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Original Research

Genotoxic and cytotoxic potential of *Alternanthera Bettzickiana*, an important ethno-medicinal plant

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Abstract: The present study was carried out to investigate the mutagenic and cytotoxic potential of n-hexane and aqueous-methanolic whole plant extracts of *Alternanthera bettzickiana*. Aqueous-methanolic and n-hexane extracts of *Alternanthera bettzickiana* extracts were assessed for the mutagenic potential with Salmonella tester strains TA-100 and TA-102 in the presence and absence of the rodent enzyme activation system and cytotoxic potential was assessed by MTT assay. Aqueous-methanolic extract showed the presence of saponins, tannins, terpenoids, flavonoids and glycosides. However n-hexane extract revealed the presence of tannins and terpenoids only. It was found that a concentration as low as 15mg/mL of both extracts was more mutagenic to the TA 102 tester strain than TA-100. Hexane whole plant extract of *Altenanthera bettzickiana* was more mutagenic than aqueous-methanolic extract considering revertant colonies of TA 100 strain. Aqueous-methanolic and n-hexane whole plant extracts of *Altenanthera bettzickiana* showed higher mutagenic potential in the presence of the enzyme activation system. Mutagenicity of aqueous-methanolic extract increased with an enzyme activation system in case of TA 100 whereas mutagenicity of n-hexane extract decreased in the presence of the enzyme activation system with TA 100 and TA 102 strains. Aqueous-methanolic and n-Hexane whole plant extracts of *Alternanthera bettzickiana* showed an IC-50 of 493 and 456 µg/mL in BHK-21 cells respectively. It can be concluded that *Altenanthera bettzickiana* exhibited mutagenic activity in a bacterial reverse mutation assay with and without enzyme activation systems. However, it showed limited cytotoxicity to BHK-21 cells.

Key words: Mutagenicity; Cytotoxicity; Phytochemicals; Medicinal plants; Ethno-pharmacology.

Introduction

Plants and herbs are considered as an inspirational source of new drug development. Plant derived medicines have been contributing to the human health for the pre-historic times. A large population in developing countries depends upon natural medicines in primary health care. Moreover, economic, clinical and pharmaceutical values of herbal medicines are increasingly recognized in industrial countries. Dependence of mankind on plants and herbs for food and medication invokes research interest in exploring and isolating phytochemicals (1). However, proper evaluation of the pharmacological and toxicological basis for their usage, ensuring efficacy and safety of herbal medicines to the patients and quality control validation and standardization of herbal drugs pose serious challenges (2).

Traditional medicines in Africa are now suggested to be included in the national drug policies (3). These drugs may become the basis for new drug development due to the presence of novel phytochemicals. Traditional medicines derived from plants contribute to the health of over 80% of global population and 70% of the population in India (4).

Alternanthera bettzickiana is a perennial herb. Its leaves and shoots are consumed as vegetable in South East Asia. It is native to South America (5). It is also known as Red Calico plant and Joyweed in South America and belongs to family Amaranthaceae. Family Amaranthaceae is comprised of several edible species. Various species are also used for different ailments in traditional medicinal systems. Several plants from Amaranthaceae family exhibit antineoplastic activity (6). *A. bettzickiana* is used as edging ornamental plant. In traditional medicines, *A. bettzickiana* is used for promoting lactation, nourishment, as a prophylaxis of dementia and to cure gastrointestinal diseases (7). It is also used an effective antipyretic agent. Decoction of whole plant is used for the treatment of arthritis. It improves blood circulation, reduces menstrual pain and acts as emmenagogue (8).

Evaluating the carcinogenic and mutagenic properties of fruits, vegetables and herbal drugs has attained a recent interest owing to the rigorous safety requirements (9). Uncontrolled proliferation occurs due to the mutation in genes involved in regulated cellular growth and tumor specific promoters. Accumulation of the genetic errors, permanent DNA damage and mutations in tumor suppressor genes also result in carcinogenesis. Mutagenic potential of drugs and toxicants can be demonstrated

with Ames Reverse Mutation Assay. Ames test determines the base pair substitutions of DNA, frame shift mutations and oxidant induced DNA damage in genetically modified bacterial strains (10). A positive result in the Ames test depicts the mutagenic nature of drugs and toxicants. It predicts the possible carcinogenic nature of test chemicals as the most mutagens are correlated with the cancer. Methyl-Thiazole Tetrazolium (MTT) assay, a colorimetric technique, has gained wide popularity among in-vitro toxicology assays (11). MTT assay demonstrates the in-vitro cell viability and may show cellular activation, cytotoxic and anti-cancer potential of chemicals and drugs (12).

In the present study, mutagenic potential of the n-hexane and aqueous-methanolic extract of *A. bettzickiana* was investigated using an Ames Reverse Mutation assay and cytotoxic potential was assessed through MTT assay in Baby hamster kidney cell line (BHK-21). The phytochemical evaluation of aqueous-methanolic and n-hexane whole plant extracts of *A. bettzickiana* was also carried out.

Materials and Methods

All the chemicals were obtained from Sigma Aldrich®, BDH® and thermo fisher Scientific®. Fresh plant was collected from Central Punjab region during August 2013. The plant was identified from by a taxonomist of the University of Agriculture, Faisalabad with voucher number 520-1-13 and the specimen was deposited in the herbarium.

Collection and extraction

In this study, whole plant of *A. bettzickiana* was investigated. The plant was shade dried and powdered. Extraction was carried out with aqueous-methanol (30:70) and n-hexane by maceration technique. Extracts were dried with a rotary evaporator and stored in refrigerator at -20°C until further use.

Preliminary phytochemical study

Phytochemical analysis of both the extracts was carried out through the methods described previously (13). Both the extracts were investigated for the detection of phyto-constituents such as glycosides, tannins, terpenoids, saponins, flavonoids and alkaloids.

Evaluation of mutagenicity

Reverse mutation assay for the estimation of mutagenic activity of the whole plant extracts of *A. bettzickiana* was carried out in *Salmonella typhimurium* TA-100 and TA-102 strains. These *Salmonella* strains were acquired from environmental bio-detection products inc. Canada (bdpi). These genetically modified strains were evaluated for their dependence on histidine and biotin amino acids and their resistance to ampicillin (10). Sodium azide (5µg/ petri plate) and Hydrogen peroxide (35% V/V) served as positive control for TA 100 and TA 102 respectively. 2-Aminoanthracycline (2AA) served as a positive control when metabolic activation system was used to determine the mutagenicity of plant extract metabolites. Distilled water served as negative control (14). Whole plant extracts of *A. bettzickiana* were separately dissolved in distilled water with 150 mg/mL concentration. Extracts were diluted with distilled water with the tenfold dilution method.

TA 100 and TA 102 were inoculated in nutrient broth and incubated at 37°C for 24 hours before mutagenicity testing. The optical density of nutrient broth was adjusted to 0.1 - 0.4 so that each 0.1 mL of nutrient broth comprised of 2 X 10^8 bacteria. Previously prepared whole plant extract (1 mL) of *A. bettzickiana* was mixed with 0.1 ml each of TA 100 and TA 102 strains separately. These pre-mixed solutions were incubated at 37°C for 1 hour. Glucose minimal agar was prepared with purified agar, Vogel-Bonner (VB) salt solution, glucose, traces of histidine and biotin and distilled water. The solutions of tester strain and plant extracts were admixed with glucose minimal agar to prepare top agar layer. Top agar was poured on the lower agar plates and incubated for 48 hours at 37°C (15). Mutations in TA 100 and TA 102 strains resulted in revertant colonies which were counted manually. Mutagenic index was calculated by dividing the number of revertant colonies in test sample plates by the number of revertant colonies in negative control plates.

The whole plant extracts was considered as non-mutagenic if the value of mutagenic index was less than 2. When the value of mutagenic index was 2 or more, it showed the possible mutagenic nature of *A. bettzickiana* whole plant extracts. The mutagenic index of 3 or more suggested a significant mutagenicity of *A. bettzickiana*. The value of mutagenic index more than 4 was suggestive of high mutagenicity of *A. bettzickiana* whole plant extracts.

Cytotoxicity Assay

In-vitro cytotoxicity assay was performed on BHK-21. MTT assay was used to assess the viability of BHK-21 cells upon exposure to *A. bettzickiana* whole plant extracts. BHK-21 cell line was acquired from Quality Operation Laboratory (QOL), UVAS, Lahore. Dulbecco's minimum essential medium (DMEM) was used as cell culture medium. Fetal bovine serum (10%) was added to the cell culture medium for optimum cell growth, whereas 1% fetal bovine serum was added to maintenance medium. Cryopreserved BHK-21 cells were defrosted, decontaminated with 70 % ethanol and thawed to 37°C. Fresh DMEM medium was added for washing and dilution of BHK-21 cells after removal of old medium by centrifugation at 2000 rpm for 3 minutes (16). BHK-21 cells were incubated in a 25 cm^2 cell culture flask at 37°C.

Control for cell viability was DMEM whereas Dimethylsulfoxide (DMSO) was solvent control. The number of BHK-21 cells was accustomed to 10^5 cells/ml. The dilutions of BHK-21 cells were prepared with DMEM. BHK-21 cell suspension (0.1ml) was dispensed in each well of 96 well plates and incubated at 37°C in a CO_2 incubator for 48 hours for attaining confluence. After 90% confluence was obtained, the growth medium was removed. BHK-21 monolayers were washed with phosphate buffer saline. *A. bettzickiana* whole plant extracts were dissolved in 10 % DMSO to maintain a concentration of 6.4 mg/ml. *A. bettzickiana* whole plant extracts were diluted with twofold dilution method. All the dilutions of *A. bettzickiana* whole plant extracts were assessed for cell viability in triplicate wells. Each well

comprised of 200 µl of *A. bettzickiana* whole plant extract. Freshly prepared 20µl MTT dye solution (0.25% w/v) was added 48 hours post incubation to each well. 96 well plates were once again incubated at 37˚C in CO_2 incubator for 3 hours. Cell culture medium was removed 3 hours post incubation and 100µl DMSO was used to dissolve any formazan crystals in 96 well plates (17). Cell viability was determined through measurement of purple color with a micro-plate reader at 570nm. Cell survival percentage was determined according to the method previously described (16). IC-50 was calculated by plotting log concentration of *A. bettzickiana* whole plant extracts versus percentage cell survival.

Statistical analysis

Revertant colonies of Salmonella TA-100 and TA-102 exhibited by aqueous-methanolic and n-hexane extracts were evaluated by one way ANOVA followed by Tukey's test and presented as mean and standard deviation. The data were analyzed by Prism pad®. Percentage viability of BHK-21 cells at various concentrations of *A. bettzickiana* whole plant extracts were appraised by regression analysis by MS Office, 2013.

Results

Phytochemical evaluation

Preliminary phytochemical evaluation of n-hexane and aqueous-methanolic whole plant extracts of *A. bettzickiana* revealed the presence of tannins and terpenoids in both extracts. Saponins, anthraquinone glycosides and flavonoids were found in the aqueous-methanolic extract only. Alkaloids were absent in both the extracts.

Mutagenicity of Alternanthera bettzickiana

Aqueous-methanolic and n-hexane extracts of *A. bettzickiana* whole plant were subjected to mutagenicity testing using genetically modified *Salmonella* strains TA-100 and 102. It was found that both the aqueous-methanolic and n-Hexane whole plant extracts of *A. bettzickiana* displayed the mutagenic potential to varying degree in Salmonella strains. There was a concentration dependent amplification in revertant colonies. Aqueous-methanolic and n-hexane extracts of *A. bettzickiana* exhibited a higher number of revertant colonies of Salmonella TA 102 than TA 100.

It was further revealed that the 15mg/ml concentration of aqueous-methanolic or n-hexane extracts were more mutagenic in TA 100 tester strain than its counterpart. N-hexane whole plant extract of *A. bettzickiana* was more noxious than its aqueous-methanolic extract considering revertant colonies of TA 100 strain. It was also found that the aqueous-methanolic and n-hexane whole plant extracts of *A. bettzickiana* were highly mutagenic in salmonella TA 102 even at 15µg/ml concentration. Revertant colonies and Mutagenicity Index of whole plant extracts of *A. bettzickiana* extracts are demonstrated in the table 1 and 2 respectively.

The study also demonstrated that the aqueous-methanolic and n-Hexane whole plant extracts of *A. bettzickiana* had shown mutagenic potential in the presence of the enzyme activation system. Mutagenicity of aqueous-methanolic whole plant extract of *A. bettzickiana* increased with an enzyme activation system in case of TA 100 whereas mutagenicity of n-Hexane extract decreased in the presence of the enzyme activation system with TA 100 and TA 102 strains. Revertant colonies and Mutagenicity Index of whole plant extracts of *A. bettzickiana* in the presence and absence of the enzyme activation system are depicted in the table 3 and 4 respectively.

Cytotoxicity Assay

Aqueous-methanolic and n-hexane whole plant

Table 1. Revertant colonies of Salmonella TA-100 and TA-102 strains exhibited by various concentrations of aqueous-methanolic and n-Hexane whole plant extracts of *Alternanthera bettzickiana.*

Aqueous-methanolic Extract (µg/plate)	Revertant Colonies		n-Hexane Extract (µg/plate)	Revertant Colonies	
	TA 100	TA 102		TA 100	TA 102
1.5×10^5	558 ± 32*	2400 ± 103*	1.5×10^5	3404 ± 119*	8832 ± 731*
1.5×10^4	2200 ± 63*	1492 ± 62*	1.5×10^4	416 ± 74*	4992 ± 264*
1.5×10^3	322 ± 37*	1252 ± 41*	1.5×10^3	330 ± 54*	3578 ± 98*
1.5×10^2	200 ± 28*	935 ± 16*	1.5×10^2	218 ± 07*	2824 ± 33*
1.5×10^1	152 ± 17*	804 ± 11*	1.5×10^1	143 ± 08*	1728 ± 48*
Aqueous-methanolic solution as negative control	101 ± 04	195 ± 06	Aqueous-methanolic solution as negative control	101 ± 04	195 ± 06

Data was shown as mean ± standard deviation. Where * shows a statistically significant difference from negative control.

Table 2. Mutagenic Index exhibited by various concentrations of aqueous-methanolic and n-Hexane whole plant extracts of *Alternanthera bettzickiana.*

Aqueous-methanolic Extract (µ/plate)	Mutagenic Index		n-Hexane Extract (µg/plate)	Mutagenic Index	
	TA 100	TA 102		TA 100	TA 102
1.5×10^5	5.52^a	12.30^a	1.5×10^5	33.70^a	45.29^a
1.5×10^4	21.78^a	7.65^a	1.5×10^4	4.11^a	25.60^a
1.5×10^3	3.18^b	6.42^a	1.5×10^3	3.26^b	18.34^a
1.5×10^2	1.98^d	4.79^a	1.5×10^2	2.15^c	14.48^a
1.5×10^1	1.50^d	4.12^a	1.5×10^1	1.41^d	8.86^a

Where "a" showed highly mutagenic. " b" showed significantly mutagenic, "c" showed possible mutagenic,"d" showed non mutagenic.

Table 3. Revertant colonies of Salmonella TA-100 and TA-102 exhibited by various concentrations of aqueous-methanolic and n-Hexane whole plant extracts of *Alternanthera bettzickiana.*

Aqueous-methanolic Extract (µg/plate)	Revertant Colonies		n-Hexane Extract (µg/plate)	Revertant Colonies	
	TA 100	TA 102		TA 100	TA 102
1.5×10^5	2845 ± 56*	-	1.5×10^5	-	-
1.5×10^4	2534 ± 43*	1936 ± 107*	1.5×10^4	396 ± 48*	5440 ± 462*
1.5×10^3	766 ± 76*	1422 ± 82*	1.5×10^3	311 ± 49*	4120 ± 170*
1.5×10^2	498 ± 48*	1145 ± 17*	1.5×10^2	217 ± 31	3468 ± 94*
1.5×10^1	322 ± 31*	842 ± 26*	1.5×10^1	133 ± 22	1942 ± 43*
Aqueous-methanolic solution as negative control	146 ± 26	168 ± 23	Aqueous-methanolic solution as negative control	146 ± 26	168 ± 23

Where * shows a statistically significant difference from negative control.

Table 4. Mutagenic Index of various concentrations of aqueous-methanolic and n-Hexane whole plant extracts of *Alternanthera bettzickiana in the* presence of enzyme activation system

Aqueous-methanolic Extract (µg/plate)	Mutagenic Index		n-Hexane Extract (µg/plate)	Mutagenic Index	
	TA 100	TA 102		TA 100	TA 102
1.5×10^5	19.48^a	-	1.5×10^5	-	-
1.5×10^4	17.35^a	11.52^a	1.5×10^4	2.71^c	32.38^a
1.5×10^3	5.24^a	8.46^a	1.5×10^3	2.13^c	24.52^a
1.5×10^2	3.41^b	6.81^a	1.5×10^2	1.48^d	20.64^a
1.5×10^1	2.20^c	5.01^a	1.5×10^1	0.91^d	11.55^a

"a" showed highly mutagenic, " b" showed significantly mutagenic , "c" showed possible mutagenic, "d" showed non mutagenic.

extracts of *A. bettzickiana* demonstrated the concentration dependent cytotoxic potential in BHK-21 cells. Aqueous-methanolic whole plant extract of *A. bettzickiana* exhibited a decline in Cell Survival Percentage (CSP) of BHK-21 with an increase in concentration. N-hexane whole plant extract of *A. bettzickiana* also displayed dose dependent increase in CSP. Aqueous-methanolic and n-hexane whole plant extracts of *A. bettzickiana* showed an IC-50 of 493 and 456 µg/ mL respectively. Thus, n-hexane whole plant extract of *A. bettzickiana* showed higher cytotoxic potential than its aqueous-methanolic counterpart. The cytotoxic potential of aqueous-methanolic and n-hexane whole plant extracts of *A.bettzickiana* against BHK-21 cells are indicated in the figure 1 and 2 respectively.

Statistical analysis of revertant colonies of TA 100 and TA 102 exhibited by aqueous-methanolic and n-hexane whole plant extracts of *A. bettzickiana* indicated the statistically significant difference between the number of revertant colonies at $P \leq 0.05$ and mutagenic potential decreased with a decrease in concentration of the extract. CSP decreased with an increase in the concentration of both the extracts.

Discussion

Regulatory authorities recommend various in-vitro toxicity testing techniques due to their robust, time efficient and highly specific nature. Drugs and chemicals are evaluated vigorously through in-vitro toxicological tests for their predictable and unexpected outcomes. Medicinal products of natural origin are also increasingly subjected to these interventions to avoid unnecessary adverse events (16).

Medicinal use of plants for the treatment of various diseases can be attributed to the presence of various primary and secondary metabolites (18). The occurrence of various secondary metabolites in aqueous-methano-

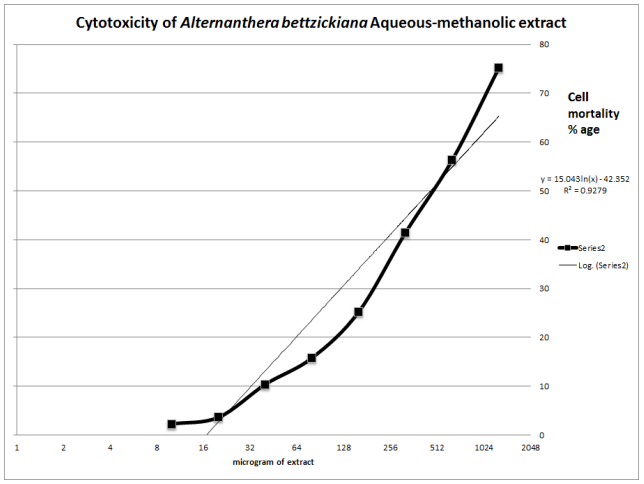

Figure 1. Cytotoxic potential of aqueous-methanolic whole plant extract of Alternanthera bettzickiana.

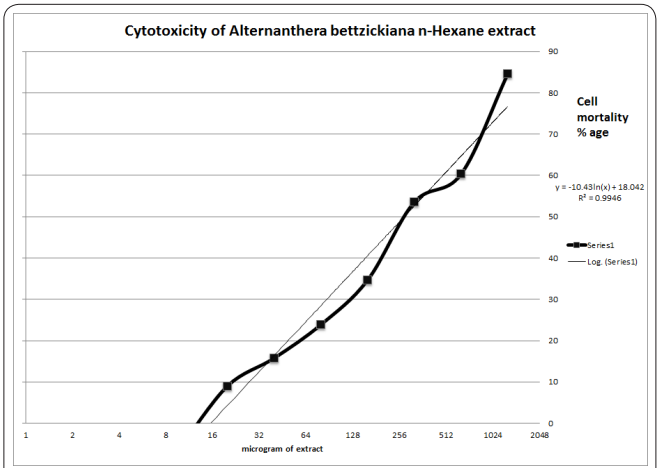

Figure 2. Cytotoxic potential of n-Hexane whole plant extract of Alternanthera bettzickiana.

lic and n-hexane extracts revealed the potential medicinal and toxicological properties of *A. bettzickiana*. The occurrence of flavonoids in medicinal plants has been linked to the lipid lowering, psychoactive, antiulcer, cardiotonic, hepatoprotective, antineoplastic, antioxidant, antimicrobial and hypoglycemic potential of medicinal plants. Flavonoids attenuate the free radical activity, prevent diabetic and cardiovascular complications and reduce the risk of chronic degenerative disorders. These are biologically active against viruses, other microbes, toxins and tumors (19). Plant terpenoids have gained extensive use for their aromatic properties and are under investigation for their antineoplastic and antibacterial potential (20). Tannins are known to exhibit potential antibacterial, antiviral and anti-neoplastic properties and are useful in dysentery and diarrhea (21). Reaction of tannins with proteins is vital in the treatment of ulcerative and inflamed tissues (22).

Evidences support that heavy metals such as cadmium (Cd) and lead (Pb) tend to accumulate in *A. bettzickiana* due to its bioremediation properties. Genotoxicity is mainly attributed to the presence of heavy metals (23). Mutagenicity of *A. bettzickiana* may be attributed to the presence of various heavy metals especially in its aqueous-methanolic extract (24). A previous study on *Alternanthera sessilis* showed that it did not exhibit any antimutagenic activity (25). Our findings suggest that *A. bettzickiana* exhibits strong mutagenic characteristics.

This study also demonstrates the cytotoxic potential of *A. bettzickiana* in BHK-21 cells. It was found in a previous study on *Alternanthera sessilis*, a plant of genus *Alternanthera*, that it exhibited 40 percent inhibition in the viability of HELA cells at a concentration of 500 µg /mL (26). Aqueous-methanolic and n-Hexane extracts of *A. bettzickiana* showed IC-50 at 456 and 493 µg/ml concentrations respectively which were comparative to the cytotoxic potential of *Alternanthera sessilis*. It can be speculated that the presence of phytochemicals such as terpenoids, tannins, saponins, anthraquinones and flavonoids would have contributed to the cytotoxic potential of *A. bettzickiana* (27),(28).

The present study conclusively demonstrates that *A. bettzickiana* exhibited mutagenic activity in a bacterial reverse mutation assay with and without enzyme activation system possibly due to the presence of various phytochemicals like saponins, tannins, terpenoids, flavonoids and glycosides. However, it showed limited cytotoxicity in BHK-21 cells.

Conflict of interest

The authors declare that they have no conflict of interests.

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