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Antitumor and Phytotoxic Activities of Leaf Methanol Extract of *Oldenlandia diffusa* (Willd.) Roxb

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Abstract: *Oldenlandia diffusa* (Willd.) Roxb., a well known medicinal plant in Bangladesh has been identified for antitumor properties through *Agrobacterium tumefaciens* infection using potato disc bioassay and phytotoxic effects on radish root and seed. Significant tumor inhibition was observed at 100ppm and 1000ppm of leaf methanol extract. Maximum tumor inhibition 40.98, 41.93 and 41.89% were observed at 1000ppm for the accessions of the *Agrobacterium tumefaciens* AtTa0112, AtAc0114 and AtSl0105, respectively. Phytotoxicity assay shows significant root length inhibition by the extract at the concentrations of 1000ppm and 10000ppm. Similarly, seed germinations were also significantly inhibited at the concentrations 1000ppm and 7500ppm extracts. Overall results supported that *Oldenlandia diffusa* might be a potential source of antitumor agent that could be used for further drug development for tumor treatment in human.

Key words: *Oldenlandia diffusa* · Potato disc bioassay · Antitumor

INTRODUCTION

Cancer is one of the most life-threatening diseases and serious public health problems in both developed and developing countries. It is a group of diseases characterized by the disregulate proliferation of abnormal cells that invade and disrupt surrounding tissues [1]. Due to the toxic and adverse side effects of synthetic drugs as well as conventional treatments are being failed to fulfill their objectives (tumor control), for these consequence herbal medicine has made a comeback to improve the fulfillment of our present and future health needs [2]. The development of efficient anticancer agents, such as vinblastine and vincristine isolated from the *Catharanthus roseus* (L.) G. Don, provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents [3].

Oldenlandia diffusa (Rubiaceae) is well known medicinal plant for its tremendous use all over the world including Bangladesh. It has long been use in China for the treatment of hepatitis, tonsillitis, sore throat, appendicitis, urethral infection and malignant tumors of the liver, lung and stomach [4]. Isolated chemical constituents i.e. Oleanolic acid and ursolic acid from this plant species are effective as antibiosis, anticancer, liver protection and transaminase degrading agents [5-7]. Pharmacological studies have demonstrated that *O. diffusa* has antitumor, immunomodulatory [8-10], anti-inflammatory, hepatoprotective [11], anti-oxidative [12, 13] and neuroprotective [14] activities. The aqueous extract of this plant is effective in inhibiting the growth of eight different cancer cell lines and inducing apoptosis reported by Gupta, *et al*. [15].

Different bioassays offer vast advantages for screening of medicinal plant extracts for different purposes i.e. antitumor, antibacterial, antioxidant, phytotoxic properties. Potato disc bioassay is one of them that are developed based on *Agrobacterium tumefaciens* infection on potato disc is useful for checking antitumor properties of plant extract. The rationale for the use of the bioassay is that the tumorogenic mechanism initiated in plant tissues by *Agrobacterium tumefaciens* is in many ways similar to that of animals [16]. According to Kempf, *et al*. [17], *Bartonella henselae*, a tumor causing bacteria in human shares a similar pathogenicity strategy with plant pathogens *A. tumefaciens*. Several scientists have used this method 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 [18]. According to leading

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background of antitumor potential of *O. diffusa*, the present study was under taken to assess specifically leaf methanol extracts for antitumor and phytotoxic properties.

MATERIALS AND METHODS

Plant Material: Fresh leaves of *Oldenlandia diffusa* were collected from Rajshahi University campus. This plant species was taxonomically identified by Dr. A.H.M. Mahbubur Rahman (Taxonomist), Assistant Professor, Department of Botany, Rajshahi University, Bangladesh.

***Agrobacterium* Strains:** Three *A. tumefaciens* strains namely AtTa0112, AtAc0114 and AtSl0105 {isolated from crown gall sample and identified in the Biotechnology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Bangladesh using standard procedure [19-22]} were used during antitumor and antibacterial assay.

Bacterial Culture Preparation: *Agrobacterium* strains were cultured on Luria-Bertani (LB) agar medium which was prepared using the following compositions: 10g of yeast extract+10g of bacto peptone+5g of NaCl+20g of agar dissolved in 1 liter of water according to Devi, *et al*. [23]. Single colony was transferred into LB broth and incubated at 30°C for 48 h. Six to seven loops of bacterial suspensions (1.0×10^9 cfu) were transferred into sterilize phosphate buffer saline (PBS) and this was used during antitumor assay as inoculums.

Extract Preparation: Collected plant materials directly crushed into small pieces and sun drying until reducing water content followed to pulverize using electric blender (Nokia, Osaka-Japan). Fifty gram powder was dipped into 250ml methanol into a conical flask with rubber corks and left for two days with occasional shaking. Filtration was done through Teton cloth and Whatman No. 1 filter paper, respectively. Filtrates were collected in a beaker and dried up to semisolid using Water bath (4 holes analogue, Thermostatic water bath, China). Semi solid extracts were then dissolved into solvent and prepared particular concentration for antitumor (10ppm, 100ppm and 1000ppm) and relevant study (antibacterial assay-250mg/ml and Phytotoxicity assay-1000ppm, 7500ppm and 10000ppm).

Antibacterial Assay: Before antitumor study, antibacterial assay of leaf methanol extract of *O. diffusa* was performed to check viability of *Agrobacterium* strains using agar disc diffusion assay [24, 25]. Sterilized Whatman No. 1 filter paper discs (6mm in diameter) were impregnated with 10µl of tested extract (250mg/ml) as well as antibiotics (Kanamycin (30µg/ml), Cefotaxime (30µg/ml) followed by air dried and placed on seeded LB agar plates and incubated at 37°C for 24 hours. 20µl of bacterial suspension (1.0×10^9cfu) was used for preparing seeded LB agar plates and negative controls were prepared using solvent only. After incubation, the antibacterial activity of *O. diffusa* was determined by measuring zone of inhibition against all the studied *Agrobacterium* strains.

Antitumor Potato Disc Assay: Methanol extract of *O. diffusa* was evaluated for antitumor properties using potato disc bioassay described by Turker and Camper [26] and Hussain, *et al*. [27]. Sterilized potatoes (*Solanum tuberosum* L., Solanaceae) were cut into 5mm×8mm in size from the center of potato tissue by sterilize cork borer. Each potato disc was overlaid with 50µl of appropriate inoculums. Following design was used for preparing inoculums: 600µl test extract+150µl Double Distilled Water (DDW)+750µl *A. tumefaciens* in PBS. Camptothecin (30ppm) was used as positive control replacing test extract. After inoculation, Petri dishes were sealed by parafilm and incubated at 27-30°C for 3 weeks. Tumors were observed on potato discs after 21 days under stereo microscope followed by staining with Lugol's iodine (10% KI and 5% I_2) after 30 minutes, where the tumors cells lack starch [28] and each experiment was carried out in triplicate. Percentage of tumor inhibition was calculated using standard formula described by Hussain, *et al*. [27]. Usually, =20% inhibition of tumor is considered as a significant value for plant extracts [29]. Data were statistically analyzed using MSTAT software version 2.10 (Russel, D. Freed, Michigan State University, USA).

Radish Seed Phytotoxicity Assay: To evaluate phytotoxic properties of leaf methanol extract of *O. diffusa*, radish seed phytotoxicity assay was performed [26]. Two types of determination were done for this purpose:-

For Root Length Determination: Whatman No. 1 filter paper kept on Petri dish and 5 ml extracts (1000ppm and 10000ppm) were added separately. Filter paper was dried at room temperature for reducing extra solvent. 5 ml DDW was added and then 20 radish seeds were placed on Petri dishes followed by tightly sealed and incubation at 23±2°C. Root length was measured after 1, 3 and 5 days of interval. Only DDW containing Petri dish was used as control. Each assay was carried out in three times.

For Seed Germination Determination: This part of the determination is similar to that of earlier determination except for the extract concentrations and number of seeds. Here two different concentrations (1000ppm and 7500ppm) and 100 radish seeds were used. Germinated seeds were counted after every day up to 5 days. Each experiment was carried out in three times.

RESULTS AND DISCUSSION

Drug discovery is the key attempt of our age to overcome many life-threatening diseases like cancer. Plant-based compounds have been playing an important role in the development of several clinically useful anti-cancer agents i.e. including taxol, vinblas-tine, vincristine, the camptothecin derivatives, topotecan and irinotecan and etoposide derived from epipodophyllotoxin [30]. *Oldenlandia diffusa* is the valuable medicinal plant for its multipurpose uses. The present study has been undertaken to identify this plant as a source of phytotoxic and antitumor agent, based on the previous antitumor [8-10], as well as anticancer [15] reports. Specifically leaf methanol extract of *O. diffusa* has been studied using different bioassay for this purpose.

Antibacterial Assay: This portion of this study was done to justify whether plant extract is lethal for gene transformation system of *Agrobacterium tumefaciens* or has no effect because genetic transformation system is required for finally induction tumor. Results of this assay confirmed extract has no effect on the viability of *Agrobacterium* strains because no zone of inhibition zone was recorded against all the studied *A. tumefaciens* strains. Whereas, inhibition zone was recorded for only studied antibiotic kanamycin and Cefotaxime (positive control), negative control did not show any visible zone of inhibition (Figure not shown) as expected. Other studies [31, 32] also represented that *Agrobacterium* is susceptible to kanamycin and Cefotaxime.

Antitumor Potato Disc Assay: Results showed that leaf methanol extract of *O. diffusa* inhibit tumor growth in highly significant way in a concentration dependent manner across the strains (Table 1 and Table 2). Significant tumor inhibition was observed at 100ppm and 1000ppm concentrations, but not at 10ppm. Maximum 40.98, 41.93 and 41.89% and minimum 13.66, 14.28 and 13.43% tumor inhibition were recorded for AtTa0112, AtAc0114 and AtSl0105 *Agrobacterium* strains,

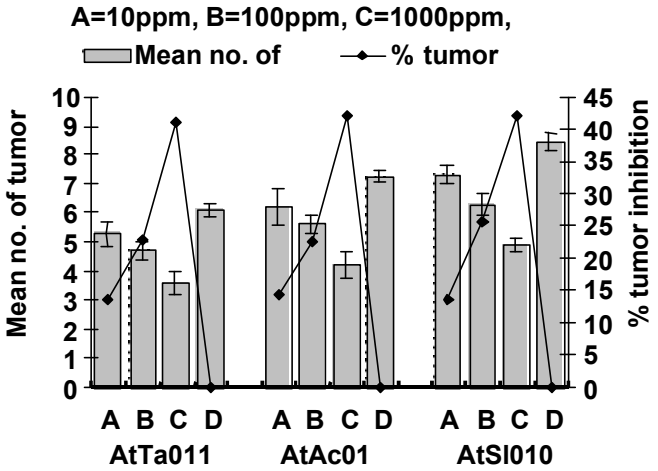

Fig. 1: Figure shows percentage of tumor inhibition by the leaf methanol extract of *O. diffusa* on potato disc at different concentrations (10ppm, 100ppm and 1000ppm)

Table 1: Statistical analysis of tumor inhibition by the leaf methanol extract of *O. diffusa* and tumor induction by *A. tumefaciens* strains on potato discs

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value	Prob
Strains (S)	2	19.62	9.81	23.71	0.0000
Concentration (C)	3	43.71	14.57	35.22	0.0000
S×C	6	1.01	0.16	0.40	
Error	24	9.92	0.41		
Total	35	74.27			

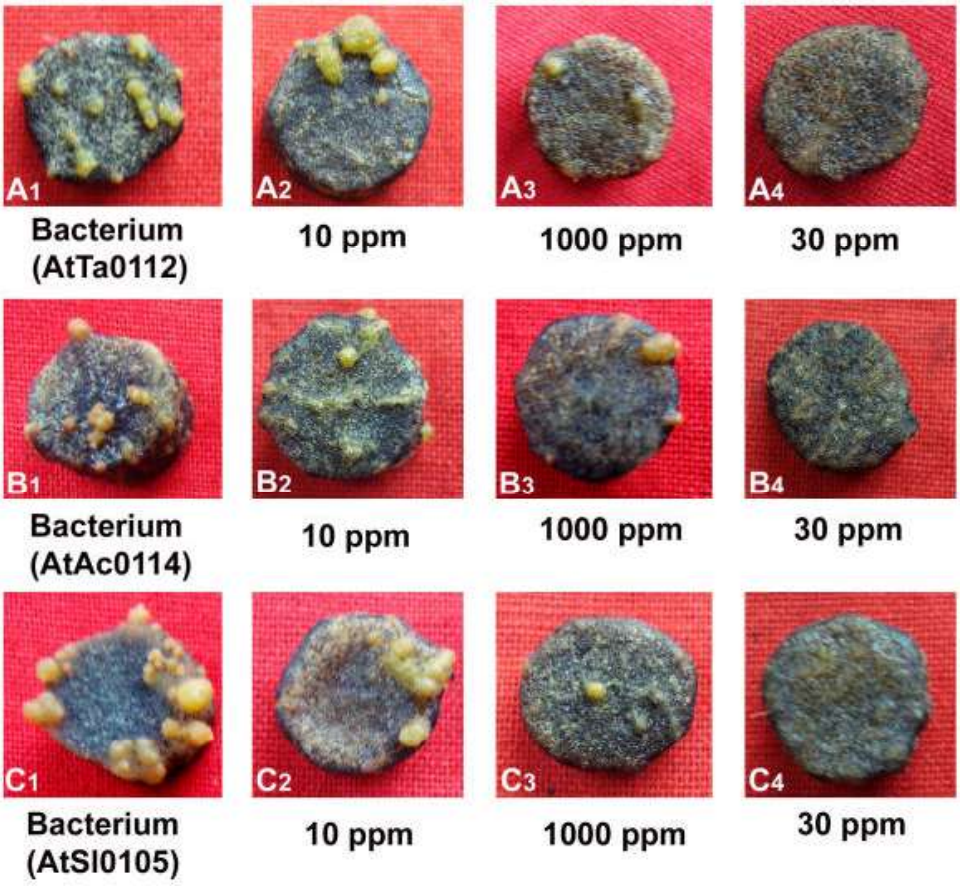

Plate 1: Photographs show gradual tumor inhibition by the leaf methanol extract of *O. diffusa* on potato discs in a concentration as well as strain dependent manner. Data were compared with control. A_1 B_1 and C_1 as negative control and A_2 B_2 and C_2 as 10ppm, A_3 B_3 and C_3 as 1000ppm plant extract and A_4 B_4 and C_4 as 30ppm Camptothecin (positive control)

Table 2: Analysis of mean data of antitumor activity of leaf methanol extract of *O. diffusa*

Variables	Mean no. of Gall
Strains	
AtSl0105	6.72 A
AtAc0114	5.80 AB
AtTa0112	4.91 B
LSD value	0.9390
Concentration	
Negative control	7.25 A
10ppm	6.25 AB
100ppm	5.52 B
1000ppm	4.23 C
LSD value	1.084

Means followed by different letter (S) down the column are significantly different among the concentration as well as days at $p<0.05$. Data values are means of three replicates

respectively (Fig. 1). It was also observed that *A. tumefaciens* AtSl0105 strains was more prominent for producing tumor (8.4±0.32) than other strains AtAc0114 (7.2 ± 0.20) and AtTa0112 (6.1 ± 0.25) suggests their differential sensitivity (Fig. 1 and Plate 1). Hussain, *et al*. [27] have also shown that tumor inhibition rate on potato discs are dependent on concentration of plant extract and also tumor producing *A. tumefaciens* strains. In the present investigation, Camptothecin served as a positive control and 100% tumor inhibition was observed. Similar phenomenon was observed by Turker and Camper [26]. This result may be attributed due to its DNA damaging activities. Camptothecin is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I (topo I) isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca, Happy tree) [33].

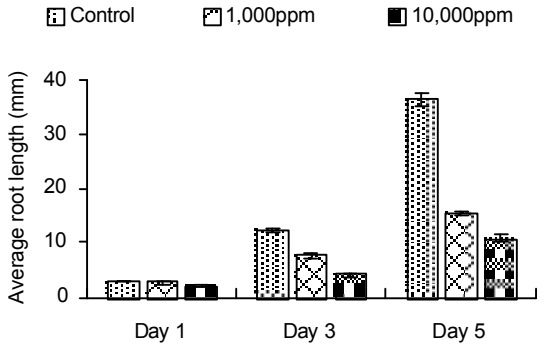

Fig. 2: Histogram shows regular root length inhibition by the leaf methanol extract at two different concentrations (1000ppm and 10000ppm) of *O. diffusa*. Data compared with control.

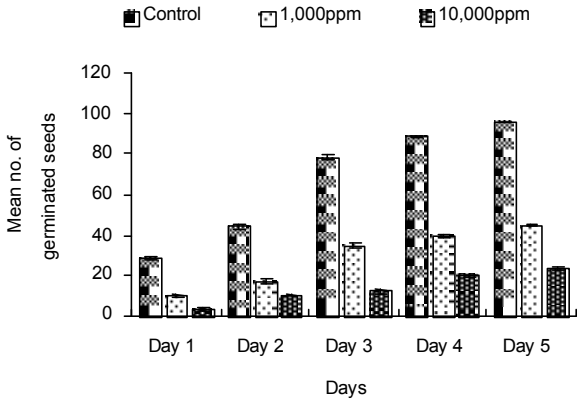

Fig. 3: Graph shows phytotoxicity assay on radish seed germination percentage at two different concentrations (1000ppm and 7500ppm) of leaf methanol extract of *O. diffusa*. Data compared with control.

Radish Seed Phytotoxicity Assay: Statistical analysis shows that root length were significantly inhibited by the extracts at both the concentrations 1000ppm and 10000ppm (Fig. 2 and Table 3). In another cases, seed germinations were also significantly inhibited by the both concentrations 1000ppm and 7500ppm (Fig. 3 and Table 4). These results were analyzed compared with control. These observations may be accomplished due to the presence of active biological compounds. Different studies have shown many secondary metabolites as a source of bioactive compounds with allelochemical potential have great chemical diversity and are involved in many metabolic and ecological processes [34-36]. In drug discovery, the major secondary metabolites (terpenoids, phenolics and alkaloids) are of potential

Table 3: Analysis of mean data of root length inhibition by leaf methanol extract of *O. diffusa*

Variables	Root length (mm)
Concentration	
Control	17.30 A
1,000ppm	8.722 B
10,000ppm	5.711 C
LSD value	1.973
Days	
Day 1	2.589 C
Day 3	8.131 B
Day 5	21.01 A
LSD value	2.547

Means followed by different letter (S) down the column are significantly different among the concentration as well as days at $p<0.05$. Data values are means of three replicates.

Table 4: Analysis of mean data of seed germination, inhibition by leaf methanol extract of *O. diffusa*

Variables	% seed germination
Concentration	
Control	67.00 A
1,000ppm	30.00 B
7,500ppm	15.07 C
LSD value	1.973
Days	
Day 1	14.00 E
Day 2	26.22 D
Day 3	42.67 C
Day 4	49.33 B
Day 5	54.56 A
LSD value	2.547

Means followed by different letter (S) down the column are significantly different among the concentration as well as days at $p<0.05$. Data values are means of three replicates

medicinal interest. The mentioned structure diversity is reflected in a variety of biological activities as, for instance, inhibitors of enzymes and antitumor, immunosuppressive and antiparasitic agents [37, 38].

Above mention discussions have provided knowledge that *O. diffusa* might be potential source of antitumor and phytotoxic properties. These findings may be attributed to the nature of biological active compounds and their strong solubility with appropriate solvent. In the present investigation, plant extract was prepared using methanol as a solvent. It is well documented that alcohols (ethanol, methanol) used as a solvent for plant extract

preparation for their strongly extraction power. However, sometimes it is often better to use alcohols (methanol, ethanol) or hydroalcoholic solutions after partial lipid removal [39]. Many researchers have already been used methanol or ethanol as a solvent for evaluating cytotoxicity, phytotoxicity, antibacterial, antitumor activity in several plant species [26, 27, 40].

Crown gall is a neoplastic disease of plants causative agent of this disease is the Gram-negative bacterium *A. tumefaciens* [41]. 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 [42]. Galsky, *et al*. [41] first demonstrated that inhibition of Crown Gall tumor distribution on potato discs correlated with compounds and plant extracts known to be active in the 3PS leukemic mouse tumor assay. The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals [43-45]. The use of this bioassay has resulted in many short lists of plants with anti-cancer activity and has helped with the discovery of novel compounds from plants [27, 29, 46, 47].

CONCLUSION

Tumor inhibition on potato disc and significant phytotoxicity by the leaf methanol extract of *O. diffusa* providing knowledge that it might be a potential source of antitumor agent. Further study is required for isolating specific compound.

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