Toxicological Assessment of Ethanolic Leaves Extract of *Kalanchoe pinnata* in Rats

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Abstract

Kalanchoe pinnata is a perennial plant that is widely used in the folklore treatment of kidney and gall stones and urinary insufficiency. The present study aims to evaluate the potential toxicity of the leaf extract of this plant upon acute and sub-acute (28 day) exposure in Wistar rats when administered orally. In acute oral toxicity(n=3) female Wistar rats were treated with a single dose of 2000mg/kg b.wt and observed for 14 days and sub-acute toxicity(n=6) male and female Wistar rats were treated with the dose of 500 and 1000mg/kg b.wt of leaf extract for 28 days consecutively. No mortality, morbidity or adverse clinical signs of toxicity were observed during the experimental period. No significant changes in the body weight was observed. Gross necropsy did not indicate any treatment-related pathological changes in any of the animals in the acute and sub-acute toxicity studies. Histopathology of the liver, heart and kidney did not show any remarkable lesions that could be related to the administration of the leaf extract. Ethanolic leaf extract of *Kalanchoe pinnata* is relatively safe with an LD_{50} value greater than 2000 mg/kg. From the sub-acute study, the no-observed adverse effects level (NOAEL) of the extract can be derived as 1000 mg/kg b.wt in male and female Wistar rats.

Keywords: *Kalanchoe pinnata, traditional medicine, safety, acute toxicity, sub-acute toxicity.*

Introduction

Kalanchoe pinnata (*K. pinnata*) is a medicinal plant of ethnomedical importance and used by the Asian folklore for various ailments.[1] The leaf extracts of this plant have been routinely used for ailments like microbial infections, kidney stones and wound healing. A number of active compounds, including flavonoids, glycosides, triterpenoids, steroids, bufadienolides and organic acids have been identified in *K. pinnata* that have been shown individually to possess variety of biological activities.[2-6]

Rajsekhar et al.[7] reviewed the pharmacological potentials of this plant which included wound-healing, antioxidant, anti-cancerous, anti-proliferative, antimicrobial, antiviral, anti-protozoal, anti-leishmanial, anthelmentic, insecticidal, anti-allergic, analgesic, antinociceptive, anti-edematogenic, anti-inflammatory, muscle-relaxant, antipyretic, anticonvulsant, antidepressant, sedative, antilithiatic, hepatoprotective, gastroprotective, antidiabetic, nephroprotective, haemoprotective, antihistamine, anti-hypertensive and immunosuppressant activities. Fernandes et al.[8] illustrated the presence of novel bioactive molecules in *K. pinnata* against bothropic venom. Zakharchenko et al.[9] explored the fungicidal potential of transgenic *K. pinnata* extract containing CecP1 and recommended their use as a candidate drug for treatment of wounds infected with *Candida albicans*.

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Despite the fact that medicinal plants such as *K. pinnata* have immense therapeutic value, the safety profiling needs to be performed using validated protocols so that it can be continuously utilized in folklore treatment.[10] To address this, a safety evaluation study of the ethanolic leaf extract of *K. pinnata* was conducted in Sprague-Dawley rats following the methodology described in the OECD guidelines for testing for acute (OECD 423) and repeated dose toxicity (OECD 407) as an initial screening for safety.

Materials and Methods

Plant material

Fresh leaves of *Kalanchoe pinnata* were collected from ITWWS (Irula Tribe Women's Welfare Society) Thandarai village, Chengalpattu, Tamil Nadu, India. The plant material was authenticated by Dr.P. Jayaraman, Director, Plant Anatomy Research Center, West Tambaram, Chennai. [PARC/3213]. The voucher specimen was deposited in the herbarium of PG and Research, Department of Plant biology and plant Biotechnology, Presidency collage, Chennai.

Extraction

The extraction was performed according to the method adopted by Azwanida.[11] Fresh leaves of *K. pinnata* were washed to remove any impurities present and shade-dried. The dried leaves were pulverized and the preparation was taken for extraction. The coarse particles were cold macerated with ethanol and kept in a shaker for seven days at 37 ºC and later filtered (using Whatman filter paper No.1 (11 µm pore size)) and dried on the hot plate at 40ºC. The crude extract was stored in refrigerator (2 -8ºC) until use.

Animals

Healthy young adult animals (8 to 12 weeks old) of Wistar strain were obtained from Sainath agencies, Hyderabad, India. The females were certified to be nulliparous and non-pregnant. The health status of the animals was provided by the vendor. All the animals were found to be health and active. The animals were acclimatized for a period of six days and housed individually in polypropylene cages with stainless steel top grill. Sterilized paddy husk bedding was provided to the animals. Standard laboratory diets (Rodent pellet) and purified drinking water was provided *ad libitum* throughout the experiment except when fasting was necessary. The animals were maintained under controlled environmental conditions at a temperature of 22°C (± 3°C) and relative humidity of 36-64% with 12 h light and 12 h dark cycle. Individual animals were identified by tail marking with unique numbers.

Acute oral toxicity

The evaluation of acute toxicity in a limit dose test was performed using the procedures described by the Organization for Economic Cooperation and Development (OECD 423). A limit test at one dose level (2000mg/kg body weight) was carried out with 6 female animals (3 animals per step). The ethanolic leaf extract of *K. pinnata* was administered as a single dose orally to overnight fasted rats. Food was withheld for a further 3 hours after dosing. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days. Individual weights of animals were determined shortly before the test substance is administered and weekly thereafter. All test animals were subjected to gross necropsy. Gross pathological changes (if any) were recorded for each animal.[12]

Repeated dose toxicity

Following the acute toxicity study, the effect of 28-day repeated oral dosing of the extract in Wistar rats was evaluated following the OECD 407 Test Guideline. The method used provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time, including effects on the nervous, immune and endocrine systems.

In the repeated dose oral toxicity study, 18 animals were randomly allocated to the control and treatment groups with 3 males and 3 females in each group. The animals in the treatment group were dosed with the ethanolic extract of *K.pinnata* at 500 and 1000mg/kg body weight daily 7 days each week for a period of 28 days. Animals in the vehicle control group received purified drinking water at 10ml/kg body weight. The extract/vehicle was administered in a single dose to the animals using a suitable intubation cannula.[13]

General clinical observations were made at least once daily. Mortality and morbidity observations were made twice daily. Body weight and feed measurements were made weekly. At the end of the 28-day treatment period, animals were euthanised. At gross necropsy, blood samples were collected from the retro-orbital sinus for haematology and biochemistry analyses.

Hematology and Biochemistry

Blood was collected from the retro-orbital sinus under mild anaesthesia using diethyl ether, into heparinized and nonheparinized tubes for hematology and biochemistry analyses respectively. Hematological parameters analyzed include Hematocrit, Clotting time, Hemoglobin concentration(Hb), Red blood cell count(RBC), White blood cell count(WBC), Differential count(DC) and Platelet count. Serum samples were analysed for the following Biochemical parameters: Sodium, Potassium, Glucose, Total cholesterol, Urea nitrogen, Creatinine, Total protein, Albumin, Total bilirubin, Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST).

Histopathology

The animals were later euthanised by CO_2 asphyxiation. Gross necropsy (External and internal) was performed; Major organs (Liver, Heart and Kidney) were collected for histopathological evaluations.

Statistical analysis

Data obtained were subjected to paired Student's *t*-test to evaluate the difference between the treated and control groups. P value was set at 0.05. Values were represented as Mean ± SD.

Results

Acute oral toxicity

No mortality/morbidity was observed any of the animals treated with the ethanolic extract of *K. pinnata* at 2000mg/kg body weight during the 14-day observation period. All animals showed an increase in body weight relative to that recorded prior to treatment. Based on the observations, the median lethal dose (LD_{50}) of *K. pinnata* extract in female rats was estimated to be greater than 2000 mg/kg body weight.

Repeated dose toxicity

Repeated oral administration of the extract did not induce any adverse clinical signs of toxicity in any of the treated animals in both the sexes. No mortality or morbidity was observed during the course of the treatment period until termination. No significant changes in body weight or feed consumption were observed. Gross necropsy did not reveal any treatment-related pathological lesions. All treated animals gained appreciable body weight relative to that recorded prior to dosing (Table 1).

Table 1. Repeated dose toxicity study – Mean Weekly Body weight (in grams)

Treatment	Days					BWC
	0	7	14	21	28	
Control 10mL/kg b.wt	128.25 ± 2.12	134.3 ± 1.71	141.3 ± 2.08	146.65 ± 3.06	152.75 ± 3.70	24.5 ± 2.78
KPEE 500mg/kg b.wt	127.27 ± 3.40	133.73 ± 2.53	139.95 ± 2.52	145.62 ± 2.31	152.43 ± 2.91	25.1 ± 1.49
KPEE 1000mg/kg b.wt	125.73 ± 2.43	132.25 ± 2.27	138.87 ± 2.59	144.53 ± 2.12	152.75 ± 3.70	24.5 ± 2.78

Values are expressed as Mean ± S.D, N=6(both 3male&3female), KPEE – *Kalanchoe pinnata* Ethanolic Extract, BWC - Body Weight Change

Hematology and Biochemistry

The effects of sub acute oral administration of *K.Pinnata* extract on hematological and biochemical parameters are presented in Tables 2 and 3. No significant changes were observed in the levels of Hematocrit, Clotting time, Hemoglobin concentration(Hb), Red blood cell count(RBC), White blood cell count(WBC), Differential count(DC), Platelet count were observed with respect to the untreated control. No statistically significant changes were observed in the biochemical parameters assessed.

Table 2. Effect of ethanolic extract from *K. pinnata* leaves on Hematological parameters

Hematological Indices		Control 10mL/kg b.wt	KPEE Treated 500 mg/ kg b.wt	KPEE Treated 1000 mg/ kg b.wt
RBC(106 / **µL)**		8.22 ± 0.24	8.21 ± 0.48	7.88 ± 0.59
Hb(g/dL)		13.73 ± 0.38	13.82 ± 0.35	13.33 ± 0.29
HCT(%)		44.37 ± 0.86	43.92 ± 2.21	43.36 ± 1.37
WBC(103/**µL)**		11.3 ± 0.34	11.02 ± 0.35	10.68 ± 0.65
PLT(103/**µL)**		949.94 ± 21.91	944.7 ± 31.94	947.33 ± 16.14
CT(s)		133.01 ± 6.7	132.97 ± 8.64	136.92 ± 3.01
Differential Count	L (%)	74.35 ± 2.37	72.17 ± 2.21	73.18 ± 0.83
	M (%)	5.48 ± 0.32	5.46 ± 0.43	5.36 ± 0.28
	G (%)	22.54 ± 1.37	21.62 ± 2.27	20.33 ± 0.71
MCH(pg)		16.03 ± 0.84	15.95 ± 0.45	16.55 ± 0.59
MCHC(g/dL)		29.55 ± 1.48	30.19 ± 1.47	30.61 ± 0.69
MCV(fL)		54.31 ± 1.35	55.01 ± 2.19	55.71 ± 1.93

N=6(both 3male&3female), KPEE – *Kalanchoe pinnata* Ethanolic Extract. Values are expressed as Mean ± S.D; Hb, hemoglobin; RBC, red blood cell count; HCT, hematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; WBC, white blood cell count; L, lymphocyte percentage; M, monocyte percentage; G,granulocyte percentage; PLT, platelet count; CT, clotting time.

Table3. Effect of ethanolic extract from *K. pinnata* leaves on Biochemical parameters

Biochemical Indices	Control 10mL/kg b.wt	KPEE Treated 500mg/kg b.wt.	KPEE Treated 1000mg/kg b.wt.
Sodium(mEq/L)	145.41 ± 2.05	144.95 ± 2.35	146.84 ± 1.34
Potassium(mEq/L)	4.89 ± 0.29	5.17 ± 0.31	5.32 ± 0.44
Glucose(mg/dL)	106.32 ± 2.86	105.11 ± 1.82	105.01 ± 2.29
Cholesterol(mg/dL)	99.35 ± 2.19	97.54 ± 3.76	98.05 ± 3.88
Total Bilirubin(mg/dL)	0.37 ± 0.07	0.42 ± 0.04	0.34 ± 0.05
Total Protein(g/dL)	6.89 ± 0.18	6.89 ± 0.37	6.44 ± 0.47
Albumin(g/dL)	3.73 ± 0.32	3.97 ± 0.28	3.84 ± 0.36
Urea(mg/dL)	16.6 ± 0.53	16.39 ± 0.75	16.79 ± 0.29
Creatinine(mg/dL)	0.56 ± 0.05	0.63 ± 0.11	0.58 ± 0.09
AST(IU/L)	75.53 ± 2.52	75.41 ± 2.35	76.91 ± 1.09
ALT(IU/L)	19.2 ± 1.11	19.34 ± 1.13	20.5 ± 0.89

N=6(both 3male&3female), KPEE – *Kalanchoe pinnata* Ethanolic Extract Values are expressed as Mean ± S.D; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase

Euthanasia and Gross necropsy:

The animals were euthanized by carbon dioxide asphyxiation and gross necropsy of the external and internal organs did not reveal any treatment-specific changes.

Histopathological Examination

Figures 1 represent the microscopic images of the stained tissue sections of liver, kidney and heart in the control group (A) and treatment groups (B, 500mg/kg b.wt and C & D, 1000mg/kg b.wt). No treatment-specific lesions, inflammation or pathological changes related to were observed in the organs of the animals from the treatment group compared to the untreated group. In general, microanatomy of liver, kidneys and heart did not present any treatment-related adverse toxicological effects in the animals that received 500mg/kg b.wt and 1000mg/kg b.wt.

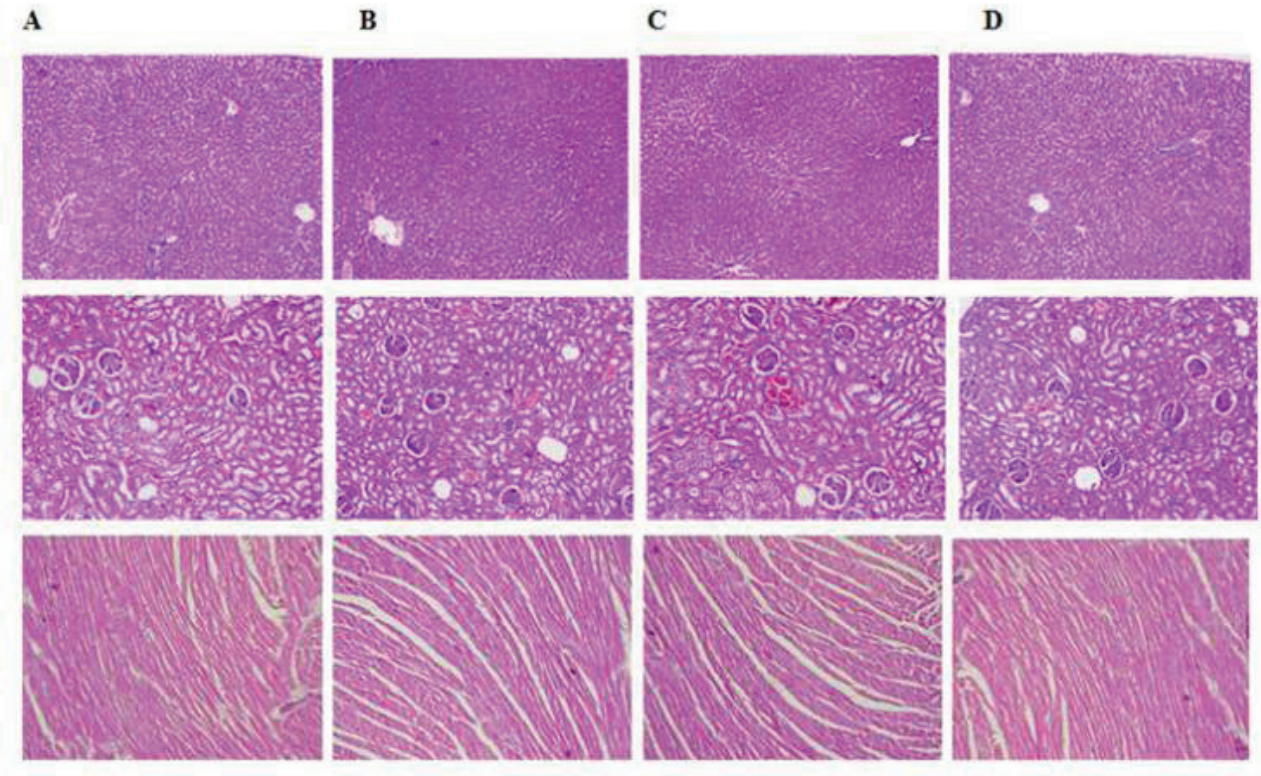

Figure 1: Hematoxylin and eosin stained cross sectional liver, kindney and heart views of control(A) and KPEE treated 500 mg/kg b.wt(B) and 1000 mg/kg b.wt (C and D) in sub-acute toxicity study in male and female rats (magnification, x40)

Discussion

Medicinal plants are an important source of substances which are claimed to induce anti-inflammatory and antioxidant effects. *K. pinnata* found in the temperate and tropical regions and largely used in Indian system of medicine(Folk, Siddha and Ayurvedic) for the treatment of kidney stones, gastric ulcers, pulmonary infections, rheumatoid arthritis.

Ozolua et al.[14] studied acute and sub-acute oral toxicity of the aqueous extract of *K. pinnata* in Sprague Dawley rats. No mortality was observed at the acute oral dose of 5 g (5000 mg) per kg body weight by the oral route. The medial lethal dose following intraperitoneal administration was 1.8 g (1800mg) per kg body weight. Sub acute treatment did not significantly alter animal weights, organ-to-body weight ratios, fluid intake, hematological indices and the levels of AST, ALP and albumin. ALT level was significantly reduced in the treated group. Total bilirubin and conjugated bilirubin levels remained unaffected.

Zakharchenko et al.[9] indicate that ethanol extracts have become increasingly popular form of *Kalanchoe* application in therapy. The therapeutic efficiency is attributed to the presence of lipophilic constituents such as the bufadionolides, polyphenols and flavonoids.

Nayak et al.[15] studied the wound healing potential of the ethanolic extract of *K. pinnata* leaf in rats and concluded that there was increased wound retraction and hydroxy proline content in the extract treated groups indicating a significant wound healing potential consistent with the traditional healing practice. Mathew et al.[6] reported the analgesic and anti-inflammatory potential of the ethanolic extract of the stem of *K. pinnata* in rat models, anti-depressant potential in mice, and anti-diabetic potential in alloxan-induced diabetic rats. In all these studies, the extract as such which served as the control did not exhibit any adverse effects in the treated animals, indicating its safety.

Nevertheless, an independent safety evaluation of the ethanolic extract has not been reports so far. The

present study attempted to evaluate the safety of the ethanolic extract of *K. pinnata* leaves during acute and sub-acute treatments in Wistar rats. The results obtained are similar to those obtained for the aqueous extracts performed by Ozolua et al.[15] in terms of the absence of mortality and the haematological and biochemical indices studied. In addition, histopathological evaluation of the major tissues (Liver, Heart and Kidney) did not indicate any remarkable lesions that could be correlated to the repeated dosing of the plant extract. The results therefore indicate the safety of the ethanolic extract of *K. pinnata* leaves complementing its use in traditional medicine.

Conclusion

Toxicological evaluation of the ethanolic extract obtained from leaves of *K. pinnata s*howed no signs or symptoms of acute toxicity. The median lethal dose (LD_{50}) value of the extract was derived to be higher than 2000 mg/kg b.wt indicating that the extract is non-toxic. Repeated dose toxicity study with the extract did not induce any adverse clinical signs of toxicity in the treated animals; in addition, hematology and biochemistry parameters remained unaffected in the treated animals. No histopathological changes (in the organs studied) related to treatment with the plant extract were recorded. The extract is therefore considered safe for repeated administration up to 1000mg/kg/day. In conclusion, the ethanolic extract of *K. pinnata* does not contain any toxic phytochemical constituents as evidenced by the lack of adverse clinical effects in acute and repeated dose toxicity studies in rats and can therefore be concluded as safe for oral consumption.

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Conflicts of Interest - The authors declare that no conflict of interest.

Ethical Clearance - This study has been approved by the Institutional Ethical Committee of GLR Laboratories Chennai.

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