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Research Article

EVALUATION OF GENOTOXIC EFFECTS OF ALCOHOLIC EXTRACT OF *KALANCHOE PINNATA* USING SINGLE CELL GEL ELECTROPHORESIS

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ABSTRACT

Objective: The objective of the study was to evaluate the genotoxic and mutagenic potentials of *Kalanchoe pinnata* alcoholic leaves extract.

Methods: Single cell gel electrophoresis (COMET assay) was adopted to investigate genotoxicity potential of standardized ethanolic leaves extract at the concentration of 10, 50, and 100 µg/ml, respectively, using human lymphocytes.

Results: *K. pinnata* leaves extract did not completely damage DNA in human lymphocytes but low, medium, and high-level damage was found in with positive control. The DNA damage level decreases in low, medium, and high (12±1, 6±1, and 1±1) without positive control at the concentration of 100 µg/ml.

Conclusion: It was concluded that the parent material did not induce mutagenic and genotoxic potentials at the concentrations 10, 50, and 100 µg/ml but its metabolites are inducing genotoxicity in various models.

Keywords: COMET, Ethanolic extract, *Kalanchoe pinnata,* Lymphocytes, Mutagenic.

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INTRODUCTION

Plants are considered as raw materials for alternative medicine and have antimutagenic effects against chemicals and environmental factors. On the other hand, these extracts may also have mutagenic effects on different organisms [1,2]. Medicinal plants are most important and commonly used in treatment for human diseases such as gastrointestinal disorders, cardiovascular disease, pain, diarrhea, fungal, and bacterial infections and cancer. The needs for reliable information on herbal medicinal products are considerable [3,4]. On the other hand, most of the traditional medicinal plants have never been the subject of exhaustive toxicological tests such as is required for modern pharmaceutical compounds. Based on their traditional use for long periods of time, they are often assumed to be safe. However, research has shown that many plants which are used as food ingredients or in traditional medicine have *in vitro* mutagenic or toxic and carcinogenic properties. Within this context, it is also important to screen medicinal plants for their mutagenic properties [5,6]. *Kalanchoe pinnata* (Family: Crassulaceae) commonly known as "Ranakalli" "Miracle leaf," "Wonder of the World." It is a perennial herb grows up to about 1–2 m tall containing a wide range of active compounds including triterpenes, alkaloids, glycosides, flavonoids, steroids, bufadienolide, and lipids. *K. pinnata* is an important plant which is largely used in folk medicine in India for the treatment of kidney stones, diabetes mellitus, and hypertension, wound healing, and diarrhea [7-10]. The aim of this study was to investigate the antimutagenic property of alcoholic extract of *K. pinnata* in human lymphocytes using single cell gel electrophoresis (SCGE) (COMET assay). Human lymphocytes are the standard model for individual eukaryotic cells that can be vitally evaluated cytologically by phase contrast and differential contrast microscopy. They can be assessed both cytochemically and biochemically for various perturbation affecting nuclear DNA including various form of DNA damage and repair [11]. In general, COMET assay is an economic, fast, sensitive, reliable, and rapid method. The SCGE/COMET assay combines the simplicity of biochemical techniques for detecting DNA single-strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross-linking with the single cell approach typical of cytogenetic assays. This technique can be applied to proliferating and non-proliferating cells and the cells of those tissues, which are the first sites of contact with mutagens/carcinogens. What makes this assay even more valuable is the specificity for detecting genotoxicity. Like in other tests, DNA effects induced due to cytotoxicity is a big challenge. Data showed that cytotoxicity can be detected (dead cells show specific kinds of comets called "clouds") and distinguished from genotoxic effects, therefore, should have no confounding effects on results [12,13].

METHODS

Fresh leaves of *K. pinnata* were collected from Thandarai village Chengalpattu, Kancheepuram district, Tamil Nadu, India. The plant material was authenticated by Dr. P. Jayaraman, Retired Professor, Department of Botany Presidency College, Chennai, Tamil Nadu, India, and Director Plant Anatomy Research Centre West Tambaram, Chennai, Tamil Nadu, India.

Preparation of plant extracts

Fresh leaves of *K. pinnata* were washed with sterile distilled water (Milli-Q) to remove any impurities present and were dried under the sunshade. The dried leaves were powdered in a motor and pistil followed by the extraction process. 100 g of powder was soaked in 600 ml of ethanol and kept in a shaker for 7 days at 37°C. After 7 days, the extract was filtered and dried on the hot plate [14]. A crude extract of 6 g was obtained. This extract was stored under aseptic conditions.

SCGE (COMET assay)

COMET assay was carried out with human blood lymphocytes. The glass slides were coated with normal melting agarose (NMA) by dipping the slide into melted NMA, and the slide was wiped clearly on one side with sterile absorbent cotton. The slide was then allowed to dry overnight. 3 milliliters of heparinized whole blood was mixed with 3 ml of RPMI 1640 medium. To this 3 ml of lymphocyte separation medium

(LSM) was added to the centrifuge tube. The diluted 3 ml blood sample was carefully layered on LSM so that the sample did not mix up with LSM. It was then centrifuged at 400 RPM for 30–40 min at 20°C. The upper layer of the centrifuged sample was drawn using a clean, sterile micropipette, leaving the lymphocyte layer undisturbed at the interface. The cell viability was counted and checked with Trypan blue staining method. The cells that exceed 90% viability were used in genotoxicity assay. 0.9 ml of the cell suspension was mixed with 0.1 ml of *K. pinnata* extract at the concentration of 10, 50, and 100 µg/ml and incubated at 37°C for 30 min. The cells were then collected by centrifugation. The cell suspension (100 µl) was mixed with 200 µl of 1% low melting point agarose prepared in phosphate buffer saline and was immediately pipetted onto a 1% NMA in distilled water pre-coated, and the slide was dried overnight. The slide was placed at 4°C for 10 min to get solidified. Over this agarose layer melting agarose was added as the third layer and the slides were kept at 4°C for 10 min and allowed to complete the polymerization of agarose and immersed in freshly made lysis solution (2.5 M-Nacl, 100 mM-EDTA, 10 mM Tris HCl, 10% dimethyl sulfoxide, 1% Triton X-100 pH-10) at room temperature for 2 h to remove cellular proteins. The slides were then rinsed with distilled water and placed in a horizontal gel electrophoresis containing buffer (300 mM NaOH, 1 mM EDTA at pH-13) and the electrophoresis was started at 25V, 200 mA for 20 min. The slides were washed 2 times for 5 min with 0.4 M Tris HCl -pH-7.4 at 4°C. After second neutralization, the slides were dried and stained with Ethidium Bromide (10 µg/ml) and examined using a fluorescent microscope. A total of 100 cells from each of the two replicate slides were examined per sample. Using computerized image analysis system, the data were analyzed. By the ratio of tail to head length the extent of DNA damages was scored as follows: <5% - no damage, 5–20% low damage, 21–40% medium damage, 41–95% high damage, and >95% complete damage [15-18].

RESULTS AND DISCUSSION

At the concentration 10 µg/ml, 53±1 cells were found to have no damage; low damage was found in 31±1 and medium damage was found in 9±1. *K. pinnata* extract caused high damage in 7±1 cells. At the concentration 50 µg/ml, 60±2 cells were found to have no damage; low damage was found to be in 28±1, and medium damage was found in 10±3. *K. pinnata* extract caused high damage in 2±0 cells. At the concentration 100 µg/ml, 43±3 cells were found to have no damage; low damage was found to be in 46±3, and medium damage was found in 8±3. *K. pinnata* extract caused high damage in 3±1 cells. The results are shown in Table 1 and Fig. 1.

The leaf extracts showed DNA damage with positive control. Hence, 100 µg/ml of leaf extract was selected for secondary comet assay. Two trials of comet assay were conducted without positive control. At the concentration 100 µg/ml, A 80±3 cells were found to have no damage; low damage was recorded in 12±1 and medium damage was observed in 6±1. *K. pinnata* extract caused high damage in 2±1 cells. At the concentration 100 µg/ml, B 79±10 cells were found to have no damage; low damage was recorded in 17±6 and medium damage was observed in 3±1. *K. pinnata* extract caused high damage in 1±1 cells. The results are shown in Table 2 and Figs. 2 and 3.

Medicinal plants are used for home remedies and are widely used in developed and developing countries. They are a diverse source of nutrients and bioactive compounds that are valuable for human health. Many ethanobotanical surveys and traditional knowledge of medicinal plant have been proved their significance in curing various diseases [19]. Therefore, it is necessary to do tests to evaluate their quality and quantity of exposure. Medicinal plants have become popular during the years, but there is limited knowledge about the safety and effectiveness of medicinal properties and their usage by physicians, and some evidence demonstrate that these medicinal plants can be dangerous [20]. This study was performed to investigate the genotoxicity of alcoholic extract of *K. pinnata* in human blood lymphocytes. Many plant species express genotoxic activities with the incidence of tumors; therefore, the understanding of the potential toxicity of medicinal plants is highly important. Photochemical present in some traditional medicinal plants are potentially carcinogenic and may have genotoxic potentials.

K. pinnata contain photochemical such as triterpenes, alkaloids, glycosides, flavonoids, steroids, and bufadienolide [21,22]. Genotoxicity studies on *K. pinnata* extract in human lymphocytes were assessed by SCGE (COMET assay) are a very sensitive test for the quantification of DNA damage and provides the direct determination of single-double strand breaks in individual cells. Cells were treated with hydrogen peroxide as positive control and different concentration of *K. pinnata* extract (10–100 µg/ml) results showed that there was low, medium, and high damage at concentration 10–100 µg/ml in preliminary comet assay it may be due to difference in cell physiology like cell cycle [23- 27]. The *K. pinnata* extracts showed DNA damage with positive control. In secondary comet assay, two trials were conducted without positive control at the concentration 100 µg/ml. Results showed that there was a decrease in cell damage compared with the preliminary COMET assay with positive control. On the other hand, another study revealed that the ethanolic extracts of *K. pinnata* show significant wound healing potential. *K. pinnata* extracts showed DNA damage in a dose-dependent manner with COMET assay. *K. pinnata* is medicinal plant, traditionally used in the treatment of several diseases showed no complete DNA damage with COMET assay. This confirms original parent molecule is not genotoxic in nature, but its metabolites are producing genotoxicity in various models. All though it possess the antimutagenic property and it requires further investigation.

CONCLUSION

K. pinnata is Indian traditional medicinal plant it contains various phytochemical widely used in siddha, Ayurveda and folk medicine

Table 1: Genotoxicity of ethanolic extract of *K. pinnata* in human lymphocyte in the COMET assay with positive control

Sample	Concentration (µg/ml)	No	Low	Med	High	Compl.
K. pinnata extract	10	53±1	31±1	9±1	7±1	0
	50	60±2	28±1	10±3	2±0	0
	100	43±3	46±3	8±3	3±1	0
Positive control		55±1	32±1	9±2	2±2	2±1
Negative control (blank)		90±3	9±1	1±0	0	0

No: No damage, Low: Low damage, Med: Medium damage, High: High damage, Compl: Complete damage, *K. pinnata: Kalanchoe pinnata*

Table 2: Genotoxicity of ethanolic extract of *K. pinnata* in human lymphocyte in the confirmatory comet assay without positive control

Sample	Concentration	No	Low	Med	High	Compl.
K. pinnata extract	100 µg/ml A	80±3	12±1	6±1	2±1	0
	100 µg/ml B	79±10	17±6	3±1	1±1	0
Negative control		95±4	4±2	1±1	0	0

No: No damage, Low: Low damage, Med: Medium damage, High: High damage, Compl: Complete damage, *K. pinnata: Kalanchoe pinnata*

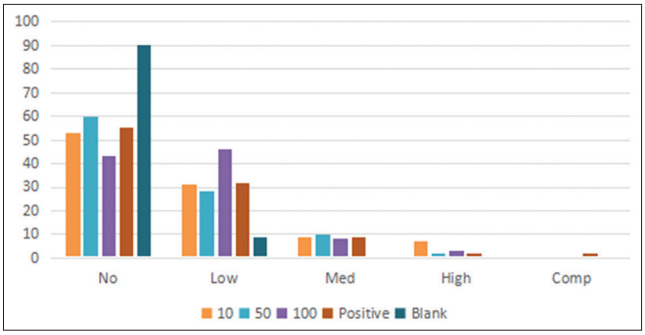

Fig. 1: Distribution of cells in various DNA damage categories for leaf extracts with positive control

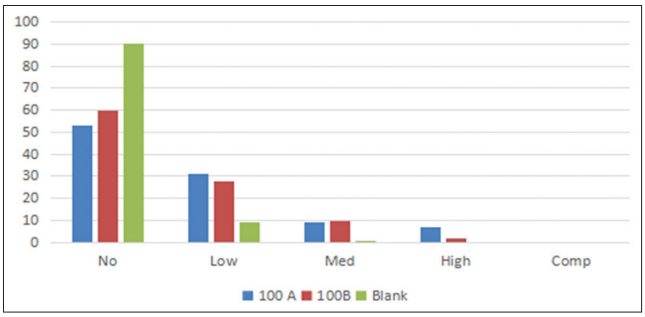

Fig. 2: Distribution of cells in various DNA damage categories for leaf extracts without positive control

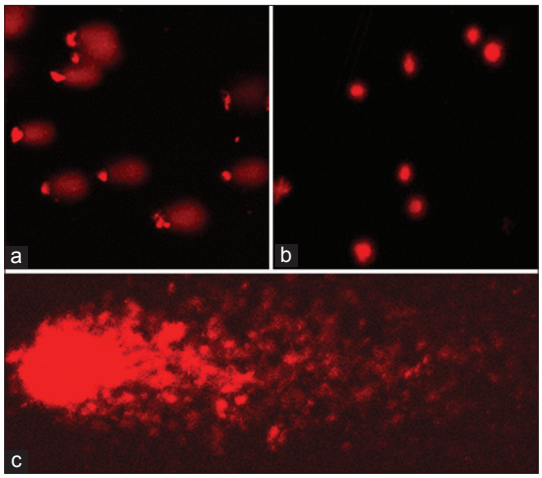

Fig. 3: Photomicrographs of stained DNA of human lymphocyte for alkaline COMET assay. (a) Treated with *Kalanchoe pinnata* extract (100 µg/ml) showing DNA damage in COMET tail. (b) Untreated showing no DNA damage, (c) Tail moment

for the treatment of various diseases such as diabetes mellitus, kidney stones, and wound healing. In this study design to examine the genotoxic effects of alcoholic extract of *K. pinnata* at various concentration using SCGE (COMET assay). These results suggest that alcoholic extract of *K. pinnata* showed DNA damage at low, medium, and high level. However, no complete DNA damage was observed in both preliminary and secondary comet assay at all the concentration using human lymphocytes. These indicate the original parent molecule is not mutagenic, but its metabolites are producing mutagenic.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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