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In vitro antimicrobial and haemolytic studies of *Kalanchoe pinnata* and *Callistemon viminalis*

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Abstract

The present study was carried out to determine the *in vitro* antimicrobial and haemolytic potential of *Kalanchoe pinnata* and *Callistemon viminalis* leaves extracts. The disc diffusion and resazurin microtitre-plate assays were used to evaluate the antimicrobial activity by measuring zone of inhibition and minimum inhibitory concentration (MIC) of the plants extracts. The both plants showed significant antimicrobial activity. The extracts of *K. pinnata* exhibited zone of inhibition 5.5-30 mm for tested strains while 95% and 90% methanolic extracts showed inhibitory activity only against *G. lucidum*. *C. viminalis* extracts showed the 2.5-25 mm zone of inhibition against tested strains. The minimum inhibitory concentration (MIC) of *K. pinnata* and *C. viminalis* active extracts ranged from 1.45-22.7 and 0.52-12.0 mg/ml respectively. The haemolytic activity of the plants *K. pinnata* and *C. viminalis* against human blood erythrocytes (RBCs) was studied and the % lysis of RBCs was found to be in the range (1.38-8.13%) and (1.95-6.33%) respectively. Together these data suggested hat both plants analyzed with significant activities could be a potential source of therapeutic drugs.

Key words: Antimicrobial, *Callistemon*, Hemolytic activity, Inhibition, *Kalanchoe*, leaves.

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1. Introduction

Kalanchoe pinnata (Pathorkuchi) (Family: Crassulaceae) is a succulent plant found in Bangladesh. In traditional medicine the leaves of this plant have been reported to exhibited antimicrobial, anti-ulcer, anti-inflammatory, analgesic, potent anti-histamine and anti-allergic activity [1, 2]. Leaves of this plant are eaten for diabetes control, used as diuretic, for dissolving kidney stones, and to treat respiratory tract infections, headache, cough, fever, chest pain, furuncle, ulcers, rheumatism, hypertension, to improve menstrual irregularities and to cure other skin diseases, as applied to wounds, boils and insect bites and the bark of this plant has been used as a purgative [3]. Ali and coworkers [4] reported the presence of various medicinally important compounds such as alkaloids, tritepenes, glycosides, flavonides, steroids lipids and bufadienolides and also evaluated the antibacterical activity of leaves extracts of *K. pinnata*. This plant has a high phytotherapeutic potential, as shown by its anti-inflammatory, anti-ulcer, hepatoprotective, antileishmania, immunomodulatory activities, tocolytic effectiveness, antithromobotic effect and used for cardiovascular treatment [5-7]. *Callistemon viminalis* (Family: Myrtaceae) is an ornamental plant commonly known as Bottle brush that is found in many areas with the exception in extremely cold and dry areas. It is also found along the streets and in the botanical gardens [8, 9]. *C. viminalis* leaves are a tea substitute and have a delightfully refreshing flavour and fragrance. Antihelminthic and antibacterial activities of *C. viminalis* various parts have already been reported [10]. Chemical Composition of the essential oils of *C. viminalis* from various countries has been previously reported [10-13]. The antibacterial activity showed by the plant *C. viminalis* could be attributed to the presence of some major components such as α-pinene, 1, 8-cineole and α-terpineol, along with other components in trace amounts such as, linalool and β-pinene, which were previously known to exhibit the bacteriostatic and antimicrobial activities. In China various *Callistemon* species, especially *C. viminalis*, are reported to be used in Chinese medicines for treatment of hemorrhoids. *Callistemons* are also used to control the weeds and as bioindicators for environmental management. As our efforts to explore the medicinal importance of

various plants [14-18] *C. viminalis* and *K. pinnata* has been to biologically validate the folk use of these both plants against various human ailments.

2. Material and Method

2.1. Collection, identification and processing of Plant Materials

The fresh leaves of the plants *C. viminalis* and *K. pinnata* were collected in April 2011 from the Botanical Garden, University of Agriculture, Faislabad, Pakistan and further identified by a taxonomist, Dr. Mansoor Hameed from Department of Botany, University of Agriculture Faisalabad, Pakistan where voucher specimens have been deposited. The plants leaves were washed with distilled water and then dried in shade. The grinded fine powders of plants leaves (1000g) were extracted with petroleum ether (2×3L) for seven days at room temperature. After filtering the extracts were concentrated through rotary vacuum evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Japan). This process was repeated thrice to obtain a sufficient quantity of petroleum ether extract. The remaining plants residues were further extracted with other different polarity based solvents and obtained successively chloroform, ethylacetate, *n*-butanol, absolute methanol, 95% methanol (95:5, methanol:water, v/v) and 90% methanol (90:10, methanol:water, v/v) extracts. All obtained extracts after drying were stored at -4°C till further analysis.

2.2. Antimicrobial assay

2.2.1. Test microorganisms

Alternaria alternata ATCC 20084, *Ganoderma lucidum* locally isolated, were used as the fungal strains and *Pasturella multocida* locally isolated, *Escherichia coli* ATCC 25922, *Bacillus subtilis* JS 2004, *Staphylococcus aureus* API Staph tac 6736153 were used as the bacterial tested organisms. The bacterial and fungal strains were collected from the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. The bacterial strains were cultured overnight at 37 °C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28 °C using potato dextrose agar (Oxoid).

2.2.2. Disc diffusion method

Antimicrobial activity of the plants various extracts was determined by using the disc diffusion method [19]. All samples (dry residue) were dissolved in 10% sterile dimethyl sulfoxide. The discs (6 mm diameter) were impregnated with 10 mg/mL extracts (100 µL/disc) placed aseptically on the inoculated agar. Discs injected with 100 µL of respective solvents served as a negative controls, Rifampicin (100 µL/disc) (Oxoid) and Fluconazole (100 µL/disc) (Oxoid) were used as positive control for bacteria and fungi, respectively. The petri dishes were incubated at 37±0.1 °C for 20-24 h and 28±0.3 °C for 40-48 h for bacteria and fungi, respectively. At the end of period, the inhibition zones formed on the media were measured. The positive antimicrobial activity was read based on growth inhibition zone.

2.2.3. Resazurin microtitre-plate assay

The minimum inhibitory concentration (MIC) of the plants extracts was evaluated by a modified resazurin subjected to *In vitro* antimicrobial and haemolytic activities microtitre-plate assay as reported by Sarker and coworkers [20] with modification. Briefly, a volume of 100 µL of each extract and fractions solution was transferred into the first row of the 96 well plates. To all other wells, 50 µL of nutrient broth and Muller Hinton broth for bacteria and fungi respectively were added. Two-fold serial dilutions were performed using a multichannel pipette such that each well had 50 µL of the test material in serially descending concentrations. To each well, 10 µL of resazurin indicator solution (prepared by dissolving 270 mg resazurin tablet in 40 mL of sterile distilled water) were added. Finally, 10 µL of bacterial/fungal suspension were added to each well. Each plate was wrapped loosely with aluminum foil. Each plate had a set of controls: a column with broad spectrum antibiotics as positive control, a column with all solutions with the exception of the test samples, a column with all solutions with the exception of the bacterial/fungal solution adding 10 µL of broths instead and a column with respective solvents as a negative control. The plates were prepared in triplicate, and incubated at 37 ± 0.1 °C for 20–24 h and 28 ± 0.3 °C for 40–48 h for bacteria and fungi, respectively The absorbance was measured at 620 nm by micro quant for fungus and at 500 nm for bacteria. The color change was then assessed visually. The growth was indicated by color changes from purple to pink or colorless. The lowest concentration at which color change appeared was taken as the MIC value.

2.3. Haemolytic activity

Haemolytic activity of the plants various extracts was checked by following the already reported method [21]. Three milliliter of freshly obtained heparinized human blood was gently mixed, poured into a sterile 15 mL polystyrene screw-cap tube and centrifuged for 5 min, at 850 *g*. The supernatant was poured off and the viscous pellet washed three additional times with 5 mL of chilled (4 °C) sterile isotonic phosphate-buffered saline (PBS) solution, adjusted to pH 7.4. The washed cells were suspended in a final volume of 20 mL chilled, sterile PBS and the cells counted on a haemacytometer. The blood cell suspension was maintained on wet ice and diluted with sterile PBS to 7.068 × 108 cells mL−1 for each assay. Aliquots of 20 µL of plant extract/fractions were aseptically placed into 2.0 mL microfuge tubes. For each assay, 0.1% Triton X-100 was used as the positive, 100% lytic control and PBS as the negative, 0% lytic control. Aliquots of 180 µL diluted blood cell suspension were aseptically placed into each 2-mL tube and gently mixed three times with a wide mouth pipette tip. Tubes were incubated for 35 min at 37 °C with agitation (80 revolutions per minute). Immediately following incubation, the tubes were placed on ice for 5 min, then centrifuged for 5 min at 1310 *g*. Aliquots of 100 µL of supernatant were carefully collected, placed into a sterile 1.5 mL microfuge tube, and diluted with 900 µL chilled, sterile PBS. All tubes were maintained on wet ice after dilution. Then 200 µL were placed into 96 well plates, and three replicates was taken in well plate which contain one positive and one negative. Absorbance at 576 nm was then measured on a microquant. The experiment was done in triplicate. Percent hemolysis was calculated by following formula: % hemolysis = Abs (sample absorbance)/Abs (control absorbance) × 100

2.4. Statistical Analysis

All the experiments were conducted in triplicate form and data were presented as mean values ± standard deviation (SD). Unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of difference $p < 0.05$ was considered to denote a statistically significance.

Table 1: Antimicrobial potential of *K. pinnata* and *C. viminalis* leaves by disc diffusion method.

Various organic extracts	Plants used	Diameter of inhibition zone (mm)					
		B. subtilis	*P. multocida*	*S. aureus*	*E. coli*	*A. alternata*	*G. lucidum*
Petroleum ether	*K. pinnata*	11.2±0.82^{c}	n.d	n.d	22.5±1.11^{b}	23±1.58^{b}	23.5±1.11^{b}
	C.viminalis	18.5±0.86^{c}	17.4±1.00^{b}	21.5±1.5^{b}	15.5±0.86^{e}	13.1±1^{e}	23±1.00^{b}
Chloroform	*K. pinnata*	29.5±1.11^{a}	30.2±0.70^{b}	10.7±0.82^{e}	28.7±0.82^{a}	14.2±1.92^{d}	17.5±1.13^{cd}
	C. viminalis	23.0±1.00b	17.7±0.43^{b}	22.5±0.86^{b}	17.1±1.00^{e}	22.0±41^{b}	14.7±0.43^{d}
Ethylacetate	*K. pinnata*	n.d	30±0.70^{b}	30±1.87^{a}	13.2±0.70^{d}	23.0±1.58^{b}	23±1.27^{b}
	C. viminalis	14±1.41^{ef}	2.5±2.51^{c}	13.1±1.00^{d}	20.7±0.82^{d}	15±1^{d}	24±1.41^{b}
n-butanol	*K. pinnata*	22.2±4.14^{b}	26±1.41^{c}	22.5±1.11^{c}	20.2±0.82^{c}	20.7±2.68^{bc}	16.5±1.11^{d}
	C. viminalis	18.7±1.29^{c}	n.d	17.2±1^{c}	17.5±1^{e}	24.5±0.86^{a}	17.5±0.86^{c}
Absolute metanol	*K. pinnata*	5.5±1.11^{d}	17.5±1.11^{d}	12.7±0.82^{d}	15.7±0.82^{d}	22.7±1.92^{ab}	n.d
	C. viminalis	15.5±0.51^{de}	17.5±1.65^{b}	21.2±1.00^{b}	23.0±1.00^{c}	14.5±0.5d^{e}	12.7±0.82^{e}
95% methanol	*K. pinnata*	n.d	n.d	n.d	n.d	n.d	22.5±1.12^{b}
	C. viminalis	7.5±7.14^{f}	n.d	n.d	25±1^{b}	n.d	22.5±0.86^{b}
90% methanol	*K. pinnata*	n.d	n.d	n.d	n.d	19±1.58^{c}	19.5±1.10^{c}
	C. viminalis	17.2±0.82^{cd}	n.d	13.5±1.65^{c}	n.d	22.5±0.86^{b}	n.d
Control	*K. pinnata*	30±1.00^{a}	34±1.58^{a}	30.0±1.65^{b}	30.5±1.65^{a}	26±1.87^{a}	26.2±1.40^{a}
	C. viminalis	40±1.08^{a}	38±1.08^{a}	39.2±0.82^{a}	30.2±1.08^{a}	32.2±1.57^{a}	27.2±2.38^{a}

Data are expressed as the mean ± standard deviation of three separate experiments; values having different letters differ significantly ($p < 0.05$) (n.d. = not detected). Rifampicin and fluconazole were used as positive control for bacterial and fungal strains respectively.

3. Results and Discussion

3.1. Antimicrobial activity of K. pinnata and C. viminalis

The Results of disc diffusion assay of the mentioned medicinally important plants are summarized in Table 1. Leaves extracts of *K. pinnata* showed significant antimicrobial activity. Petroleum ether extract of *K. pinnata* leaves showed antimicrobial activity that ranged from 11.2-23.5 mm zone of inhibition in diameter in which high activity was shown against *G. lucidum* (23.5 mm) and *E. coli* (23 mm). his extract did not show any notable antimicrobial activity against *P. multocida* and *S. aureus*. Chloroform extract of this plant showed significant antimicrobial activity with 10.7-30.2 mm zone of inhibition in diameter, against *P. multocida* the extract showed highest activity (30.2 mm). Ethylacetate and *n*-butanol extracts of *K. pinnata* showed strong activity against *P. multocida* and *S. aureus* with larger inhibition zones (30, 30 mm) and (26, 22.5 mm) "respectively" for both extracts. Ethyl acetate extract showed no activity against *B. subtilis.* Absolute methanol extract showed strong activity against *A. alternata* (22.7 mm) and least activity was exihibited against *B. subtilis* with small inhibition zone (5.5 mm). Methanolic extract (95%) showed growth inhibition action only against *G. lucidum* (22.5mm). This extract did not show notable antimicrobial activity against all other tested strains.

Similarly 90% methanolic extract was active only against *A. alternata* (19 mm) and *G. lucidum* (19.5 mm).

Table 2: Minimum inhibitory concentration (MIC) of *K. pinnata* and *C. viminalis* leaves against some microbial strains.

Various organic extracts	Plants used	MIC (mg/ml)					
		B. subtilis	*P. multocida*	*S. aureus*	*E. coli*	*A. alternata*	*G. lucidum*
Petroleum ether	*K. pinnata*	14.8±0.62	n.d	n.d	6.83±0.68	4.77±0.51	6.83±0.68
	C.viminalis	1.89±0.09	6.00±0.01	1.76±0.003	6.00±0.17	12±0.16	7.45±0.12
Chloroform	*K. pinnata*	1.45±0.356	5.56±0.09	11.3±1.07	1.48±0.35	7.21±0.59	4.43±0.81
	C. viminalis	0.52±0.04	4.09±0.09	0.80±0.006	5.12±0.17	7.24±0.23	10.0±0.08
Ethylacetate	*K. pinnata*	n.d	7.63±0.07	2.79±0.62	13.6±0.49	5.5±0.43	9.81±0.62
	C. viminalis	3.78±0.32	8.65±0.03	5.83±0.007	4.5±0.217	9.02±0.22	8.82±0.09
n-butanol	*K. pinnata*	5.81±0.59	8.67±0.05	5.78±0.70	8.71±0.58	6.21±0.36	22.7±0.81
	C. viminalis	1.23±0.46	n.d	3.98±0.009	4.76±0.50	6.02±0.07	8.82±0.08
Absolute metanol	*K. pinnata*	47.8±0.66	13.4±0.37	10.6±0.53	11.7±0.60	5.89±0.87	n.d
	C. viminalis	2.98±0.03	5.09±0.03	3.12±0.004	4±0.17	10.1±0.11	11.0±0.11
95% methanol	*K. pinnata*	n.d	n.d	n.d	n.d	n.d	8.82±0.63
	C. viminalis	4.11±0.0	n.d	n.d	3.02±1.04	n.d	11.5±0.22
90% methanol	*K. pinnata*	n.d	n.d	n.d	n.d	6.11±0.99	10.8±0.54
	C. viminalis	2±0.09	n.d	4.12±0.22	n.d	6.08±0.07	n.d
Control	*K. pinnata*	1.76±0.554	1.36±0.23	2.64±0.50	1.54±0.01	1.55±0.40	1.53±0.35
	C. viminalis	0.32±0.02	3.5±0.32	0.76±0.003	1.23±0.32	4.02±0.32	4.17±0.45

Data are expressed as the mean ± standard deviation of three separate experiments ($p < 0.05$) (n.d. = not detected). Rifampicin and fluconazole were used as positive control for bacterial and fungal strains respectively.

Table 3: Haemolytic activity, as a percentage of haemolysis caused by *C. viminalis* and *K. pinnata* leaves.

Plants various extracts	*C. viminalis*	*K. pinnata*
Petroleum ether	2.48±0.13	5.72±0.13
Chloroform	6.33±0.19	4.58±0.19
Ethylacetate	5.72±0.22	2.44±0.01
n-butanol	1.95±0.19	6.84±0.30
Absolute methanol	4.41±0.06	5.25±0.21
95% methanol	4.81±0.16	8.13±0.17
90% methanol	5.18±0.22	1.38±0.20
Phoshate Buffer Saline (PBS)	0±0	_
Triton X-100	99.9±1.37	99.8±1.37

The values are the average of triplicate experiments (n=3) ± S.D., ($p < 0.05$)

All extracts of *C. viminalis* leaves showed considerable antimicrobial activity against all tested strains (Table 1). Petroleum ether extract of the plant leaves showed antimicrobial activity that ranged from 13.1-23.5 mm zone of inhibition in which high activity was shown against *G. lucidum* and least activity was exhibited against *A. alternata*. Chloroform extract of the plant showed strong growth inhibitory effect against *B. subtilis* (23mm) and least activity was shown against *G. lucidum* (14.7 mm). Ethylacetate and *n*-butanol extracts showed marked antimicrobial activity ranging from 2.5-24 mm 17.2-24.5mm zone of inhibition in diameter, respectively. Ethylacetate extract showed high activity against *G. lucidum* (24 mm). Ethylacetate and *n*-butanol extracts showed no inhibitory action against *B. subtilis* and *P. multocida* respectively. Absolute methanol extract showed strong activity against E. coli (23 mm) and *S. aureus* (21.2 mm). *P. multocida, S. aureus, A. alternata* showed resistance to 95% methanolic extract with no noticeable activity. 90% methanolic extract was inactive against *P. multocida, E. coli, G. lucidum*. The rifampicin and fluconazole were used as positive control for bacterial and fungal strains respectively. The standard drugs showed higher activity on the organisms than the tested extracts of experimental plants (Table 1). The standard antibiotics were refined industrial products so there activity was more as compared to crude extracts of plants.

Minimum inhibitory concentration of the plants leaves extracts was determined against a set of bacterial and fungal strains (Table 2). The MIC values of the *K. pinnata* extracts against various strains was found in range 1.45-22.7 mg/ml and in case of *C. viminalis* the MIC values were in range of 0.52-12 mg/ml. The MIC values disclosed that greater the growth inhibition zone by disc diffusion method, lower would be the value of MIC. Several studies have been surveyed to confirm that bacterial and fungal strains used in the current study are disease causing pathogens [22, 23]. There are alarming reports of fungal infections [24] which explain that the resistance of the organisms increased due to the use of commercial antimicrobial drugs commonly used to cure infectious diseases. This condition forced the scientists to search for new antimicrobial agents from different sources including medicinal plants [24]. Our research results revealed that medicinal plants *K. pinnata and C. viminalis* can play an important role in fighting the bacterial and fungal resistance. The applications of natural antimicrobial agents are likely to grow steadily in the future because of greater consumer demands for minimally processed foods and those containing naturally derived preservation ingredients [25].

Akinpelu [26] reported the antibacterial activity of *K. pinnata* leaves methanolic extract and results of this report matched with our findings with some variations. Oyedeji and coworkers [27] reported the antimicrobial activity of *C. viminalis* essential oil against various strains. Delahaye and coworkers [28] investigated the antimicrobial activity of methanol, *n*-hexane and aqueous extracts of *C. viminalis* against various strains the methanol extract was found to be most effective. These results of our study support the ethnomedicinal claim that the both plants exhibited potenet antimicrobial activity so these plants may be used in future to cure the diseases caused by bacteria and fungi.

3.2. Haemolytic activity

Haemolytic activity was analyzed against human red blood cells (RBCs) using Triton X-100 as positive control. The % lysis of RBCs was observed in *C. viminalis* and *K. pinnata* extracts that ranged from (1.95-6.33%) and (1.38-8.13%) respectively (Table 3). The order of % haemolysis of various extracts of both plants was found to be different.

***C. viminalis*:** Chloroform> Ethylacetate> 90% methanol> 95% methanol> Absolute methanol> Petroleum ether> *n*-butanol

***K. pinnata*:** 95% methanol> n-butanol> petroleum ether> Absolute methanol > Chloroform> Ethylacetate> 90% methanol

The stability of erythrocytic membrane is a good sign of the effect of different *in vitro* studies by the various compounds for the screening the cytotoxic effects. The percentage haemolysis of human erythrocytes was below 5.0 % for all samples except 95% methanolic extract of *C. viminalis,* so it can be expected that all extracts of the both plants have a minor cytotoxity [21, 29].

Conclusion

Data collected in the present study confirmed the considerable antimicrobial and haemolytic activities possessed by *C. viminalis and K. pinnata* leaves. The presence of biologically important phytochemicals in the plant extracts may contribute to their medicinal value and potential sources for useful drugs. The both investigated plants may be processed for natural and pharmaceutical therapies to cure the ailments in human beings.

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