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Comparative study on antitumor activity of three pteridophytes ethanol extracts

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The extracts from twigs of three Pteridophytes (locally available) viz. *Selaginella ciliaris* (Retz.), *Marsilea minuta* (L.) and *Thelypteris prolifera* (Retz.) were investigated for antitumor properties. Among the species, highest percentage of tumor inhibition was found in *M. minuta* (82.32%) followed by *S. ciliaris* (80%) and *T. prolifera* (75.68%) at 1000ppm, and significant tumor inhibition was observed at 10, 100 and 1000 ppm of plant extracts on potato disc induced by *Agrobacterium tumefaciens* indicating their presence of tumor inhibitor metabolites. These metabolites may serve an important role in developing antitumor drugs for human beings, as there is a similarity between human and plant tumor formation mechanism.

Key Words: Antitumor activity, Pteridophytes, potato disc bioassay, *Agrobacterium tumefaciens, A. tumefaciens* sensitivity test.

Introduction

During the course of infection, a defined portion of the Ti- plasmid (T-DNA) is stably transferred to plant cell genome where it is integrated and expressed (Thomashow *et al.*, 1980) and expression of the integrated portion leads to the formation of neoplastic cell which forms the crown-gall disease (Al-Momani *et al.*, 2006). The relevance of the crown-gall tumor system to the general cancer problem has been thoroughly reviewed (Cloud *et al.*, 1974). 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 (Lippincorr and Heberlein, 1965).

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Crown-gall is a neoplastic disease of plants caused by *A. tumefaciens* following by the transfer and expression of its special type of DNA segment (T-DNA) in the plant genome through type IV secretion system (T4SS) (Zupan *et al.*, 2000). T4SS is also used by other pathogenic bacteria to deliver macromolecules detrimental to the host like plant, animal and human (Cascales and Christie, 2003). Among those, *Bartonella henselae* (Kempf *et al.*, 2002) and *Helicobacter pylori* (Raderer *et al*., 1998), tumor causing bacteria in human share a similar pathogenicity strategy to plant pathogen *A. tumefaciens* (Zhu *et al.*, 2000). The above mentioned relation and previous studies have documented the similarities between crown-gall tumors and animal cancer, especially the correlation between antileukemic activity and inhibition of crown-gall tumor formation on potato discs by some medicinal herbs (Anderson *et al.*, 1988). Potato disc is a useful test for monitoring the inhibition of crown-gall tumors (McLaughlin, 1991). The inhibition of crown-gall tumor initiation on potato discs showed good agreement with compounds and plant extracts known to be active in the 3PS (*in vivo*, mouse leukemia) antitumor assay (Galsky *et al.*, 1980) and it also the inhibition of tumors growth agrees well with 3PS activity (Galsky *et al.*, 1981).

Plant derived drugs serves as a prototype to develop more effective and less toxic medicines (Aswar *et al.*, 2008). So, if the effective plant extract would be find out for the inhibition of tumor forming mechanism, it would be used in drug developmental research for tumor treatment in human. Some medium polarity extracts of different *Pteris* species showed effective antitumor and antibacterial activity (Gong *et al*., 2007) and *Dryopteris* spp. are generally characterized by the presence of phloroglucinol derivatives (flavaspidic acids, triflavaspidic acids, dryocrassins, albaspidins and filixic acids). Chemical investigation of more than 30 species of the Pteridaceae has been reported (Chen *et al.*, 2008). So, considering of its tremendous importance, it is very necessary to identify the native effective plant's extract for tumor treatment in human.

Materials and methods

Plant material

Three Pteridophytes (Fig.1): *Selaginella ciliaris* (Retz.), *Marsilea minuta* (L.) and *Thelypteris prolifera* (Retz.) collected form different places of Rajshahi University Campus, were used as plant material. Taxonomic identification of these plants was confirmed by Dr. Sabrina Naz, Professor, Department of Botany, Rajshahi University, Rajshahi-6205, Bangladesh. Twigs were used for solvent extraction.

Preparation of plant extracts

It was carried out according to plant extraction method (Ahmad and Beg, 2001) with some modification. Collected twigs were rinsed well with tap and distilled water (DW), and kept under shade still drying. Dried material powdered coarsely using mortar and pestle followed by oven dry and further reduced to fine powder using electric blender (Nokia, Osaka-Japan) and stored in air tight glass container. Powder (50 g) was then dissolved in ethanol by allowing to sediment at room temperature (27-30 ^{0}C) for 7 days with occasional shaking. For the filtration of materials, teton cloth and Whatman no. 1 filter paper were used, respectively. Filtrates were then transferred into glass beaker and dried into semi-solid material using water bath (4 holes analogue, Thermostatic water bath, China). Particular concentrations (10 ppm, 100 ppm and 1,000 ppm; Note: 1 ppm = 1 mg L^{-1}) of the plant extracts were prepared using ethanol for antitumor activity test, and 250,000 ppm was prepared for *A. tumefaciens* sensitivity test.

Antitumor potato disc bioassay

Antitumor assay of plant extracts was performed according to standard potato disc bioassay (Hussain *et al.*, 2007). *A. tumefaciens* strain named AtTp0120 (isolated and identified in our laboratory) was 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 into 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 sterilized distilled water (SDW) + 750 µl *A. tumefaciens* in PBS. Camptothecin (Conc. 30 ppm) 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. Potato tubers were cut into 8 mm diameter in size cylindrical pieces using cork borer and transferred into SDW containing conical flask. After washing, the cylindrical segments were cut into 5 mm × 8 mm disc and placed onto agar (15 g/L) plates (10 discs per plate) and 50 µl of appropriate inoculum were placed on the surface of each potato disc. The peti plates were sealed with parafilm and incubated at room temperature at 27-30 ^{0}C. 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 three times. Percent inhibition of tumors was calculated (McLaughlin and Rogers, 1998). More than 20% tumor inhibition is

considered significant (Ferrigni *et al.*, 1982). Data were analyzed using MS excel-2003.

Sensitivity test of A. tumefaciens (as a partial assay)

Disc diffusion assay (Kirby-Bauer Method) was used to screening *A. tumefaciens* sensitivity test by studied plant ethanol extracts (Barry, 1980). Ethanol (75%) was used as negative control, and Kanamycin (30 µg ml^{-1}), Cefuroxime (30 µg ml^{-1}), Tetracycline (30 µg ml^{-1}) and Rifampicin (10 µm ml^{-1}) were used as positive control. Discs (Whatman No. 1 filter paper) were impregnated with 10 µl of the extracts (250 mg ml^{-1}= 250,000 ppm), antibiotics and Ethanol 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 ^{0}C for 24 hours. The sensitivity test was evaluated by the measurement of inhibition zone's diameter (mm) against *A. tumefaciens* strain (AtTp0120). Each assay was carried out in triplicates.

Results

Results of antitumor activity of studied three plant (Fig.1) extracts are described under the following heads

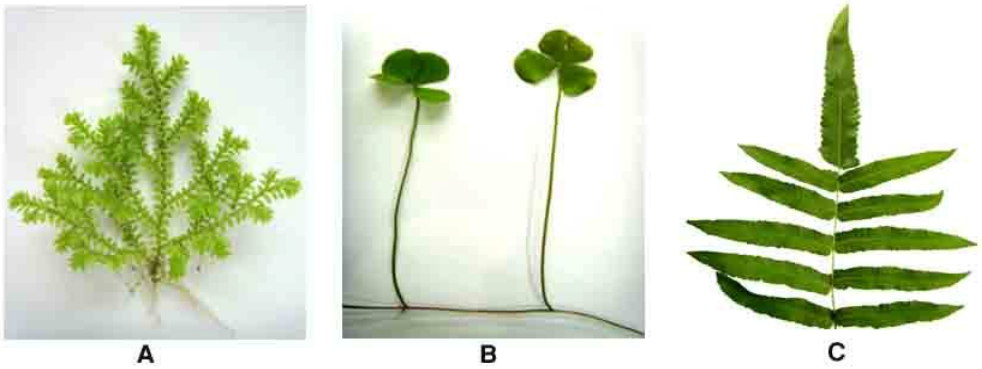

Fig 1. Vegetative parts of studied Pteridophytes: A. *Selaginella ciliaris* (Retz.), B. *Marsilea minuta* (L.), C. *Thelypteris prolifera* (Retz.).

Sensitivity test of A. tumefaciens (as a partial assay)

Before antitumor activity test, the sensitivity test was performed against *A. tumefaciens* strain (AtTp0120) to check out its viability against plant extracts. Very high concentration (250 mg ml^{-1} or 250,000 ppm) of the studied

plant ethanol extracts and comparatively low concentration 30 µg ml^{-1} for kanamycin, 30 µg ml^{-1} for Cefuroxime, 10 µg ml^{-1} for rifampicin and 30 µg ml^{-1} for tetracycline were used for antibacterial assay.

Studied three Pteridophytes ethanol extracts showed no effect on the viability of *A. tumefaciens* strain (AtTp0120), because there was no inhibition zone observed against plant extracts. On the other hand, *A. tumefaciens* strain (AtTp0120) was susceptible to kanamycin and Cefuroxime, but, was resistant to rifampicin and tetracycline during the study of *A. tumefaciens* sensitivity test (Fig.2).

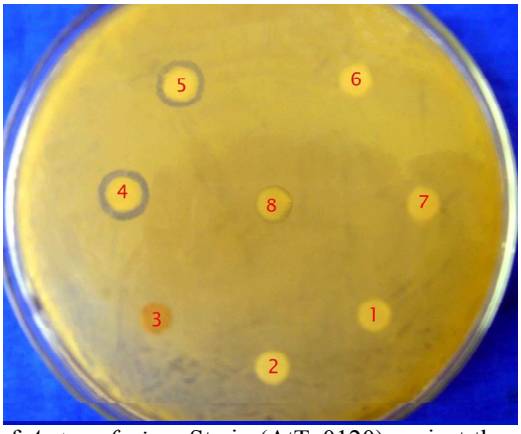

Fig 2. Sensitivity test of *A. tumefaciens* Strain (AtTp0120) against three pteridophytes ethanol extracts and four antibiotics. Note: Plant extracts (250 mg ml^{-1}or 250,000 ppm): 1:*Selaginella ciliaris* (Retz.), 2:*Marsilea minuta* (L.), 3:*Thelypteris prolifera* (Retz.); Antibiotics: 4:Kanamycin (30 µg ml^{-1}), 5:Cefuroxime (30 µg ml^{-1}), 6:Rifampicin (10 µg ml^{-1}), 7:Tetracycline (30 µg ml^{-1}), 8:Ethanol (75%).

Antitumor potato disc bioassay

Comparatively low concentrations (10, 100 and 1000 ppm) of plant extracts were used for antitumor activity test. Significant tumor inhibition was observed at 10, 100 and 1000 ppm for all the studied plant extracts. 100% tumor inhibition was observed in camptothecin (positive control) (Fig.4).

Among the studied three Pteridophytes, *Marsilea minuta* and *Thelypteris prolifera* showed the highest (82.32323%) and lowest (75.67568%) percentage of tumor inhibition, respectively at 1000 ppm concentration of ethanol extracts (Table 1; Fig.3, 4).

Table 1. The effect of three Pteridophytes (viz. *Selaginella ciliaris, Marsilea minuta and Thelypteris prolifera*) ethanol extracts on crown gall tumor formation by *A. tumefaciens* Strain (AtTp0120) on potato discs

Name of the Pteridophytes	Concentrations (ppm)	Percentage of Tumor Inhibition (%)	Mean no. of Gall	Standard Error (SE)
S. ciliaris	10	28.15	6.47	0.49
	100	51.48	4.37	0.47
	1000	80	1.8	0.12
M. minuta	10	29.29	4.67	0.22
	100	41.92	3.83	0.12
	1000	82.32	1.17	0.17
T. prolifera	10	28.10	4.43	0.29
	100	37.29	3.87	0.59
	1000	75.68	1.5	0.29
Control (average value)	Bacterium (negative control)	0	7.23	0.26
	30 ppm of Camptothecin (positive control)	100	0	0.20

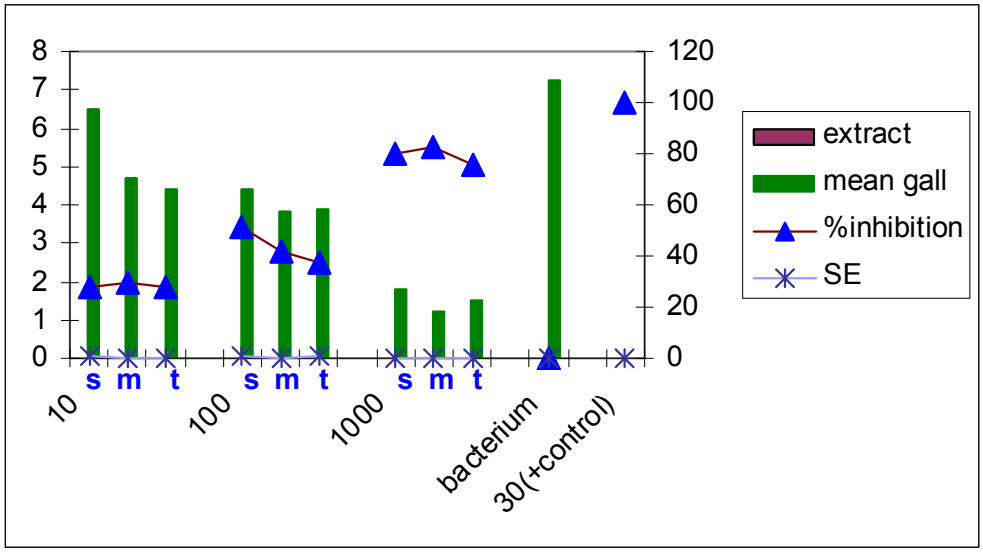

Fig 3. Graphical presentation showing the effect of three Pteridophytes ethanol extracts (Concentrations: 10, 100 and 1000 ppm) against the crown gall tumor formation by *A. tumefaciens* strain (AtTp0120). Note: s: *Selaginella ciliaris*, m:*Marsilea minuta*, t:*Thelypteris prolifera*.

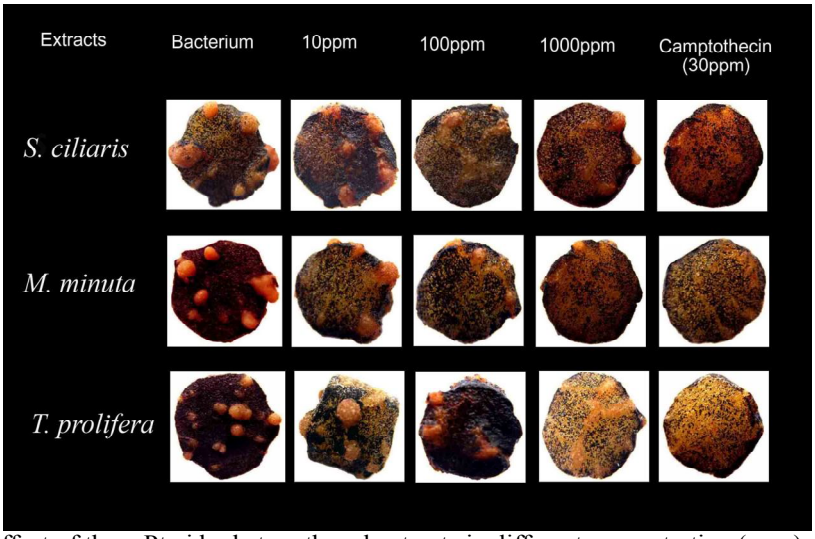

Fig 4. Effect of three Pteridophytes ethanol extracts in different concentration (ppm) on crown gall tumors produced by *A. tumefaciens* strain (AtTp0120) on potato discs including with negative (bacterium) and positive (camptothecin) control.

Discussion

Considering the tremendous medicinal value and ethnobotanical uses of Pteridophytes, and their biodiversity, ecological adaptation, local availability in Bangladesh it is justified to work their aspect. Nowadays it is very much necessary to find out the effective medicinal value of native plants species. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (Dubey *et al.*, 2004). Antitumor properties of three wild species of Pteridophytes have been proven through *A. tumefaciens* infection using potato disc bioassay.

A. tumefaciens sensitivity assay of tested plants extract was conducted before checking their antitumor activity. For this very high concentration (250,000 ppm) of the plant extract was used and no inhibition zone was recorded. This was further confirmed by using antibiotics viz. Kanamycin (30 µg ml^{-1}), Cefuroxime (30 µg ml^{-1}), Rifampicin (10 µg ml^{-1}) and Tetracycline (30 µg ml^{-1}) and Ethanol (75%). *A. tumefaciens* strain (AtTp0120) was susceptible to kanamycin and Cefuroxime. This result indicated that there was no killing or inhibitory effect of plant extracts on *A. tumefaciens* growth. So these results revealed that tumor formation was decreased only for the plant extracts not for the other factors. Such types of result were observed by Turker and Camper (2002), Hussain *et al.* (2007) and Inayatullah *et al.* (2007). Generally antibacterial assay is performed for sensitivity test of bacteria on

plant extracts or others materials. Hussain *et al.* (2007) demonstrated antibacterial assay against *A. tumefaciens* to check whether extracts are lethal for bacteria or are inhibiting at any level that is necessary for the genetic transfer mechanism and finally induction of tumor.

Camptothecin (CPT) is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase-I (topo I) and Wall *et al*. (1966) discovered it in systematic screening of natural products for anticancer drugs and it was isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca, Happy tree), a tree native in China. CPT showed remarkable anticancer activity in preliminary clinical trials but also showed low solubility and adverse drug reaction. Because of these disadvantages researchers have made numbers of derivatives to increase the benefits of the chemical, with good results. Two CPT analogues have been approved and are used in cancer chemotherapy today, topotecan and irinotecan (Wall *et al.*, 1966). In the present investigation, camptothecin served as a positive control and inhibited tumor production at all the concentrations tested. Similar result was also observed by Turker and Camper (2002).

Plant extracts were prepared using ethanol (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 (ethanol, methanol) or hydroalcoholic solutions after partial lipid removal (Marston and Hostettmann, 1991). Many researchers have already been used ethanol or methanol as a solvent for evaluating cytotoxicity, phytotoxicity, antibacterial, antitumor activity in several plant species (Turker and Camper, 2002; Hussain *et al.*, 2007; Inayatullah *et al.*, 2007).

During the study of antitumor activity test, it was observed that tumor formation was observed when *A. tumefaciens* strain (AtTp0120) was 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. This result indicates that living substrates (cell) with *A. tumefaciens* is very much needed for finally induction tumor on potato disc. The attachment of the bacterium to a tumor-binding site is completed within 15 min following inoculation (McLaughlin *et al.*, 1993). Turker and Camper (2002) described that inhibition of tumor formation in the potato disc assay could result from either anti-tumorigenesis or by affecting the viability of *A. tumefaciens*. Results of *A. tumefaciens* sensitivity test showed that studied extracts had no effect on the viability of the bacterium. So tumor formations were inhibited by the plant extracts only for the presence of bioactive compound.

Antitumor potato disc assay is a valuable tool that indicates antitumor activity of test compound by their inhibition of characteristic crown galls formation 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). The potato disc assay demonstrates the inhibition of tumor formation on potato discs; materials 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). 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). 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 (Ahsan *et al*., 2007; Islam *et al*., 2010). These metabolites from three tested plants would be further elucidated the chemical structures to know the bioactive pure compounds.

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References

Ahmad, I. and Beg, Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 74: 87-91.

Ahsan, U., Ansari, F.L., Ihsan-ul-Haq, Nazir, S. and Mirza, B. (2007). Combinatorial synthesis, lead identification, and antitumor study of a chalcone-based positional-scanning library. Chemistry and Biodiversity 4: 203-214.

Al-Momani, F., Albasheer, S. and Saadoun, I. (2006). Distribution of *Agrobacterium tumefaciens* Biovars in Jordan and Variation ofVirulence. Plant Pathology Journal 22(4): 318-322.

Anderson, W.K., Heider, A.R., Raju, N. and Yucht, J.A. (1988). Vinylogous carbinolamine tumor inhibitors. 23. Synthesis and antileukemic activity of bis[[(carbamoyl)oxy]methyl]-substituted pyrrolo[2,1-a]isoquinolines, pyrrolo[1,2-a]quinolines, pyrrolo[2,1-a]isobenzazepines, and pyrrolo[1,2-a]benzazepines. Journal of Medicinal Chemistry 31(11): 2097-2102.

Aswar, M., Aswar, U., Wagh, A.B., Watkar, M.V. and Gujar, K.N. (2008). Antimicrobial activity of Ficus Benghalensis. Pharmacologyonline 2: 715-725.

Barry, A.L. (1980). Procedure for testing antimicrobial agent in agar media. In: Lorian V (ed) Antibiotica in laboratory medicines, Willims and Wilkins Co. Baltimore, USA.
Cascales, E. and Christie, P.J. (2003). The versatile bacterial type IV secretion systems. Nature Reviews Microbiology 1: 137-150.
Chen, Y., Chang, F., Lu, M., Hsieh, P., Wu, M., Du, Y. and Wu, Y. (2008). New Benzoyl Glucosides and Cytotoxic Pterosin Sesquiterpenes from *Pteris ensiformis* Burm. Molecules 13: 255-266.
Cloud, W. (1974). Stalking the wild crown-gall. In S Wilson, R Roe (eds) Readings in the Life Sciences Biology Anthology. West Publishing Co, (New York).
Coker, P.S., Radecke, J., Guy, C. and Camper, N.D. (2003). Potato tumor induction assay: a multiple mode of action drug assay. Phytomedicine 10: 133-138.
Dubey, N.K., Kumar, R. and Tripathi, P. (2004). Global promotion of herbal medicines: India's opportunity. Current Science 86: 37-41.
Ferrigni, N.R., Putnam, J.E., Anderson, B., Jacobsen, L.B., Nichols, D.E., Moore, D.S. and McLaughlin, J.L. (1982). Modification and evaluation of the potato disc assay and antitumor screening of Euphorbiaceae seeds. Journal of Natural Products 45: 679-686.
Galsky, A.B., Kozimor, R., Piotrowski, D. and Powell, R.G. (1981). The crown-gall potato disc bioassay as a preliminary screen for compounds with antitumor activity. Journal of the National Cancer Institute 67: 689-692.
Galsky, A.B., Wilsey, J.P. and Powell, R.G. (1980). Crown-gall tumor disc bioassay: a possible aid in the detection of compounds with antitumor activity. Plant Physiology 65: 184-185.
Gong, X.L., Chen, Z.H. and Liang, N.C. (2007). Advances in study on chemical constituents and pharmacological activities of plants of genus *Pteris*. Zhongguo Zhong Yao Za Zhi 32: 1382-1387.
Hussain, A., Zia, M. and Mirza, B. (2007). Cytotoxic and antitumor potential of *Fagonia cretica* L. Turkish Journal Biology 31: 19-24.
Inayatullah, S., Irum. R., Ateeq-ur-Rehman, M., Chaudhury, F. and Mirza, B. (2007). Biological evaluation of some selected plant species of Pakistan. Pharmaceutical Biology 45(5): 397-407.
Islam, M.S., Rahman, M.M., Rahman, M.A., Qayum, M.A. and Alam, M.F. (2010). *In vitro* evaluation of Croton bonplandianum Baill. as potential antitumor properties using *Agrobacterium tumefaciens*. Journal of Agricultural Technology 6(1): 79-86.
Jerry, L.M. and Lingling, L.R. (1998). The use of biological assays to evaluate botanicals. Drug Information Journal 32: 513-524.
Kempf, V.A.J., Hitziger, N., Riess, T. and Autenrieth, I.B. (2002). Do plant and human pathogens have a common pathogenicity strategy? Trends in Microbiology 10(6): 269-275.
Lippincorr, J.A. and Heberlein, G.T. (1965). The quantitative determination of the infectivity of *Agrobacterium tumefaciens*. American Journal of Botany 52: 863.
Marston, A. and Hostettmann, K. (1991). Plant saponins: chemistry and molluscicidal action. In: Harborne JB, Tomas-Barberan FA (Eds), Ecological Chemistry and Biochemistry of Plant Terpenoids. Clarendon Press, Oxford. pp. 265-266.
McLaughlin, J.L. (1991). Crown gall tumors on potato discs and bine shrimp lethality: Two single bioassays for plant screening and fractionation. In Hostettmann K (ed) Methods in Plant Biochemistry, Academic Press, London. 6: 1-31.
McLaughlin, J.L. and Rogers, L.L. (1998). The use of biological assays to evaluate botanicals. Drug Information Journal 32: 513-524.

McLaughlin, J.L., Chang, C.J. and Smith, D.L. (1993). Simple bench-top bioassay (brine shrimp and potato discs) for the discovery of plant antitumor compounds. In: Kinghorn AD, Balandrin MF (eds) Human Medicinal Agents from Plants. ACS Symposium. Washington, D.C., American Chemical Society 534: 112-137.

Raderer, M., Wrba, F., Kornek, G., Maca, T., Koller, D.Y., Weinlaender, G., Hejna, M. and Scheithauer, W. (1998). Association between *Helicobacter pylori* infection and pancreatic cancer. Oncology 55: 16-9.

Thomashow, M.F., Nutter, R., Montoya, A.L., Gordon M.P. and Nester, E.W. (1980). Integration and organization of Ti-plasmid sequences in crown gall tumor. Cell 19: 729-739.

Turker, A.U. and Camper, N.D. (2002). Biological activity of common mullein, a medicinal plant. Journal of Ethnopharmacology 82: 117-125.

Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.I. and Sim, G.A. (1966). Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminate. Journal of the American Chemical Society 88: 3888-3890.

Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K. and Winans, S.C. (2000). The bases of crown gall tumorigenesis. Journal of Bacteriology 182(14): 3885-3895.

Zupan, J., Muth, T.R., Draper, O. and Zambryski, P. (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant Journal 23: 11-28.

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