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Evaluation of the analgesic, sedative-anxiolytic, cytotoxic and thrombolytic potentials of the different extracts of *Kalanchoe pinnata* leaves

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ABSTRACT

Objective: To evaluate the analgesic, neuropharmacological, cytotoxic and thrombolytic potentials of the aqueous, ethanol and ethyl acetate extracts of *Kalanchoe pinnata* leaves.
Methods: At the dose of 400 mg/kg body weight, the analgesic activity of the extracts were evaluated by the acetic acid-induced writhing and formalin-induced persistent pain tests while neuropharmacological activity was evaluated by the open field, hole cross and elevated plus maze tests. The cytotoxic potential was observed by brine shrimp lethality bioassay and the thrombolytic potential was investigated by clot lysis test.
Results: The aqueous extract significantly suppressed the number of writhing (96.78%) as well as the formalin-induced persistent pain on the early phase (46.92%) and on the late phase (40.98%). Again in case of hole cross and open field tests, the locomotor activity was decreased significantly ($P < 0.001$) mostly by the ethyl acetate extract. Furthermore, the sedative-anxiolytic activity was supported by the increased percent ($P < 0.01$) of frequency into the open arm on elevated plus maze test. Besides, the extracts showed moderate lethality and thrombolytic activity.
Conclusions: The findings showed that activities are comparable to the standards and in some cases are stronger than the standards. Therefore, based on the results, it is evident that it has great analgesic and sedative-anxiolytic activity with moderate cytotoxic and thrombolytic potential.

1. Introduction

Kalanchoe pinnata (Lam.) Pers. (*K. pinnata*), an ornamental plant, belongs to the family of Crassulaceae and is a straight, juicy, perennial shrub, about 1.5 m tall and reproduces through seeds or propagates through stems or leaf cutting. It has fresh dark green leaves[1]. *K. pinnata* is also locally known as "pathorkuchi" in Bangladesh. It is rich in alkaloids, triterpenes, glycosides, flavonoids, steroids and lipids. It is used traditionally for the treatment of earache, burns, abscesses, ulcers, insect bites, whitlow, diarrhea, lithiasis, skin fungus, fever, worms, acute and chronic bronchitis, pneumonia, asthma and also effective as sedative wound-healer, diuretic and cough suppressant[1,2]. In Ayurvedic, it is used as astringent, analgesic, carminative and also used for the treatment of nausea and vomiting[3]. In Bangladesh, it is locally used for cough, jaundice, indigestion and gonorrhea. The plant exhibits many pharmacological activities against otitis, headache, inflammations, convulsions and general debility[4] and posses antihistamine and anti-allergic[5], anthelmintic[3], immunosuppressive and anti-urolithiatic activity[6]. Cancer chemo preventive potential of bufadienolides and the protective effect in fatal anaphylactic shock has also been reported[7,8]. However, no such studies were performed on aqueous, ethanol and ethyl acetate extracts of *K. pinnata* leaves for analgesic, central nervous system (CNS), cytotoxic and thrombolytic activities. The aim of this study was therefore to investigate the analgesic, anxiolytic, cytotoxic and thrombolytic potentials of different extracts of *K. pinnata* leaves.

2. Materials and methods

2.1. Collection of plant materials

K. pinnata leaves were collected from Chittagong Hill forest on October 2011. After collection, they were thoroughly washed with

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water and dried at room temperature for 7 days. The sample specimen was sent to National Herbarium (Accession No. 39519).

2.2. Extract preparation

Dried *K. pinnata* leaves were powdered (1 000 g) and macerated with water (KP AQ), ethanol (KP EOH) and ethyl acetate (KP EA) at normal temperature for 7 days with intermittent shaking. Then, it was filtered and the filtrate was condensed by rotary evaporator to obtain a viscous extract. It was kept dry under a ceiling fan to get the dried extract (about 10%). For the *in vivo* tests involving mice, 400 mg/kg body weight of leaf extract was used.

2.3. Animals

Swiss albino mice of both sexes weighing 22–28 g were collected from International Centre for Diarrheal Disease Research, Bangladesh and were kept at the laboratory animal house, Department of Pharmacy, East West University, Bangladesh. The animals were housed in standard cages with free access to modified fish pellet and sufficient water. The animals experienced a 12-hour light/dark cycle regularly. For analgesic and neupharmacological experiments, animals were divided (n = 5) in control, standard and three test groups. Control always received vehicle (1% Tween in water) and test groups received aqueous, ethanol, ethyl acetate extracts at 400 mg/kg dose.

2.4. Drugs and chemicals

Diclofenac sodium and diazepam were obtained from Square Pharmaceuticals Ltd., Bangladesh. Tween 80 and acetic acid were obtained from Merck, Germany. Formalin was purchased from Central Drug House, India. Analytical grade chemicals were used for all experiments.

2.5. Acute toxicity test

The test groups received the aqueous, ethanol and ethyl acetate extracts of *K. pinnata* leaves orally at the doses of 500, 1 000, 1 500, and 2 000 mg/kg. Then, the animals were kept in separate cages. The animals were observed for any behavioral alteration, allergic reactions and death for the next 72 h[9].

2.6. Analgesic screening

2.6.1. Acetic acid-induced writhing test

The antinociceptive potential of the samples was studied with writhing method[10,11]. Tested samples and vehicle were administered orally. Thirty minutes later, 0.7% 0.1 mL/10 g acetic acid solution was intraperitoneally injected to the mice. Positive control diclofenac was administered 15 min before acetic acid injection. After 5 min, mice were observed for writhing (defined contraction of body) for the next 20 min[12]. The decrease in number of writhes was considered as the evidence of analgesic effect.

2.6.2. Formalin test

On the day of experiment, animals were dosed orally 1 h before formalin challenge. Then 20 μL of 5% formalin was injected into the dorsal lateral surface of the left hind paw. The time spent on licking was recorded for 30 min immediately after formalin injection in the first phase (0–5 min) and the second phase (16–30 min). The decrease in licking time was considered as antinociception[13].

2.7. Tests for the sedative and anxiolytic activities

2.7.1. Hole cross test

Hole cross test was performed with a specially designed cage having a wooden dividing wall fixed in the middle of the cage (30 cm × 20 cm × 14 cm) with a 3 cm dia hole at 7.5 cm height on that partition[14]. The tested samples and standard drug diazepam (1 mg/kg body weight) and vehicle was orally administered to the respective groups and the animals were immediately placed in the cage for observation. The number of crossing for a mouse through the hole from one compartment to the other was counted for 3 min at 0, 30, 60, 90 and 120 min.

2.7.2. Open field test

Like the hole cross test, animals in positive control group received diazepam (1 mg/kg body weight). The floor area of the open field (half square meter) was divided into a series of squares. The number of line crossed (distance travelled) by the animals was counted for 3 min at 0, 30, 60, 90, and 120 min after the oral feeding of the tested samples and the standard[15].

2.7.3. Elevated plus maze (EPM) test

The EPM apparatus is made up of two open arms (5 cm × 10 cm) and two closed arms (5 cm × 10 cm × l5 cm)[16]. Sixty minutes after oral administration of the tested samples and the standard, mice were placed at the middle of the maze to move in free directions. The number of open and closed arm entries along with the duration in open and closed arms was recorded for 5 min[17].

2.8. Test for the cytotoxic activity by brine shrimp lethality bioassay

Brine shrimp (*Artemia salina* Leach) eggs were collected and hatched in a large beaker with a temperature of 37 ˚C and continuous oxygen supply and allowed to hatch and mature for the next 2 days. Stocks were prepared by dissolving extracts in pure dimethyl sulfoxide. Four milliliters of seawater taken and specific volume of sample were transferred from the stock solution to the vials to get final sample concentrations of 0.00, 7.82, 15.62, 31.25, 62.50, 125.00, 250.00, 500.00, 750.00, and 1 000.00 μg/mL. Ten living nauplii were put with Pasteur pipette to each vials. After 24 h, the number of nauplii survived in the vial was counted. The percentage of lethality was calculated as % mortality = number of dead nauplii/initial number of live nauplii × 100. The LC_{50} was determined from the log concentration versus % mortality curve[18].

2.9. Test for the thrombolytic activity by clot lysis test

A 100 mg/10 mL extract suspension of distilled water was vigorously shaken and overnight soaked to get a soluble supernatant which was carefully decanted and finally filtered by 0.22 μ syringe filter. Then, 100 μL of it was transferred to the alpine tube containing the blood clots which was previously prepared by drawing 500 μL venous blood from 10 healthy volunteers and incubated at 37 ˚C for 45 min. These alpine tubes were again weighed to determine the clot weight (clot weight = weight of clot containing tube - weight of tube alone). A volume of 100 μL of streptokinase and 100 μL of distilled

water were served as positive and negative control, respectively. Tubes were then incubated for 90 min at 37 °C and observed for the thrombolysis. After incubation, tubes were again weighed after removing the obtained fluid to get the weight difference after clot disruption. This weight difference obtained before and after clot lysis was expressed as the percentage of clot lysis[19].

2.10. Statistical analysis

Statistical analysis was carried out by One-way ANOVA and followed by Dunnett's *t* test with SPSS 20 for windows. All results were compared with the control group at $P < 0.05$, 0.01 and 0.001.

3. Results

3.1. Acute toxicity test

Oral administration of all three extracts at the doses of 500–2 000 mg/kg did not produce any mortality or noticeable behavioral changes in mice within 72 h observation period. Therefore, it can be suggested that the aqueous, ethanol and ethyl acetate extracts of leaves of *K. pinnata* have low toxicity profile with LD_{50} greater than 2 000 mg/kg.

3.2. Acetic acid-induced writhing test

Table 1 shows the effect of the aqueous, ethanol and ethyl acetate extracts of leaves of *K. pinnata* on acetic acid-induced writhing in Swiss albino mice. Both aqueous and ethanol extracts showed significant reduction ($P < 0.001$) of writhing.

Table 1

Acetic acid-induced writhing test of *K. pinnata*.

Group ($n = 5$)	Number of writhing	Inhibition (%)
Diclofenac	0.100 ± 0.112***	99.70674
Control	34.100 ± 7.478	0
PK AQ	1.100 ± 0.447***	96.77419
KP EOH	2.700 ± 1.517***	92.08211
KP EA	15.000 ± 2.823**	56.01173

Value of number of writhing is presented as mean ± SEM ($n = 5$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Dunnett's *t* test compared with the control group.

3.3. Formalin test

The aqueous extract significantly inhibited ($P < 0.001$) the licking activity of both phases of formalin induced pain in mice which was comparable to the reference analgesic drug diclofenac sodium (10 mg/kg) whereas the ethanol extract showed high activity in first phase but moderate ($P < 0.05$) in late phase (Table 2).

Table 2

Formalin test of *K. pinnata*.

Group ($n = 5$)	Early phase licking time	Inhibition (%)	Late phase licking time	Inhibition (%)
Diclofenac	42.200 ± 2.219***	61.78	25.400 ± 1.924***	58.36
Control	110.400 ± 6.130	0	61.000 ± 4.472	0
PK AQ	58.600 ± 6.048***	46.92	36.000 ± 5.788**	40.98
KP EOH	64.800 ± 5.343***	41.30	40.000 ± 6.245*	34.42
KP EA	85.200 ± 5.273**	22.82	56.200 ± 5.153	7.86

Value of licking time is presented as mean ± SEM ($n = 5$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Dunnett's *t* test compared with the control group.

3.4. Hole cross test

The ethyl acetate extract showed a significant ($P < 0.001$) decrease in locomotion in all intervals throughout the experiment (Figure 1). The maximum suppression was observed at the last three intervals (60, 90 and 120 min), which was comparable to the standard.

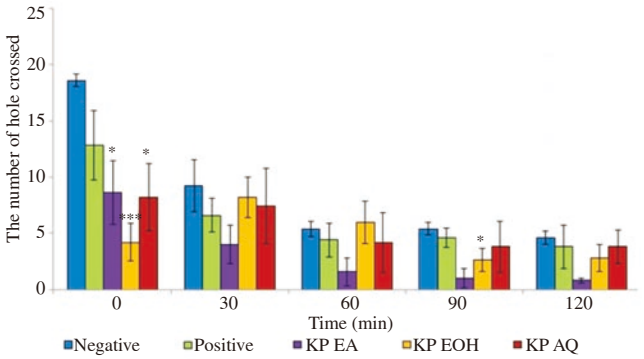

Figure 1. Effect of the aqueous, ethanol and ethyl acetate extracts of leaves of *K. pinnata* on hole cross test in mice.

Values are presented as mean ± SEM ($n = 5$). *: $P < 0.05$; ***: $P < 0.001$. Dunnett's *t* test as compared to control (vehicle = 0.5 mL/mouse).

3.5. Open field test

The number of line crossed by the animals was significantly suppressed from the second observation period by the ethyl acetate extract of the leaves. The results were statistically significant ($P < 0.001$) (Figure 2).

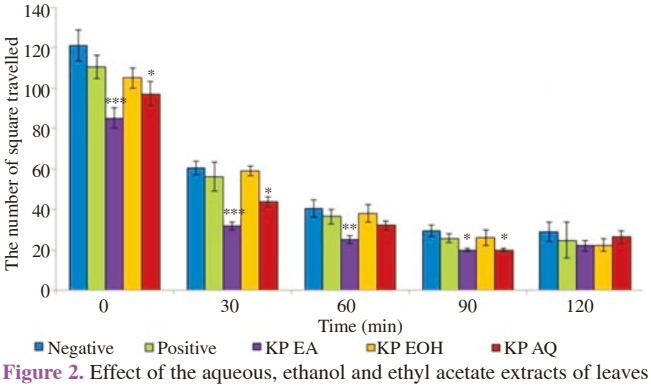

Figure 2. Effect of the aqueous, ethanol and ethyl acetate extracts of leaves of *K. pinnata* on open field test in mice.

Values are presented as mean ± SEM ($n = 5$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Dunnett's *t* test as compared to control (vehicle = 0.5 mL/mouse).

3.6. EPM test

The ethyl acetate extract of *K. pinnata* significantly increased the proportion of entries (Table 3) of mice into open arms and the proportion of the duration spent in open arms of the maze. The percentage results of these two observations were statistically significant ($P < 0.01$).

Table 3

EPM test of *K. pinnata*.

Group ($n = 5$)	Number of entries into the open arm	Time spent in the open arms
Positive	77.720 ±1.828**	80.540 ± 5.286***
Negative	53.410 ± 2.211	49.220 ± 8.119
PK AQ	71.590 ± 9.511***	74.940 ± 8.600***
KP EOH	64.720 ± 4.420***	69.180 ± 5.770***
KP EA	80.620 ± 6.920***	85.360 ± 6.490***

Each value is presented percentage as mean ± SEM ($n = 5$). **: $P < 0.01$; ***: $P < 0.001$. Dunnett's *t* test compared with control group.

3.7. Cytotoxicity test

Table 4 represents the percentage of the mortality of shrimp eggs in different concentrations of the extract. It was observed that the mortality was directly proportional to the concentrations of the extracts. The LC_{50} values of the aqueous, ethanol and ethyl acetate obtained in the present experiment were 275.42, 70.79 and 371.53 μg/mL, respectively, which demonstrated the ethanol extract's greater toxicity as compared with others.

Table 4
The percentage of the mortality of different extracts of *K. pinnata.*

Concentration (μg/mL)	Log C	Mortality of different extracts (%)		
		KP AQ	PK EOH	KP EA
1000.00	3.000	80	100	80
500.00	2.698	70	95	70
250.00	2.397	45	75	45
125.00	2.096	40	60	30
62.50	1.795	25	45	5
31.25	1.494	5	25	5
15.62	1.193	0	10	0
7.82	0.893	0	0	0
LC_{50} (μg/mL)		275.42	70.79	371.53

All concentrations are presented in log value and the percentage of the mortality of different extracts are presented accordingly.

3.8. Clot lysis test

A total of 100 μL of streptokinase (standard) showed 84.39% clot lysis whereas 100 μL of sterile distilled water (control) showed negligible clot lysis (6.90%) (Table 5). 22.33%, 21.46%, 17.10% clots were lysed after the treatment with 100 μL of aqueous, ethanol and ethyl acetate extracts, respectively. The mean percentage of clot lysis by the extracts was statistically significant ($P < 0.01$–0.001) as compared with control.

Table 5
Clot lysis activity of *K. pinnata.*

Group ($n = 5$)	Clot lysis (%)	*P*
Positive	84.39 ± 9.86	< 0.001
Negative	6.90 ± 3.05	-
KP AQ	22.33 ± 8.88	< 0.001
EOH KP EOH	21.46 ± 5.20	< 0.001
KP EA	17.10 ± 5.55	< 0.01

Each value is presented as mean ± SD. *P* value was calculated followed by two-tailed *t*-test as compared to negative control (water).

4. Discussion

Writhing movement is a sensitive method to evaluate peripheral acting analgesics by inducing pain with chemical agents such as acetic acid. Prostaglandin pathways, peritoneal mast cells and acid sensing ion channels are the responsible factors for this pain mediation[20-22]. Acetic acid in peritoneal cavity releases prostaglandins and sympathomimetic mediators such as PGE2 and PGF2[23]. These sensitize nociceptive receptors to produce abdominal constriction[24]. It is therefore possible that the extract exerts its analgesic effect by inhibiting the synthesis of prostaglandins or interfering with other mediators which are responsible for peripheral pain and that may be resulted by phytochemicals present in the extract.

A subplantar administration of formalin, a chemical irritant, induces visceral and peripheral pain in mice[25,26]. The response of the early phase is supposed to represent a direct chemical stimulation due to the irritant effect of formalin on sensory C fibers[27]. Whereas the late phase is considered to be the secondary inflammatory response by releasing allergic mediators[28]. The suppression to licking response in the early and late phases by the tested drugs signifies the analgesic effect of the extracts. The results of the study demonstrated that aqueous and ethanol extracts of *K. pinnata* leaves exerted the potential analgesic effect in experimental animal models which supported the claims by traditional medicine practitioners.

The ethyl acetate extract of the leaves of *K. pinnata* decreased the spontaneous motor activity both in hole cross and open field tests. This decrease in degree of movements could be attributed to the sedative effect of the plant extracts, since the locomotor activity is a measure of the level of excitability of the CNS[28,29]. The anxiolytic effect of the extract was also observed by the EPM test. EPM is considered as a valuable model to investigate anxiolytic effects of drugs in experimental animals[30,31]. The ethyl acetate extract displayed a significant increase in the percentage of open arm entries and the open arm duration at the maze. This result was even stronger than that of the reference diazepam.

Binding of a benzodiazepine agonist to its recognition site at GABA-A benzodiazepine receptor results in a increase of chloride ion influx, which eventually hyperpolarizes the postsynaptic membrane below spike generation level and that is why for hypnosis some GABA-A agonists are frequently used[32,33]. The leaves of *K. pinnata* have been reported to contain *p*-coumaric, ferulic, syringic, caffeic and *p*-hdroxybenzoic acids, quercetin and kaempferol, which can be hypothesized to agonize GABA-A receptor to exhibit CNS depressant effect of the leaves.

Brine shrimp lethality bioassay is considered primarily and mostly widely used indicator of cytotoxic action[34]. The extracts have cytotoxic potential which is concentration-dependent and may have potentials of tumor and cancer suppression. Antithrombotic agents activate plasminogen which unfolds cross-linked fibrin mesh to make the clot soluble and facilitate further proteolysis by other enzymes. This phenomenon brings back normal blood flow over blocked vessels establishing the treatment of myocardial infarction, deep vein thrombosis, thromboembolic strokes and pulmonary embolism, which avoids permanent damage to the perfused tissue (*e.g.* myocardium, brain, leg)[19]. *K. pinnata* has potential thrombolytic activity which can be the new source for potent lead compounds for the development of useful medicines.

The extracts of *K. pinnata* showed promising analgesic potential in different pain models. Also, *K. pinnata* leaves possessed sedative and anxiolytic potentials. Besides, the cytotoxic and thrombolytic tests showed moderate activity. Therefore, this extract may establish the alternate therapy for the treatment of anxiety, other psychiatric disorders and thrombosis and pain sensation. However, further chemical and pharmacological studies are necessary to identify bioactive compounds which can be the source of new lead molecules for drug development.

Conflict of interest statement

We declare that we have no conflict of interest.

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