ORIGINAL PAPER

PHYTOCHEMICAL SCREENING AND IN-VITRO EVALUATION OF ANTIOXIDANT, CYTOTOXICITY, ANTIFUNGAL ACTIVITIES OF *KALANCHOE PINNATA* (L)

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Abstract. *The present study was aimed to investigate phytochemical screening and to evaluate reducing power, cytotoxicity and antifungal activities of the petroleum ether and aqueous extracts of leaves and stems of Kalanchoe pinnata Linn (Crassulaceae). The qualitative phytochemical tests showed the presence of alkaloid, glycoside, gums, saponins, reducing sugar and tannins in the petroleum ether crude extract of Kalanchoe pinnata Linn while alkaloid, glycoside, steroid, saponins, tannins were present in the aqueous extract of Kalanchoe pinnata Linn. It was observed that both extracts of the studied plant possessed cytotoxic activity. In brine shrimp lethality bioassay, the LC_{50} (µg/ml) and LC_{90} (µg/ml) of the petroleum ether and aqueous extract of Kalanchoe pinnata Linn, were 25.12µg/ml& LC_{90}: 177.83µg/ml respectively. The aqueous extract of the medicinal plant also showed lethality against the brine shrimp nauplii (LC_{50}: 25.12µg/ml& LC_{90}: 173.78µg/ml). Moreover, the extracts showed significant reducing power. It was also identified that the petroleum ether and aqueous extracts of Kalanchoe pinnata Linn showed antifungal activity. 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 antifungal, anticancer agent and pesticide.*

Keywords: *Kalanchoe pinnata Linn, crassulaceae, antioxidant, cytotoxicity, antifungal.*

1. INTRODUCTION

Kalanchoe pinnata (*K. pinnata*) (Family: Crassulaceae) is an erect, succulent, perennial shrub that grows about 1.5m tall and reproduces through seeds and also vegetatively from leaf bubils [1]. The synonyms of *Kalanchoe pinnata* Linn (Family: Crassulaceae) includes *Bryophyllum calycinum, Bryophyllum pinnatum. Kalanchoe pinnata* Linn is widely distributed in Asia, Australia, New Zealand, West Indies, Macaronesia, Mascarenes, Galapagos, Melanesia, Polynesia and Hawaii [2]. K. pinnata is used in ethnomedicine for the treatment of earache, burns, abscesses, ulcers, insect bites, whitlow, diarrhea and cithiasis. In traditional medicine, Kalanchoe species have been used to treat ailments such as infections, rheumatism, and inflammation and have immunosuppressive effect as well. In South-eastern Nigeria, this herb is used to facilitate the dropping of the placenta of new born baby. The lightly roasted leaves are used externally for skin fungus. The leaf infusions are an internal remedy for fever [3]. Kalanchoe pinnata is also used to expel worms, cure acute and chronic

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bronchitis, pneumonia and others forms of respiratory tract infections such as asthma. The plant is considered a sedative wound-healer, diuretic and cough suppressant. The plant is also employed for the treatment of kidney stones, gastric ulcer and edema of legs. The plant Kalanchoe pinnata is also widely used in ayurvedic system of medicine as astringent, analgesic, carminative and also useful in nausea and vomiting [4].

2. MATERIALS AND METHODS

2.1. PLANT COLLECTION

The leaves and stem of *Kalanchoe pinnata* were selected for this project work. The plant selected for present work was *Kalanchoe pinnata* (Family: *Crassulaceae)* which was collected from Pahartolly in Chittagong town, Bangladesh in January, 2011 at day time. The plant is available in winter season and the leaves and stems were collected from the fresh plants.

2.2. PREPARATION OF CRUDE EXTRACTS

The collected plant parts were separated from undesirable materials or plant parts. Then the leaves and the stems were washed and cut into very small pieces and kept in the open air under shadow for 15 days. Then the plant parts (leaves & stems) were grinded into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark, and dry place until analysis commenced. In this project work cold extraction process with the help of petroleum ether and distilled water was used. About 100 gm of powdered materials was taken in a clean, flat bottomed plastic container and soaked in 300 ml of petroleum ether and distilled water. The container with its contents was sealed and kept for a period of 21 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of clean, white cotton materials. The filtrate obtained was evaporated under ceiling fan and in a tray until dried. It rendered a gummy concentrate of reddish black color and the gummy concentrate was designated as crude extract petroleum ether and aqueous.

2.3. APPARATUS AND CHEMICALS USED

For the phytochemical screening of *Kalanchoe pinnata* Mayer's reagent, Dragendroff's reagent, Fehling's solution A, Fehling's solution B, Benedict's reagent, Molish reagents were used [5,6]. All chemicals were analytical grade. Volumetric flasks 100ml and 250 ml, Electronic balance (Shimadza, three digit after point), UV spectroscopy (Jenway 6305 spectrophotometer), Incubators (Model-J02714, electronic heated laboratory incubator), Centrifugal, pH meter were used in this study for Screening of reducing power activity. Ascorbic acid, Ferric chloride, Trichloroacetic acid (MW-163.39gm), Qualikems fine chemical private Ltd.), Potassium ferric cyanide (MW-422.4gm, Unichem chemical reagent), Potassium hydrogen orthophosphate (MW-136.09gm), Disodium hydrogen orthophosphate (MW-141.96, Qualikems fine chemical private Ltd.) were also used for Screening of reducing power activity [7]. *Artemia salima* Leach (brine shrimp eggs from store), Table salt, NaCl (with iodine and without iodine), Small tank (glass jar) and Lamp to hatch the shrimps, Pipettes, Micro-pipette (50 µl), Volumetric flask, DMSO (Di-methyl sulfoxide), Spoon,

Electric air blower, Test tubes, Beaker and Magnifying glass were used for screening of cytotoxic activity [8,9] .

2.4. PHYTOCHEMICAL SCREENING OF KALANCHOE PINNATA

Standard method was used for the identification of different phytochemicals like test for alkaloids, glycosides, steroids, reducing sugar, flavonoids, tannins and Saponins according to reference [10,11].

2.5. ANTIFUNGAL ACTIVITY OF KALANCHOE PINNATA

The antifungal activity of the pt. ether and aqueous extract of *Kalanchoe pinnata* were performed by using the disc diffusion assay method and the whole testing procedure was the same as the antibacterial activity test. The only difference was that the period of incubation was 48 hours at room temperature.

2.5.1. FUNGI USED

The clinical fungi that are used in this test are *Aspergillus niger*, *Blastomyces dermattitidis*, *Candida albicans*, *Pityrosporum ovale*, *Trichophyton spp*, *Microsporum spp.* The strains of these organisms were collected from Department of Pharmacy, BGC Trust University Bangladesh (Pharmaceutical Microbiology Laboratory).

2.5.2. CULTURE MEDIA

Potato dextrose agar (PDA) medium was used to perform the antifungal activity and for subculture of the test organisms. The potato dextrose agar (PDA) medium consisting of potato slice(200gm), Dextrose (20.20gm),bacterial agar medium(16 gm) and distilled water(q.s. to 1000 ml) [12].

2.5.3. PREPARATION OF THE MEDIUM

The weight amount of potato slice was boiled with a little amount of distilled water for 30 minutes and applied for course filtration by the help of cotton. The required amount of dextrose and bacterial agar medium were properly mixed in a conical flask. Finally the constituents of two flasks were mixed thoroughly after the adjustment of volume by the distilled water the medium was sterilized in an autoclave.

2.5.4. PLACEMENT OF DISCS, DIFFUSION AND INCUBATION

Preparation of the test plates, preparation of the discs, preparation of the test sample, placement of the discs diffusion and incubation. Preparation of the test plates, disc, test sample, placement of the discs, diffusion and incubation process were almost same of the antibacterial activity screening. Here, only the incubation period was replaced by 72 hours at 25°C temperature.

2.6. SCREENING OF REDUCING POWER ACTIVITY

2.6.1. PREPARATION OF SAMPLES

0.1 gm of sample was taken in 100 ml volumetric flasks and adjusted the volume up to 100 ml with water. Then 1000 µg/ml, 500 µg/ml, 250µg/ml, 125 µg/ml, 50 µg/ml, 25 µg/ml, of sample was made consequently.

2.6.2. PREPARATION OF STANDARD

0.1 gm of standard (ascorbic acid) was taken in 100 ml volumetric flasks and the other part was filled with distilled water. Then preparation of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 50 µg/ml, 25 µg/ml, of standard solution as per procedure of sample.

2.6.3. PREPARATION OF SAMPLE FOR REDUCING ACTIVITY

1 ml of sample and standard (1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 50µg/ml, 25µg/ml) was in test tube. Then 2.5 ml of phosphate buffer was taken in each test tube. 2.5 ml of potassium ferric cyanide was taken in each test tube. Then the test tubes was taken in incubator at 50^0C for 20 minutes. 2.5 ml of trichloroacetic acid was taken in each test tube.Centrifuged at 3000s for 10 minutes. 2.5 ml of solutions of each test tube was separated. And taken in separate test tubes.2.5 ml of distilled water was taken in each test tube. 0.5 ml of ferric chloride was taken in each test tube. Then the absorbance of the solution each concentration was measured in 700nm [13].

2.7. IN-VITRO CYTOTOXICITY BIOASSAY

2.7.1. PREPARATION OF SEA WATER (BRINE)

19g NaCl (with iodine) and 19 g NaCl (without iodine) salt were dissolved in 1000 ml distilled water and then filtered to prepare sea water [14].

2.7.2. HATCHING OF BRINE SHRIMP

Sea water was taken in the small tank and shrimp eggs were added to the one side of the divided tank and the side was covered. The shrimps were allowed for 36 hrs to hatch and mature as nauplii. During this period constant oxygen supply and temperature (around 37°C) was maintained. The hatched shrimps were attracted to the lamp through the perforations in the dam and they were taken for bioassay.

2.7.3. IN-VITRO BRINE SHRIMP LETHALITY BIOASSAY

In this bioassay, the crude extract showed lethality indicating the biological activity as the test sample showed different mortality rates at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase order of concentrations and plot of percent mortality versus the concentration of crude extract used on the graph paper produced an approximate linear correlation between them. From the graph the concentration at which 50% & 90% mortality (LC_{50} & LC_{90}) of brine shrimp nauplii occurred were obtained by extrapolation.

3. RESULTS AND DISCUSSIONS

3.1. PHYTOCHEMICAL ANALYSIS

Results of different qualitative phytochemical tests showed the presence of alkaloid, glycoside, gums, saponins, reducing sugar and tannins in the petroleum ether crude extract of *Kalanchoe pinnata* Linn while alkaloid, glycoside, steroid, saponins, tannins were present in the aqueous extract of *Kalanchoe pinnata* Linn. Table-1 showed the results of the qualitative analysis of the petroleum ether and aqueous extract of *Kalanchoe pinnata* Linn.

Table 1. Results of phytochemical analysis of petroleum ether and aqueous crude extracts of *Kalanchoe pinnata* Linn.

Extracts of *Kalanchoe pinnata*	Alkaloid	Glycoside	Steroid	Gum	Flavonoid	Saponin	Reducing sugars	Tannin
Petroleum ether	+	+	-	+	-	+	+	+
Aqueous extract	+	+	+	-	-	+	-	+

+ = presence and – = absence

3.2. CYTOTOXIC ACTIVITY

In brine shrimp lethality bioassay, the petroleum ether and aqueous extract showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations (Tables 2 and 3). From the plot of percent mortality versus log concentration on the graph paper (Figs.1 and 2), LC_{50} (µg/ml) and LC_{90} (µg/ml) of the petroleum ether and aqueous extract of *Kalanchoe pinnata* Linn were deduced respectively (LC_{50}: 25.12µg/ml& LC_{90}: 177.83 µg/ml; LC_{50}: 25.12µg/ml& LC_{90}: 173.78µg/ml).The variation between three tests in Tables 2 and 3 were considered to be statistically significant. Because in case of t-test at 5% level of significance, the table value was 2.23 and the computed value were 3.29 (Table 2) & 8.64 (Table 3). Here, alternative hypothesis were accepted.

Table 2. Brine shrimp lethality bioassay of pt. ether extract of *Kalanchoe pinnata* Linn

Conc. (µg/µl)	Log (Conc.)	No. of alive shrimp				% of mortality	LC_{50} (µg/ml)	LC_{90} (µg/ml)
		Test-1	Test-2	Test-2	Avg.			
5	0.70	6	7	6	6.33	36.67	25.12	177.83
25	1.40	5	4	6	5.00	50.00		
50	1.70	4	3	4	3.67	63.33		
75	1.88	3	2	2	2.33	76.67		
100	2.00	3	2	2	2.33	76.67		
125	2.10	2	1	1	1.33	86.67		
150	2.18	2	1	1	1.33	86.67		
200	2.30	1	1	0	0.67	93.33		
250	2.40	0	1	0	0.33	96.67		
300	2.48	0	0	0	0.00	100.00		
400	2.60	0	0	0	0.00	100.00		
Blank	0.00	10	9	10	9.67	3.33		

Table 3. Brine shrimp lethality bioassay of aqueous extract of *Kalanchoe pinnata* Linn.

Conc. (µg/µl)	Log (Conc.)	No. of alive shrimp				% of mortality	LC_{50} (µg/ml)	LC_{90} (µg/ml)
		Test-1	Test-2	Test-3	Avg.			
5	0.70	6	4	6	5.33	46.67	25.12	173.78
25	1.40	6	4	5	5.00	50.00		
50	1.70	4	6	4	4.67	53.33		
75	1.88	3	2	3	2.67	73.33		
100	2.00	3	1	2	2.00	80.00		
125	2.10	3	2	3	2.67	73.33		
150	2.18	1	2	1	1.33	86.67		
200	2.30	0	1	1	0.67	93.33		
250	2.40	0	0	0	0.00	100.00		
300	2.48	0	0	0	0.00	100.00		
400	2.60	0	0	0	0.00	100.00		
Blank	0.00	10	8	9	9.00	10.00		

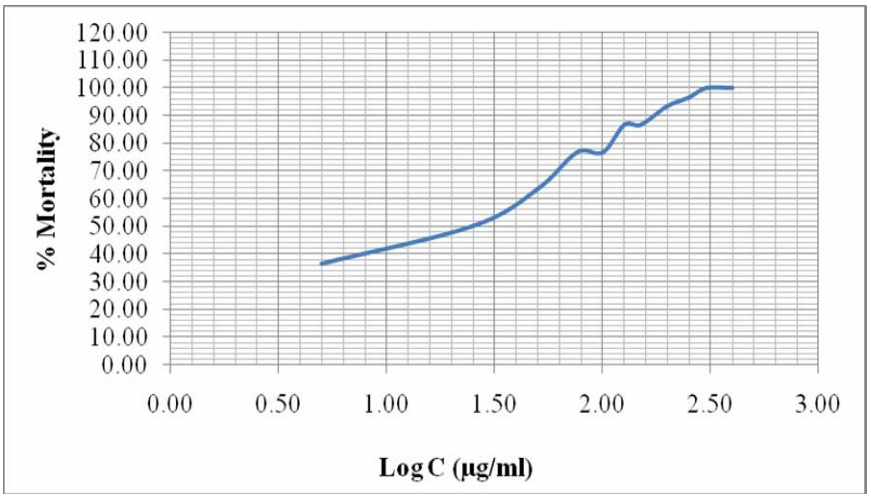

Figure 1. Graphical presentation of LC_{50} (µg/ml) and LC_{90} (µg/ml) of the petroleum ether of *Kalanchoe pinnata* Linn.

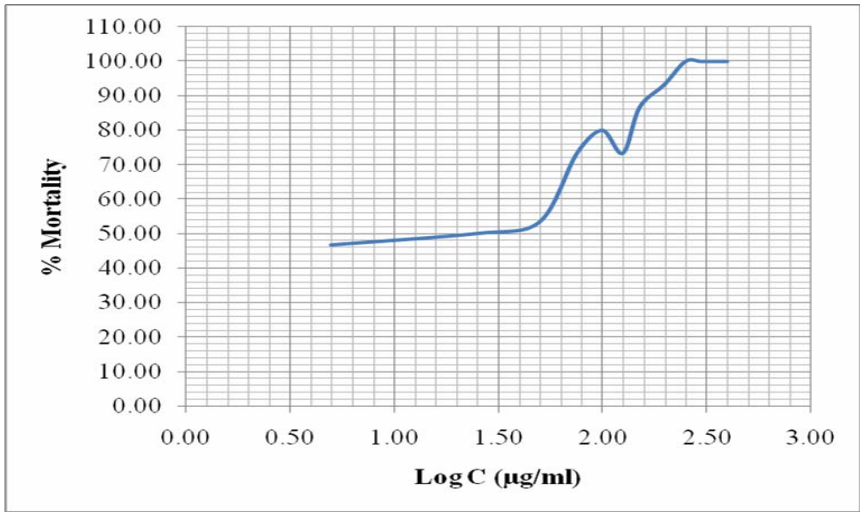

Figure 2. Graphical presentation of LC_{50} (µg/ml) and LC_{90} (µg/ml) of the aqueous extract of *Kalanchoe pinnata* Linn.

3.3. ANTIFUNGAL ACTIVITY

Tables 4 and 5 showed the results of antifungal test. The antifungal potentials of the petroleum ether and aqueous extract of *Kalanchoe pinnata* Linn. (30µg/disc) were assessed against six fungus. The results (diameter of zone of inhibition) were compared with the

activity of the standard drug, Griseofulvin (30µg/disc). At 80µg/disc, the petroleum ether extract of *Kalanchoe pinnata* Linn. Exhibited antifungal activity with small zone of inhibition ranging from 1.50 mm to 2.66 mm while at concentration of 80µg/disc of the aqueous extract of *Kalanchoe pinnata* Linn. Also showed antifungal activity with zone of inhibition ranging from 1.66mm to 2.66mm. The variation between three test in Table 2 & Table 3 were considered to be statistically significant because in case of t-test at 5% level of significance , the table value was 2.5 and the computed value were 6.8 (Table 4) & 13.74 (Table 5). Here, alternative hypothesis were accepted. So, there was significant difference between three tests.

Table 4. Anti-fungal activity of the petroleum ether extract of *Kalanchoe pinnata* Linn., standard and blank

Tested Fungi	Zone of inhibition (mm) by the crude pt. ether extract of *K. pinnata* (80µg/disc)				Standard drug, Griseofulvin (30µg/disc)	Blank
	Test-1	Test-2	Test-3	Avg		
Aspergillus niger	3.00	2.00	2.00	2.30	10.50	-
Blastomyces dermatitides	2.50	1.00	2.00	1.80	12.25	-
Candida albicans	2.00	2.00	1.50	1.83	12.45	-
Pityrosporum ovale	1.50	1.50	1.50	1.50	14.30	-
Trichophyton spp	2.50	2.00	2.00	2.16	13.75	-
Microsporum spp	3.00	2.50	2.50	2.66	11.75	-

Table 5. Anti-fungal activity of the aqueous extract of *Kalanchoe pinnata* Linn., standard and blank

Tested Fungi	Zone of inhibition (mm) by the crude aqueous extract of *K. pinnata* (80µg/disc)				Standard drug, Griseofulvin (30µg/disc)	Blank
	Test-1	Test-2	Test-3	Avg		
Aspergillus niger	2.50	2.00	2.00	2.16	10.50	-
Blastomyces dermatitides	2.50	1.50	2.00	2.00	12.25	-
Candida albicans	3.00	2.50	2.50	2.66	12.45	-
Pityrosporum ovale	1.50	2.50	2.00	2.00	14.30	-
Trichophyton spp	1.50	2.00	1.50	1.66	13.75	-
Microsporum spp	1.00	2.50	2.00	1.66	11.75	-

3.4. REDUCING POWER

From Tables 6 and 7 shows the reductive capabilities of the plant extracts compared to ascorbic acid. An increase in absorbance in the reducing power method implies that both of the crude extracts are capable of donating hydrogen atoms in a dose dependent manner [15]. The variation between three tests in Table 6 was considered to be statistically significant because in case of t-test at 5% level of significance, the table value was 2.5 and the computed value was 14.5. Here, alternative hypothesis were accepted. But in Table7, at 5% level of significant the table value was 2.5 and the computed value was 0.9. Here, null hypothesis were accepted. So, there was no significant difference between three tests.

Table 6. The absorbance of petroleum ether extract with standard ascorbic acid at 700nm

Conc (µg/ml)	Standard	Petroleum ether extract			
		Test-1	Test-2	Test-3	Avg.
25	0.16	0.009	0.008	0.006	0.008
50	0.21	0.017	0.019	0.018	0.018
125	0.33	0.019	0.020	0.020	0.020
250	0.40	0.025	0.019	0.026	0.023
500	0.55	0.026	0.025	0.022	0.024
1000	0.70	0.026	0.024	0.024	0.025

Table 7. The absorbance of aqueous extract with standard ascorbic acid at 700nm

Conc. (µg/ml)	Standard	Aqueous extract			
		Test-1	Test-2	Test-3	Avg.
25	0.16	0.006	0.005	0.006	0.006
50	0.21	0.005	0.007	0.006	0.006
125	0.33	0.007	0.010	0.010	0.009
250	0.40	0.009	0.007	0.010	0.009
500	0.55	0.011	0.011	0.010	0.011
1000	0.70	0.018	0.019	0.018	0.018

4. CONCLUSION

Petroleum ether and aqueous extracts of the leaves and stems of *Kalanchoe pinnata* Linn possess cytotoxic and antifungal activities. Both of the plant extracts also showed reducing power. It was also observed that both extracts of the studied plant possessed cytotoxic activity. In brine shrimp lethality bioassay, the LC_{50} (µg/ml) and LC_{90} (µg/ml) of the petroleum ether and aqueous extract of *Kalanchoe pinnata* Linn were 25.12µg/ml& LC_{90}: 177.83µg/ml respectively. The aqueous extract of the medicinal plant also showed lethality against the brine shrimp nauplii (LC_{50}: 25.12µg/ml& LC_{90}: 173.78µg/ml). Further investigation is required to isolate pure compounds for establishing its mechanism of action.

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