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ASSESSMENT OF CYTOTOXICITY AND ANTIBACTERIAL ACTIVITIES OF ETHANOLIC EXTRACTS OF *KALANCHOE PINNATA* LINN. (FAMILY: CRASSULACEAE) LEAVES AND STEMS

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ABSTRACT

Objective: In the present work, the leaves and stems of *Kalanchoe pinnata* Linn. (Family: Crassulaceae) have been investigated for screening phytochemicals, cytotoxicity and antibacterial activities.

Materials and methods: Brine shrimp lethality (BSL) bioassay was used as a pre-screening method for cytotoxic effect. Ethanolic extract of the plant was tested by using this method. The agar disc diffusion method was used for antimicrobial assay of the plant extract.

Results: The crude extract produced the most significant cytotoxic activity against brine shrimp *Artemia salina* (LC50: 100µg/mL and LC90: 204.17µg/mL). The ethanolic extract of *Kalanchoe pinnata* Linn. showed significant antibacterial activity against gram positive (*B. Subtilis, S. aureus)* and gram negative *(E. coli, P. aeruginosa, S. dysenteriae)* bacteria with the zones of inhibition ranging from 6.0±0.35 to 8.2±0.22 mm. The phytochemical screening of the ethanolic extracts of *Kalanchoe pinnata* Linn. showed the presence of alkaloids, glycosides, steroids, gums, flavonoids, saponins, reducing sugars and tannins.

Conclusion: The obtained results provide a support for the use of this plant in traditional medicine. The plant studied can be a potential source of biologically active compounds as antitumor agent, antibacterial and pesticide.

INTRODUCTION: *Kalanchoe pinnata* Linn. (Family: Crassulaceae) is commonly known as patharkuchi. It is a tall, erect perennial glabrous herb with woody stem and thick, succulent leaves having crenate margins, often with foliar buds and adventitious roots, grown in gardens all over the country [1].

The plant contains bersaldegenin-1, 3, 5-orthoacetate [2] and bufadienolide- bryophyllin-B [3]. Other chemical constituents from the plants bryophyllol, bryophyllone, bryophyllenone, bryophynol are also isolated [4]. The leaves and bark are bitter tonic, astringent to bowels, analgesics, carminative and useful in diarrhoea and vomiting. Antiulcer [5] activities of the leaf were also reported. Several other biological activities have been reported for *Kalanchoe pinnata* Linn.

The plant has hepatoprotective activity and is also used to increase vascular integrity [6], to treat hypertension and kidney stones[7] and to enhance the dropping of umbilical cord of a newly born baby [8]. The leaves of the plant are eaten to control diabetes. They are diuretic, and applied to wounds, boils and bites of insects.

Leaf juice is used in the treatment of coughs, bronchial affections, blood dysentery, jaundice and gout [1].

MATERIALS AND METHODS:

Plant materials: The fresh plant selected for the study was collected from Pahartolly and Satkania under the district of Chittagong division, Bangladesh from August to December in 2010 at day time. After collection, the leaves and stem of *Kalanchoe pinnata* Linn. (Family: Crassulaceae) were cut into very small pieces and dried at room temperature under shade. The leaves and stems were ground into a coarse powder with the help of a suitable grinder. The powder was then stored in an airtight container and kept in a cool, dark and dry place until the analysis was commenced.

Extraction procedures: Ground plant materials (100gm) were taken in a clean, flat bottomed plastic container and soaked in 300ml of ethanol. The container with its contents was sealed and kept for a period of 7 days accompanied by continuous shaking with the shaker. The extracts were filtered through a Millipore filter (0.25 µm). The resulting filtrate was concentrated under reduced pressure and then transferred into a well labelled sterile container.

Test organisms: The test of microorganisms included gram positive (*B. Megaterium, B. Subtilis, S. aureus)* and gram negative (*E. coli, P. aeruginosa, S. dysenteriae, S. typhi, Vibrio cholerae)* bacteria were clinical strains obtained from the stock culture of the microbiology laboratory, Department of Pharmacy, Southern University Bangladesh. Stock cultures of bacteria were maintained on Nutrient Agar and all cultures were sub-cultured monthly and subsequently stored at 4^{o}C.

Standard drug: Amoxycillin was used as a standard drug in this research work and the drug was collected from Incepta Pharmaceuticals Limited, Dhaka, Bangladesh.

Phytochemical screening of the Extracts: The phytochemical screening was done on the ethanolic extracts using standard procedures and the following qualitative tests [9] were carried out:

Test for Alkaloids: Two tests were performed to identify the presence of alkaloids in *Kalanchoe pinnata* Linn.

Mayer's Test: Under this test, 2ml of the extract and 0.2ml of dilute hydrochloride acid were taken in a test tube. Then, 1ml of Mayer's reagent was added. A yellowish buff precipitate is indicative of the presence alkaloids.

Dragendroff's Test: In case of this test, 2ml of the extract and 0.2ml of dilute hydrochloride acid were taken in a test tube. Then, 1ml of Dragendroff's reagent was added. Observation of deposits of an orange-brown precipitate was taken to indicate the presence of alkaloids.

Test for Glycosides: A small amount of ethanolic extract was taken in 1ml of water in a test tube and a few drops of aqueous NaOH were added. A yellow coloration indicates the presence of glycosides.

Test for Steroids: 1ml of ethanolic extract was taken and 1ml of sulphuric acid was later added. The formation of red colour solution indicates the presence of steroids.

Test for Gums: 5 ml of the extract was taken and then Molisch reagent and sulphuric acid were added. The formation of red-violet ring at the junction of two liquids indicates the presence of gums.

Test for Flavonoids: A few drops of concentrated hydrochloride acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour indicates the presence of flavonoids.

Test for Reducing Sugars: 2ml of an ethanolic extract of the plant material was added to 1ml of a mixture of equal volume of Fehling's solution A & B and was boiled for 5 minutes on a boiling water bath. The formation of brick-red color precipitate shows the presence of reducing sugars.

Test for Tannins: 5ml of the extract was taken in a test tube and then 2ml of 5% $FeCl_3$ solution was added. A greenish-black precipitate indicates the presence of tannins.

Test for Saponins: 1ml of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. The formation of one centimetre layer of foam indicates the presence of saponins.

Pharmacological Studies:

Cytotoxicity test: Brine shrimp lethality bioassay [10, 11] is a recent development in the assay procedure which indicates cytotoxicity as well as a wide range of pharmacological activities of the plant extracts. The eggs of brine shrimp used for cytotoxicity test were obtained by hatching 5 mg of eggs of *Artemia salina* in natural seawater after incubation at about 29°C for 48 h. The larvae (nauplii) were allowed another 48 h in seawater to ensure survival and maturity before use. Ten (10) mature larvae (nauplii) were kept in glass vials containing 10 ml of seawater.

The test bacteria dissolved in DMSO (10 mg/ml) was applied to the nauplii in each vial. However, not more than 50 µl of DMSO was added to the vials containing the shrimps. For each concentration, vials containing the same volume of DMSO plus seawater and shrimps were used as controls. After 24 h, the vials were observed for mortality. The number of survived nauplii in each vial was counted and from this data the percentage of lethality of the brine shrimp nauplii was calculated [12] and the lethal concentration (LC_{50}) and (LC_{90}) of the ethanolic extract was determined.

***In vitro* Antimicrobial Assay:** The antimicrobial assay was done using the agar disc diffusion method. The nutrient agar media and nutrient broth were used to demonstrate the antibacterial activity and to make subculture of the test organisms.

Sterilization of different equipments and media: Media, petridish and other glassware were sterilized by autoclaving at a temperature of 121^{o}C and then, all of these were kept in the laminar air flow for 30 minutes. The UV-light was also switched on before one hour working in the laboratory.

Preparation of Subculture Media and determination of zone of inhibition: The agar medium was distributed among the conical flasks and the bacteria were placed. After mixing very quickly, the mixture was placed in the respective petridish. In sterile medium, the filter paper discs were impregnated with known amount of test substances by using micropipette and dried. These discs were placed in the petridish and kept the petridishes in refrigerator for 5 hours for diffusion.

Then, the petridishes were moved to incubator at 37^{o}C for 18 to 24 hours to assure the growth of bacteria. If the sample has antimicrobial activity, it will inhibit the growth of microorganisms by giving clear, distinct zone called ‘‘zone of inhibition’’. The antimicrobial activities were determined by the width of the zone of inhibition in mm.

RESULTS:

Preliminary Phytochemical Analysis: Results of different qualitative phytochemical tests on ethanolic extract of *Kalanchoe pinnata* Linn. showed the presence of alkaloids, glycosides, steroids, gums, flavonoids, saponins, reducing sugars and tannins. **Table 1** showed the result of the qualitative analysis of the ethanolic extract of the plant.

TABLE 1: RESULTS OF PHYTOCHEMICAL ANALYSIS OF ETHANOLIC EXTRACTS OF *KALANCHOE PINNATA* LINN.

Alkaloid	Glycoside	Steroid	Gums	Flavonoids	Saponins	Reducing sugar	Tannin
+	+	+	+	+	+	+	+

(+) = present

Cytotoxic Activity: In brine shrimp lethality bioassay, the ethanolic extract showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations **(Table 2).** From the plot of percent mortality versus log concentration on the graph paper **(Figure 1),** LC_{50} (µg/ml) and LC_{90} (µg/ml) were deduced (LC_{50}: 100µg/mL and LC_{90}: 204.17µg/mL).

Antimicrobial Activities: Table 3 showed the results of antibacterial test. The antibacterial potential of the extract was assessed against eight bacterial strains (both gram positive and gram negative) at the dose of 0.5mg/disc and the results (diameter of zone of inhibition) were compared with the activity of the standard drug, Amoxycillin (0.1mg/disc).

At 0.5mg/disc, the ethanolic extract of *Kalanchoe pinnata* Linn. exhibited significant zone of inhibition against *B. Subtilis* (7.0 ± 0.55), *S. aureus* (7.1±0.45), *E. coli* (8.2±0.22), *P. aeruginosa* (6.0±0.35) and *S. dysenteriae* (6.8±0.27).

TABLE 2: BRINE SHRIMP LETHALITY BIOASSAY OF ETHANOLIC EXTRACT OF *KALANCHOE PINNATA* LINN.

Conc. of samples (µg/ml)	Log (Conc.)	No. of alive shrimp				% mortality	LC_{50} (µg/ml)	LC_{90} (µg/ml)
		Test-1	Test-2	Test-3	Average			
5	0.70	9	10	9	9.3	7		
25	1.40	6	7	8	7.0	30		
50	1.70	4	5	7	5.3	47		
75	1.88	5	5	6	5.3	47		
100	2.00	4	6	5	5.0	50	**100**	**204.17**
125	2.10	4	4	4	4.0	60		
150	2.18	2	3	2	2.3	77		
200	2.30	1	2	1	1.3	87		
250	2.40	0	0	0	0	100		
300	2.48	0	0	0	0	100		
400	2.60	0	0	0	0	100		
500	2.70	0	0	0	0	100		

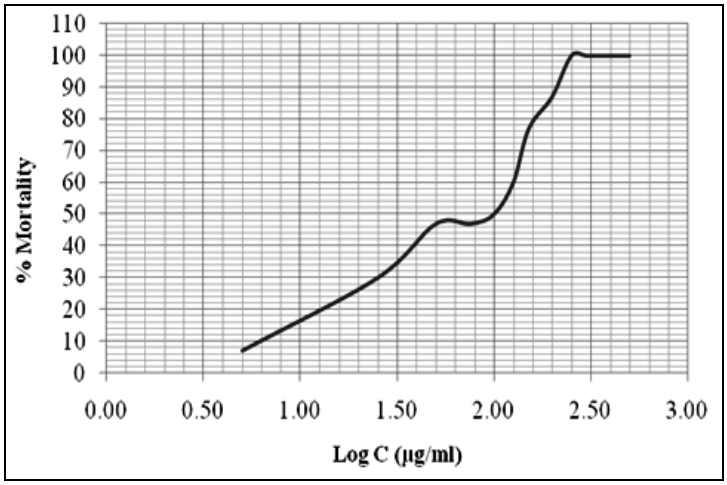

FIG. 1: GRAPHICAL PRESENTATION OF LC_{50} (µG/ML) AND LC_{90} (µG/ML) OF ETHANOLIC EXTRACTS OF *KALANCHOE PINNATA* LINN.

TABLE 3: ANTIMICROBIAL ACTIVITIES OF ETHANOLIC EXTRACTS OF *KALANCHOE PINNATA* LINN.

Test Organisms	Blank	Zone of Inhibition (mm)	
		Ethanolic extract of *Kalanchoe pinnata* Linn. (0.5mg/disc)	Standard drug, Amoxycillin (0.1mg/disc)
Gram positive			
B. megaterium	-	-	14.3±0.90
B. subtilis	-	7.0±0.55	13.5±1.08
S. aureus	-	7.1±0.45	14.0±0.20
Gram negative			
E. coli	-	8.2±0.22	15.5±0.41
P. aeruginosa	-	6.0±0.35	-
S. dysenteriae	-	6.8±0.27	10.3±0.40
S. typhii	-	-	-
Vibrio cholerae	-	-	15.0±0.82

Data were represented as Mean ± SD of triplicate determination and (-): no inhibition

DISCUSSION: Plants are employed as important source of medication in many traditional medications [13, 14, 15]. The phytochemical screening of the ethanolic extract of *Kalanchoe pinnata* Linn. showed the presence of alkaloids, glycosides, steroids, gums, flavonoids, saponins, reducing sugars and tannins. The cytotoxic activity of the ethanolic extract of *Kalanchoe pinnata* Linn. was tested by using brine shrimp lethality bioassay. The extract was found to show potent cytotoxic activity against the brine shrimp nauplii.

Therefore, the positive response obtained in this assay suggests that the extract may contain antitumor, antibacterial or pesticidal compounds. The antibacterial test was performed using the disc diffusion method. The antibacterial activity of the extract was assessed against eight bacterial strains (both gram positive and gram negative) at the dose of 0.5gm/disc and the results were compared with the activity of the standard drug, Amoxycillin (0.1gm/disc). In this experiment, the ethanolic extract of *Kalanchoe pinnata* Linn. showed significant sensitivity to the five of the test organisms both gram positive and gram negative type of bacteria except *B. Megaterium, S. typhi* and *Vibrio cholerae.*

The zone of inhibition varies within the range of 6.0±0.35 and 8.2±0.22 mm. The highest zone of inhibition (8.2±0.22 mm) was recorded against *E. coli.* and the results support the traditional uses of the *Kalanchoe pinnata* Linn. as a remedy of diarrhoea, dysentery and gastrointestinal disturbances.

CONCLUSION: Finally, it could be suggested that the ethanolic extract of *Kalanchoe pinnata* Linn. leaves and stems possess cytotoxic and antibacterial activities. The plant studied can be seen to be potential source of useful antibacterial drug. The experiment was only conducted with seven species of bacteria as test samples. Therefore, further research is essential to evaluate the sensitivity of the plant extract against other species of bacteria, fungi, virus of other microorganisms. Further studies are however recommended on the plant to determine the pharmaceutical potentialities of the plant as a medicine and to isolate and elucidate the structure of the bioactive compounds.

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