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RESEARCH ARTICLE

In Vitro Evaluation of Cytotoxic and Genotoxic Effects of *Calotropis Procera* (Aiton) Extracts on Bovine Lymphocytes

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ABSTRACT

This study was designed to determine the in vitro cytotoxic and genotoxic effects of crude extracts from the leaves and stems of *Calotropis procera* (*C. procera*) on bovine lymphocytes. The lymphocytes were cultured in the presence of extracts at various concentrations (10µg/mL, 20µg/mL, 30µg/mL, 50µg/mL, and 70µg/mL) for 24h, and the percentage of cell viability was determined by MTT assay. Based on cytotoxic assessment, concentrations of extracts were determined at which cells remained viable at 25%, 50%, and 75% (LD50/2, LD50, and 2XLD50/2). Genotoxicity of aqueous and ethanolic extracts of *Calotropis procera* was determined by culturing the cells with these three concentrations (25µg/mL, 50µg/mL, and 75µg/mL) for 24h, and DNA damage was analyzed by the Comet assay. The micronucleus assay was evaluated for nuclear and chromosomal damage. Our results indicated that out of both extracts of *C. procera* (leaves and stem), the alcoholic extract showed greater cytotoxicity in a dose-dependent pattern. In contrast, aqueous extracts were comparatively less genotoxic at concentrations against bovine lymphocytes. On further evaluation, it was observed that both extracts showed chromosomal aberrations and the formation of micronuclei. Thus, results indicated that aqueous and alcoholic extracts (stem and leaves) of *C. procera* possess in vitro cytotoxicity and genotoxicity against bovine lymphocytes.

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INTRODUCTION

Natural preparations derived from plant material are widely used by the people of rural and urban areas as therapeutics. Even the drugs prescribed by physicians are modified forms of natural products and in some cases, these are isolated from plant parts (Wang *et al.*, 2007). *Calotropis* is a small genus that includes six species of small trees or shrubs, dispersed in tropical and subtropical areas of Asia, the America, and Africa. Its two species, *Calotropis procera* and *Calotropis gigantea*, are closely related in structure and function. It is reported that the whole plant contains sterols, alkaloids, cardiac glycosides, flavonoids, and usharin (Suresh *et al.*, 2013). The plant contains many medicinal properties such as purgative, diuretic, and anti-inflammatory that has been reported in earlier study (Iqbal *et al.*, 2005). *C. procera* contains high level of basic proteins and is considered a good animal food. The plant is easily digested by the animals. Hay made from *C. procera* given to the animal results in weight gain and shiny hair (Madruga *et al.*, 2008). Latex characterization of *C. procera* gave the idea that it is produced in response to defense action against microorganisms such as fungi, viruses, and insects. This latex has adhesive properties which cause to immobilization of the insects (Silva *et al.*, 2010). Leaves of *C. procera* are helpful in water treatment, and their capability to lessen total viable

count has been reported by Shittu *et al.* (2004). In Pakistan, leaves of *C. procera* have been used for the treatment of experimentally infected cross-breed cattle with *Theileria annulata*. Butalex is being used by the farmers against *T. annulata* but the drug is costly and unaffordable for the farmers. Experimentally infected animals were given different doses of plant extract and showed no toxicity at a dose of 0.3mk/kg of oral dose and animals got cured (Durrani *et al.,* 2009). Secretions from the root bark are traditionally used in India to treat skin diseases by applying the secretions on the skin. Likewise, these secretions are also used to treat intestinal worms (Parrotta, 2001). In Senegal, cutaneous diseases are treated with the milky latex of *C. procera* including leprosy, syphilitic sores, and ringworms (Singh *et al.,* 2017). In Western Africa, doctors have stated that they have effectively used plant to treat many diseases. They used plant latex against microorganisms and found bactericidal properties in the latex of *C. procera* and provided the fact that bactericidal properties of the latex are due to the presence of active compounds such as calcactin, calotropain, and mudarin (Kareem *et al.,* 2008). The plant shows pharmacological activity due to the content of calotoxin, calotropin, and calactin, which have therapeutic properties. Plant parts are also used in the treatment of fever, leprosy, and snake bites. Due to the presence of metabolites, the plant is used in different medicines (Sharma *et al.*, 2012).

Despite these benefits, *C. procera* causes toxic effects in animals through air, consumption of the plant, and by getting in touch with plant parts in livestock. A study revealed that *C. procera* acts like a poison and is known to have cytotoxic properties, which include dermatitis and iridocylites which have lethal effects. Diarrhea and anorexia are being reported in the sheep as a result of the toxic effects of *C. procera*. Its consumption in man is as harmful as in the case of livestock (Vadlapudi and Naidu, 2010). In the semi-arid regions of Brazil, several farmers suggested that accidental ingestion of fresh leaves of *C. procera* has been toxic to many ruminants. Some studies have supported these observations in which toxic effects of latex and leaves of *C. procera* are being reported (Singh *et al.*, 2017; Singhal and Kumar, 2009). The present study aimed to evaluate the cytogenetic effects of this plant on bovine lymphocytes.

MATERIALS AND METHODS

Plant collection and preparation of crude extracts: The *C. procera* plant was collected from Lahore, Pakistan. Plant was identified by the botanist from the GC university Lahore. Plant leaves and stems were washed and air-dried for a week. Leaves and stems of *C. procera* were taken, air-dried, and powdered. Aqueous and ethanolic (95%) extracts were prepared, using grounded plant, filtered, by soxhlet method and kept at 4 ºC (Velaga *et al.,* 2017). Extracts were concentrated by drying under reduced pressure. Further extracts were completely evaporated. For testing of bovine lymphocytes, stock solutions were prepared by dissolving in 1% DMSO. For this, 5 doses were prepared with the concentration ranges 10µg, 20µg, 30µg, 50µg, and 70µg by dissolving the extract in 1% DMSO.

Growth and culturing of lymphocytes: Blood from healthy animals (n=5) was taken in sterile EDTA-coated syringes and shifted to sterile Falcon tubes. Lymphocytes were separated from the whole blood by using the histopaque-1077 (Sigma, Aldrich) technique by adding histopaque-1077 in an equal volume of blood and centrifuging at 400*g for 30 minutes at room temperature. The supernatant was carefully removed, and the middle layer containing mononuclear cells was extracted. Viable cells were analyzed under the microscope. RPMI-1640 culture media was used to culture lymphocytes with 10% FBS, 1% amphotericin-B, 1% penicillin-streptomycin, and 1% HEPES. Media filtered with a 0.2 µm syringe filter and stored at 4ºC. In 50 mL of growth media, viable cells were added under sterile conditions in a safety cabinet. Sterile culture flasks of 75cm^2 were used for the cultivation of lymphocytes and incubated at 37ºCat appropriate conditions of 5% CO_2; 2% O_2 and 95% N_2.

Cytotoxicity assessment: Cytotoxicity was assessed by using the MTT assay (Ali and Cigerci, 2017) to analyze the activity of living cells to reduce the dye 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to purple colored formazan. Cells were supplemented with growth media (RPMI 1640 medium with 20% fetal bovine serum, 25mM HEPES, 250mg/mL amphotericin B, and 0.66% penicillin at 37ºC with 5% CO_2. Cells were incubated in 96 well plates (1×10^4 cells /mL) to allow adhesion of cell growth. After 24 hours, the plant's crude extracts (aqueous and ethanolic) were separately added to each well in the concentration range of (LD 50/2 (25µg), LD 50(50µg), and LD 2XLD50/2 (75µg). After 24 hours of incubation, the supernatant was discarded and 10% MTT was added to each well. To dissolve the formazan product, 10% DMSO was added to record the absorbance at 570nm (Beckman Coulter). Pure DMSO was used as the positive control.

Genotoxicity assessment: Comet assay and micronucleus assay were used to check the genotoxicity. Comet assay was performed according to Ciğerci *et al.* (2018). Comet assay was performed only with three doses of both aqueous and ethanolic extracts of *C. procera* at 25%, 50%, and 75% of cells remained viable in MTT assay that was (LD 50/2

(25µg), LD 50(50µg), and 2XLD50/2 (75µg), respectively. Cells were incubated in 96-well plates with these lethal dose concentrations of crude extract for 24 hours. After the incubation, cells were harvested and mixed with 0.65% low-melting agarose (LMA) and spread on 1% normal agarose pre-coated slides. Slides were dipped in lysis solution (100mM Na_2 EDTA, 2.5M NaCl, 10mM Tris, pH 10 with 10% DMSO, and 1% Triton X100 freshly added) for an hour at 4ºC and then shifted to electrophoresis buffer (1mM Na_2 EDTA, 300mM NaOH, and 0.2% DMSO with pH > 13.5) for 20 minutes for DNA unwinding to occur. Electrophoresis was carried out at 25V, 300mA for 15 minutes at 4ºC. Slides were stained with 75 µl of ethidium bromide (20mg/mL). Each slide was randomly scored and examined by using the fluorescent microscope (Olympus, Japan) equipped with specific filters at 40X. DNA damage was described as the percentage of tail DNA, which specified the extent of damage to the cells (Ciğerci *et al.*, 2015).

The micronucleus assay was performed by incubating the cultured lymphocytes with LD50/2, LD50, and 2XLD50/2 of *C. procera* extracts for 24 hours. Dose-treated cells were harvested by centrifuging with 1% KCL at 1500 rpm for 15 minutes. Pellets were treated with fixative-I and fixative-II respectively centrifuging at speed of 1500rpm. On pre-cilled slides, samples were coated carefully and air dried. Slides were stained with 10% Giemsa stain and observed to identify micronuclei and binuclei at 40X using a Nikon compound microscope (Hashimoto *et al.*, 2010).

RESULTS

Cytotoxic assessment revealed that both aqueous and ethanolic extracts of *C. procera* induced cell death after 24 hours of exposure in a dose-dependent manner. The effect of increasing the concentration of aqueous extract from leaf and stem extract of *C. procera* on cultured lymphocytes using the MTT assay is shown in Fig. 1. The effective concentration of aqueous *C. procera* extract which caused 50% cell death (LD50) in bovine cultured lymphocytes is about 50µg after 24 hours of exposure to plant extract. The effect of increasing concentration of ethanolic extract on cultured lymphocytes is represented in Fig. 1. It is noticed from the results that bovine lymphocytes are less sensitive to the aqueous extract of *C. procera* than the ethanolic extract.

Results of Comet assay and micronucleus assay showed that ethanolic extract at the dose of 75µg/mL significantly (p≥0.05) caused the DNA damage maximum 41±1 as compared to control 0.33±0.57 and aqueous extract 35±1 of *C. procera* (Table 1). Similarly, maximum micronuclei and binuclei appeared in treatment of ethanolic extracts of *C. procera* as compared to aqueous and control (Table 2).

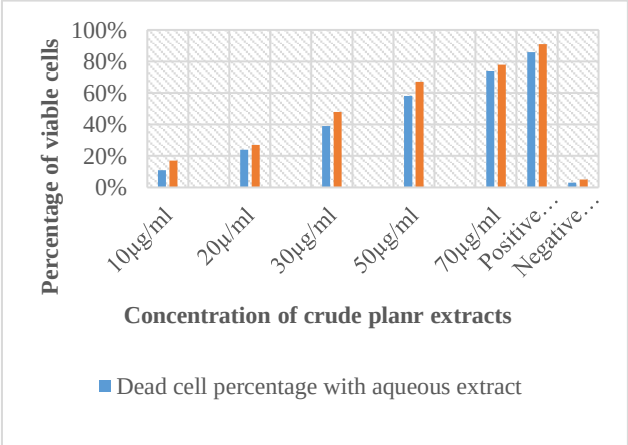

Fig. 1: Graph is representing the live and dead cell percentage in MTT assay at various concentrations of aqueous plant extract. Percentage of viable cells.

Table 1: Average score and mean ± Standard Deviation of DNA damage found in the comet assay in LD 50/2, LD 50, and 2*LD/50 doses of ethanolic and aqueous extract of *Calotropis procera.*

Treatment	Ethanolic	Aqueous	Control
LD50/2 (25µg/mL	26 ± 1.52^{c}	13 ± 1^{c}	0.33±0.57^{d}
LD50 (50 µg/mL	36 ± 1.53b	27 ± 2^{b}	
2XLD50/2(75µg/mL)	41 ± 1^{a}	35 ± 1^{a}	

Values bearing the same letters are insignificant and vice versa. Where (p<0.05)

DISCUSSION

Cytotoxicity is the ability of any compound to stimulate cell death. The in vitro cytotoxicity assays are broadly used for screening chemicals for analyzing toxicity in humans or in animals (Eisenbrand *et al.*, 2002). *C. procera* is a wild plant that is known for its cytotoxic effects on animals as well as on humans. Even accidental ingestion of fresh leaves or latex of plant can cause serious health issues (Vadlapudi and Naidu, 2010).

Table 2: Number of micronuclei and binuclei per count of 1500 cells treated with aqueous and ethanolic extract at concentrations of LD 50/2, LD 50, and 2XLD50/2.

Treatment	Micronuclei at Aqueous conc.	Binuclei at Aqueous conc.	Micronuclei at Ethanolic conc.	Binuclei at Ethanolic conc.	Micronuclei in Control
LD50/2 (25µg/mL)	11±1.00^{c}	1.6±0.5^{b}	17.6±0.5^{c}	2.6±0.5^{b}	0.33 ± 0.57^{d}
LD50 (50 µg/mL)	13±0.57^{b}	3±1.0^{ab}	20.3±1.5^{b}	4.33±0.5^{ab}	
2XLD50/2 (75µg/mL)	17±0.57^{a}	3.66±0.57^{a}	24.33±0.5^{a}	6.00±1.0^{a}	

Values bearing the same letters are insignificant and vice versa. Where, (p<0.05)

Cytotoxic effects of crude extract of *C. procera* extracts on bovine lymphocytes were assessed using MTT assay. A strong growth inhibitory effect of *C. procera* extract was found at a concentration of 75µg/mL. MTT results predicted a decrease in cell viability in dose-dependent manner on bovine lymphocytes.

Cytotoxicity of *C. procera* root extract has already been studied on human oral (KB) and central nervous system (SNB-78) cell lines (Bhagat *et al.,* 2010). They found that the cytotoxic effects of aqueous extract were less effective as compared to alcoholic extract in which they found that the *n*-butanol fraction of alcoholic extract showed 50% growth inhibition at the highest dose concentration of 100µg/mL. Free radical scavenging and antioxidant activity of plant is due to the phytochemical constituent of *C. procera* (Moustafa *et al.,* 2010). In another study, the genotoxic effects of *C. procera* root and leaf extract on the human chromosome were analyzed, in which they observed alteration in morphology of chromosome than in the control (Nidhi *et al.,* 2018).

Micronucleus and comet assays are sensitive tools extensively used to detect the genotoxic effects of chemicals found in the environment (Ali *et al.,* 2009). Comet assay offers benefit over other cytogenic analysis techniques such as chromosome aberrations and exchange of sister chromatids. While micronucleus assay is used to detect the DNA damage and mitotic activity of cells is not required for Comet assay (Bankoglu *et al.,* 2021). A wide range of in vivo and in vitro markers are used to detect the genotoxic effects of environmental pollutants but comet assay is most popular in the way that it is sensitive to detect DNA damage even at very low level (Cordelli *et al.,* 2021).

In the current study, genotoxic effect was studied in way of finding high DNA damage and formation of Micronuclei in the lymphocytes that were exposed to the different concentrations of crude extract of *C. procera* extract. Our results are in agreement with the study of Nidhi *et al.* (2018) who signified those genotoxic effects of aqueous, methanolic, and chloroform extracts of *C. procera* which altered the morphology of human chromosomes under in vitro conditions human peripheral blood culture. Dose-dependent in vitro cytotoxicity of *C. procera* was also studied against human oral and CNS cancer cell lines by exposing the cells to concentrations of 10 µg/mL, 30 µg/mL, 100 µg/mL (Bhagat *et al.,* 2010). The effect of methanolic extract of flowers of *C. procera* and high radical scavenging activity was observed along with the cytotoxicity (Pourmoard *et al.,* 2006). Genotoxic effects of leaf extract of *C. procera* extract have has also been studied, demonstrating that at a high dose concentration of 20mg/100mL chromosomal aberrations increased. Aberrations include clumped metaphase and arrested telophase observed in dividing cells which is similar to our findings (Malode and Khandare, 2010). Latex also shows toxic properties which include inflammation, iridocyclites, and irritation (Singhal and Kumar, 2009). Other toxic effects include liver damage, testicular necrosis, and cardiotoxicity (Kapoor and Adlakha, 2023). It is important to note that latex also exhibits toxic properties like irritation, inflammation, and iridocyclitis (Singhal and Kumar, 2009), cardiotoxicity, liver damage, and testicular necrosis (Kapoor and Adlakha, 2023). Genotoxic and mutagenic effects of *C. procera* latex which induced auxotrophic mutations at high concentration on *Aspergillus terreus* have also been reported (Sabir, 2010).

Conclusion

Current study provides useful information about cytotoxic and genotoxic effects of *Calotropis procera* crude extract on bovine lymphocytes. It provides insight about the acceptable and lowest possible concentration of *Calotropis procera* extracts as a medicine to limit the risk of the plant which can otherwise cause mutation at the cellular level in animals.

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Authors' contribution: MKA and FSRC provided the research idea, performed the experiments. MKA wrote the manuscript. FSRC handled the revision.

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