observed against the strain AtSl0105 (16.92-40.76 %) and AtTa0112 (9.83-37.70 %), respectively. No significant tumor inhibition was observed at 10 ppm against the studied strains. Strain AtSl0105 was more prominent for producing tumor (8.6 ± 0.23) compared to strains AtAc0114 (7.2 ± 0.20) and AtTa0112 (6.1 ± 0.25) (Fig. 1).

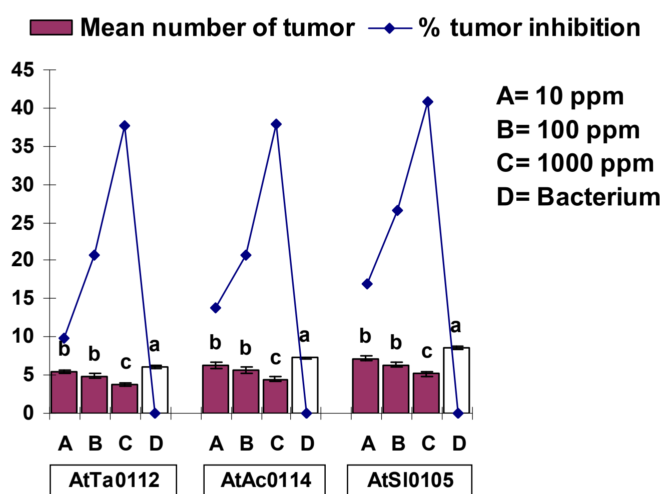

Fig. 1. Comparative graphical presentation of different concentrations of methanol extract of *Croton bonplandianum* and studied three *Agrobacterium tumefaciens* strains on crown gall tumors formation in potato discs.

Antibacterial assay

To justify the results of antitumor potential of the plant extract but not on *Agrobacterium* viability, antibacterial assay was conducted before antitumor activity test. Very high concentration (250 mg/ml) of the plant extract was used for antibacterial assay and no inhibition zone was recorded. Similar results were observed in using antibiotics.

Radish seed phytotoxicity assay

Root length and seeds germination were actively inhibited by the extracts at both concentration (1,000 ppm and 10,000 ppm) (Figs.3,4). On the fifth day, root length inhibitions 14.56±0.34 and 7.16±0.37 were recorded at 1,000 ppm and 10,000 ppm, respectively. On the other hand, seed germinations 45.66±01.20 and 26.66±0.88 were recorded at 1,000 ppm and 7,500 ppm, respectively. These results were calculated compared to the control.

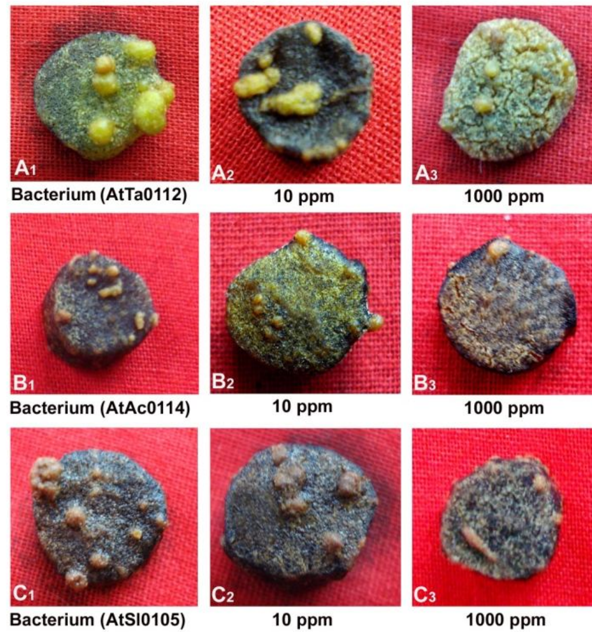

Fig. 1. Photographs showing effect of methanol extracts of *Croton bonplandianum* on crown gall tumors formations by three *Agrobacterium tumefaciens* strains on potato discs. Data were recorded after 21 days of treatment. A_1, B_1 and C_1 showing normal tumor development in abscence of plant extracts (control). A_2,B_2 and C_2 showing reduction of no. Of tumor development in presence of 10 ppm plant extract. A_3,B_3 and C_3 showing further reduction of tumor formation in presence of 1,000 ppm plant extract.

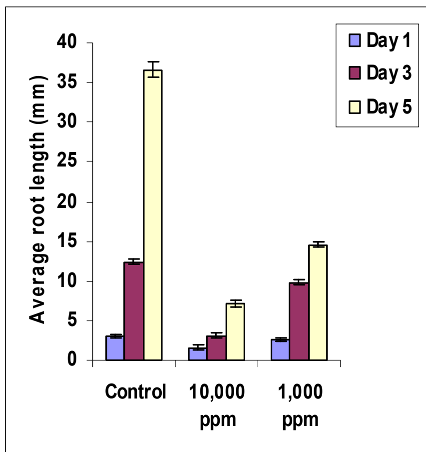

Fig. 3. Histogram showing radish seed phytotoxicity assay on root length at two concentrations of methanol extract of *Croton bonplandianum* including control.

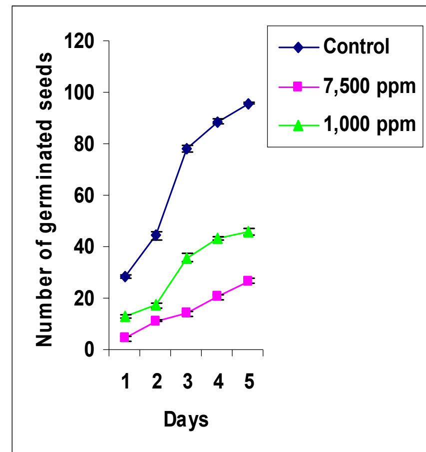

Fig. 4. Graph showing radish seed phytotoxicity assay on percentage of seed germination at two concentrations of methanol extract of *Croton bonplandianum* including control.

Discussion

Antitumor potato disc assay is a valuable tool that indicates antitumor activity of test compound by their inhibition of formation of characteristic crown galls induced in wounded potato tissues by *A. tumefaciens* (Inayatullah *et al*., 2007). This bioassay is a sensitive, bench-top antitumor assay for chemicals that disrupt the cell cycle (mitosis, S phase, etc.) regardless of their mode of action (Coker *et al*., 2003). Several scientists have used these methods over the past 15 years, and they appear to be adaptable to the purpose of standardization or quality control of bioactive compounds in such heterogeneous botanicals (Jerry and Lingling, 1998).

Considering its (*C. bonplandianum*) tremendous important in antitumor potential, the methanol extract was evaluated for antitumor activity with phytotoxic analysis. During the study of antitumor activity test, it was observed that tumor formation was observed when *Agrobacterium* strains were alive on living potato disc. Most often potato discs were damaged due to the contamination and other physiological factors when there was no tumor formation was observed. Thus successful attachment of *Agrobacteirum* on living potato disc is needed for antitumor test of plant extracts.

To justify the results of antitumor potential of the plants extract, antibacterial assay was conducted before antitumor activity test. Very high concentration (250 mg/ml) of the plant extract was used for antibacterial assay and no inhibition zone was recorded. So this result indicates that three was no effect of plant extract on the viability of *A. tumefaciens* (Hussain *et al*., 2007). Three antibiotics (erythromycin, carbenicillin and chloramphenicol) were also studied for further confirmation and similar results were found. These results revealed that tumor formation was decreased only for the bioactive compound (s) present in plant extract not for the other factors.

After successful antitumor activity test for confirmation of bioactive compounds, the methanol extract was evaluated for phytotoxic analysis. Here it was observed that root length and seed germination was actively inhibited by the plant extract even at 1,000 ppm, supported by Inayatullah *et al*. (2007). The maximum inhibition was observed at 10,000 ppm as reported earlier by Turker and Camper (2002). This is proving the similarity between the two phenomenon's (Turker and Camper, 2002; Inayatullah *et al*., 2007).

Comparison of human tumours with plant tumours reveals striking similarities. According to Kempf *et al*. (2002), *Bartonella henselae,* a tumor causing bacteria in human shares a similar pathogenicity strategy with plant pathogens *A. tumefaciens*. Therefore this experiment was carried out to justify the effect of medicinal plant extract on tumor inhibition caused by three

Agrobacterium strains, which would help further to search for new drug development for tumor treatment in human beings.

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