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EVALUATION OF THE PHYTOCHEMICAL, ANTIPROLIFERATIVE, ANTIOXIDANT, ANTIBACTERIAL ACTIVITIES OF *KALANCHOE BLOSSFELDIANA LEAVES*

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ABSTRACT

The present study describes the anticancer, hemolytic, antioxidant, antimicrobial activity, and phytochemical property of *Kalanchoe blossfeldiana.* The phytochemical screening of the petroleum ether, chloroform and methanolic extracts of *K.blossfeldiana* showed the presence of Alkaloids, Cardiac glycosides, Carbohydrates, Triterpenoids, Proteins, Flavonoids, Phenolic, Tannins, Quinones, Terpenoids, Coumarins, Anthocyanin, Acid, Steroids. The Antiproliferative activity of *K. blossfeldiana* methanolic extracts exhibited an IC_{50} value of 21.9 µg/ml on non-small lung cancer cell line A549 and no hemolysis was observed on human erythrocytes. DPPH radical scavenging activity and ABTS assay of the plant showed IC_{50} value of 54.32 µg/ml and 72.67 µg/ml respectively against standard Gallic acid. The methanolic extract of *K. blossfeldiana* showed significant antibacterial activity against *Bacillus subtilis, Klebsiella pneumonia* and *Salmonella typhi* with the zones of inhibition ranging from 9.0-16 mm and no activity was seen against *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*. These results showed that *K. blossfeldiana* can be a promising source for development of biologically active compounds as antitumor, antioxidant, antibacterial agent.

KEYWORDS: *K. blossfeldiana*, Cancer, *S. aureus, E. coli, P. aeruginosa, B. subtilis, K. pneumonia, S. typhi.*

INTRODUCTION

Kalanchoe is a genus of the Family Crassulaceae, are often referenced in folklore, and commonly used in traditional medicine worldwide. These species are also used by the Kerala tribes for treating cancer symptoms. A variety of bufadienolide compounds were isolated from various *Kalanchoe* species, which show strong anti-tumor promoting activity.[1]

Medicinal plants constitute a common alternative for cancer treatment in many countries around the world. At this time, more than 3000 plants worldwide have been reported to have anticancer properties. Globally, the incidence of the use of plant-derived products for cancer treatment is from 10% to 40% with this rate reaching 50% in Asiatic patients.[2] Medicinal Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, phenols and quinones, which have been used worldwide in traditional medicine to treat several diseases and infection.[3, 4] Many studies all over the world have been showed that these plants and their extract have multi-antimicrobial properties.[5]

Cancer is a class of diseases characterized by out-of-control cell growth. It harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected.[6] Lung carcinoma is the leading cause of cancer deaths in the United States and worldwide for both men and women.[7] Chemotherapy for non–small cell lung carcinoma (NSCLC), which accounts for approximately 85% of lung cancer cases, remains marginally effective. [2] Concerning *K.blossfeldiana,* no reports on biological activities could be traced in the available literature which prompted the biological investigation of their leaf extracts. The present study is designed to explore the Preliminary phytochemical analysis of *K. blossfeldiana* leaf, which is responsible for its anticancer properties.

MATERIALS AND METHODS

Collection of Plant Material and authentication

The plants ***K. blossfeldiana (leaf)*** were collected from Lalbagh, Bangalore authenticated by Dr. Kiran HOD, Bio-Science Department, CMR institute of management studies, Bangalore.

Chemicals and reagents

Petroleum ether, chloroform and methanol, SDS, DMSO, ABTS (2,2'-azinobis-ethyl-benzothiozoline-6-sulphonic acid), DPPH (1,1-diphenyl-2-picryl hydrazyl), PBS Phosphate Buffered Saline -125mM NaCl in 10mM Sodium phosphate buffer, pH 7.4, MTT (3-[4, 5-

dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide)- 1.2mM MTT in PBS and the solution is filtered through a 0.2 μ m filter and stored at 2–8 °C for frequent use or frozen for extended periods.

Extraction of plant Material

Plant material was shade dried, dehydrated and was powdered into coarse particles with the help of kitchen blender. Weighed 20 g dried powder of plant material and was extracted with 250 ml of methanol, petroleum ether, and chloroform using soxhlet apparatus. The extract was filtered with Whatmann filter paper and the filtrate was collected in 50 ml beaker**.** Then the filtrate was kept at 80 ºC for few hours until the extract gets completely dried and turn into semisolid form. Total yield of crude extract was obtained to b 12.33%.

Test organisms

The test of microorganisms included gram positive (*B. subtilis, S. aureus)* and gram negative (*E. coli, P. aeruginosa, K. pneumonia, S. typhi,)* bacterial stock culture maintained on Nutrient Agar at 4^{o}C were obtained from the microbiology laboratory, CMR institute of management studies.

Standard drug: Tetracycline was used as a standard drug in this research work and the drug was obtained from Sigma Aldrich, USA.

Cell culture

In this study we have used cancer cell line derived from human *non-small cell lung carcinoma* A549 obtained from the American Tissue Culture Collection (ATCC). A549 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) media supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Phytochemical screening of the Extracts

Petroleum ether, chloroform and methanolic extracts of *K. blossfeldiana* leaves were subjected to following chemical tests for the presence of various phytoconstituents. Test for the presence of Alkaloids, Cardiac glycosides, Carbohydrates, Triterpenoids, Proteins, Flavonoids, Phenolic, Tannins, Quinones, Terpenoids, Coumarins, Anthocyanin, Acid, Steroids were performed as per the standard procedure.[7]

Hemolysis Assay

Hemolytic activity is a method to study the interactions between blood and biomaterials which may induce erythrocyte lysis. In this study we have taken 50 µl of 10 dilution (100µl Erythrocytes suspension: 900µl 1X PBS) of erythrocytes suspension into 2 ml of new eppendorf tube and incubated with 100 µl of different concentration of plant extracts (10, 20, 40, 80, 160, 320µg/ml) at 37^0C water bath for 60-90 min. We used 100 µl of 1X PBS as negative control and 100 µl of 1% SDS as positive controls. Then adjusted the volume of reaction mixture to 1 ml by adding 850 µl of 1X PBS. Finally centrifuged at 300 rpm for 3 min and the resulting hemoglobin in supernatant were measured at 540 nm by Tecan micro plate reader, Magellan ^{TM}- Data Analysis Software to determine the concentration of hemoglobin. The hemolysis caused by 100 µl of 1% SDS was taken as 100 % hemolysis; and the percentage of hemolysis was calculated by the equation.[8, 9]

Percentage of hemolysis = (control-sample**)** / (control)*100

Antiproliferative assay (MTT)

The cytotoxic assay detects the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase to blue insoluble formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. Briefly 5.0 X 10^4 cells of A549 were seeded in 96 well plates (n=3) with DMEM and incubated for 24 hrs at 37^oC. Plant extracts were tested at 0, 2.5,5, 10, 20, 40, 80,160, 320µg/ml in serum free DMEM media and incubated for 24 hr in CO_2 incubator at 37°C. After incubation with plant extracts, the media was removed from the wells and 100 µl/well of the MTT reagent was added and incubated again for 3-4 hrs. After incubation, the MTT reagent was removed before adding 100μL DMSO to each well and gently shaked. Plant extracts treated cells were compared to untreated cells. Measured the absorbance at 570 nm using a Spectra fluor Tecan plate reader.[10, 11]

ABTS radical scavenging assay

ABTS radical cations are produced by reacting ABTS (7mM) and APS (2.4mM) on incubating the mixture at room temperature in dark for 16 hours. The solution thus obtained was further diluted with PBS to give an absorbance of 1.000. Different concentrations of the test sample and the reference standard (highest volume taken was 50µl) were added to 950 µl of ABTS working solution to give a final volume of 1ml, made up by adding PBS.

The absorbance was recorded immediately at 734nm. The percent inhibition was calculated at different concentrations and the IC_{50} values were calculated by Log-Probit analysis.[12]

DPPH anti-oxidant assay

Various concentrations (0.5-2.5 µg/mL) of test solution and control without test sample were added to DPPH (3.29mM) solution and volume was made upto 3mL with HPLC grade methanol. The reaction mixture was mixed and incubated at 25°C for 15 minutes. Absorbance was measured at 510 nm using semi-auto analyzer.[13] % inhibition = [Absorbance (control) – Absorbance (sample)/ Absorbance (control)]*100

Antibacterial activity

Well diffusion method: An overnight culture of *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumonia*, *B. subtilis* and *S. aureus* was standardized to contain approximately 1x 10^7cfu/ml. The gram negative and gram positive bacteria were inoculated into 20 ml of Nutrient broth. The culture medium was allowed to set.

Thereafter, all the inoculum was swabbed over the surface of the Muller Hinton agar medium using sterile cotton swab. Using a sterile cork borer of 5 mm diameter, five wells were made in solidified sterile Muller Hinton agar medium (one in the centre and four wells at the corner). The agar plugs were removed with a flamed and cooled wire loop. Then 100%, 75%, 50%, 25% of methanolic extract of *K. blossfeldiana* leaves were placed in the wells made in inoculated plates. The treatment also includes 50 µl of sterilized distilled water as control.

All the plates were incubated for 24hours at 37^{o}C and zone of inhibition if any around the well were measured in millimeter (mm). For each treatment three replicates were maintained. The same procedure was followed for standard antibiotics Tetracycline (25mg) to evaluate the efficacy of methanolic extract against test organisms.[14]

RESULTS

Preliminary phytochemical analysis

Phytochemical constituents of *K. blossfeldiana* leaf extract were tested for Alkaloids, Cardiac glycosides, Carbohydrates, Coumarins, Anthocyanin showed positive in petroleum ether, chloroform, methanol extracts. Volatile oil**,** Triterpenoids**,** Acid were present in petroleum ether, chloroform extract. Proteins, Flavonoids, Phenolics, Tannins, Quinones**,** and

Terpenoids were present in only in methanolic extract. Anthocyanin, Steroids were present in petroleum ether, chloroform respectively. (Table 1)

Table1: Phytochemical tests of *K. blossfeldiana* methanolic extract

Sl. No	Name of the test	Petroleum ether	Chloroform	Methanol
1	Alkaloids	+	+	+
2	Cardiac glycosides	+	+	+
3	Carbohydrates	+	+	+
4	Triterpenoids	+	+	-
5	Proteins	-	-	+
6	Saponins	-	-	-
7	Flavonoids	-	-	+
8	Volatile oil	+	+	-
9	Phenolic	-	-	+
10	Tannins	-	-	+
11	Quinones	-	-	+
12	Terpenoids	-	-	+
13	Coumarins	+	+	+
14	Anthocyanin	+	+	+
15	Acid	+	+	-
16	Steroids	+	-	-

The effects of methanolic extract of *k.blossfeldiana* on inhibition and proliferation of non-small cell lung carcinoma (A549).

To verify the possible anti-proliferative effect of above mentioned plant extracts as a first step towards the development of novel putative anticancer agents, we tested methanol extract of *K. blossfeldiana* (leaf) to check the capability to inhibit cell growth or viability on of A549 cancer cell line at concentrations of 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/ml. Proliferation of these cells was significantly inhibited by above extracts in a concentration-dependent manner for 24 hours, as shown in Figure 1. It showed dose dependent inhibition of growth of A549. *K. blossfeldiana* inhibited growth of A549 cells and the IC_{50} values of *(*21.9µg/ml) were obtained showed in Table 2 & Figure 1.

Table 2: Antiproliferative activity of *K. blossfeldiana* on A549 lung carcinoma

Test material	Concentation. µg/ml	Average OD at 590 nm ± S.E.M, n=3	% Inhibition	IC50 µg/ml
	Control	0.627 ±0.2		
	Vehicle	0.639±0.1	0	
K. blossfeldiana	10	0.54±0.12	15.22	21.91
	20	0.42±0.1	34.17	
	40	0.31±0.0	51.02	
	80	0.23±0.12	64.30	
	160	0.18±0.0	70.96	
	320	0.10±0.3	83.46	

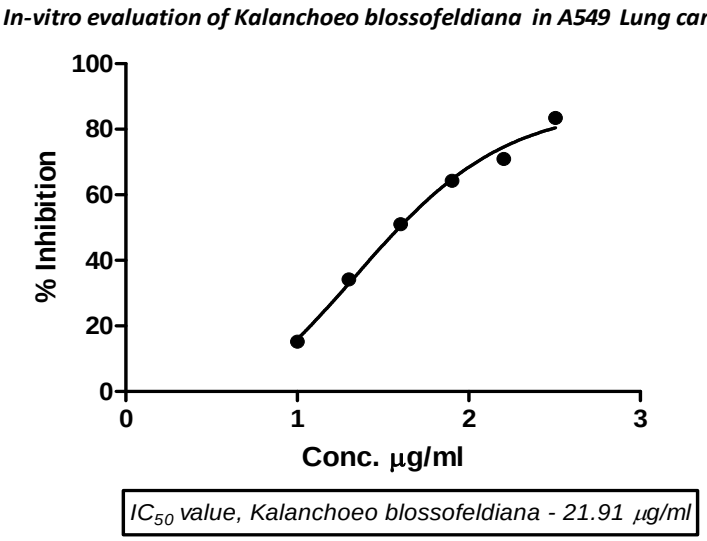


Figure I Cytotoxic effect of *k. blossfeldiana* in non-small cell lung carcinoma (A549).

Cells were treated with various concentrations (0, 2.5,5,10, 20, 40, 80, 160 and 320 µg/ml) of above mentioned plant extracts for 24 hrs grown in a serum free media. The percentage of cell death induced was determined using MTT assay.

The effects of methanolic extract of *k.blossfeldiana* on human erythrocytes.

Hemolytic assay method is suited to evaluate the hemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002. As these extracts showed anticancer activity in above mentioned human cancer cells, here we have tested 0-320 µg/ml of *K.blossfeldiana* extract effect on human erythrocytes. *K.blossfeldiana* extracts showed 0 to 8 % hemolysis at concentration of the 0-320µg/ml as shown in Table 3 & Figure II.

Table 3: Absorbance values of methanolic extracts of *K. blossfeldiana* plants by Hemolysis assay

Plants materials	Concentation µg/ml	Absorbance at 590nm ± S.E.M, n=3	% Hemolysis
	Control	0.2971±0.3	0
	1% SDS	0.071±0.2	76.03
K. blossfeldiana	10	0.295±0.1	0.706
	20	0.294±0.0	1.04
	40	0.278±0.3	6.42
	80	0.276±0.22	7.10
	160	0.275±0.13	7.43
	320	0.271±0.11	8.78

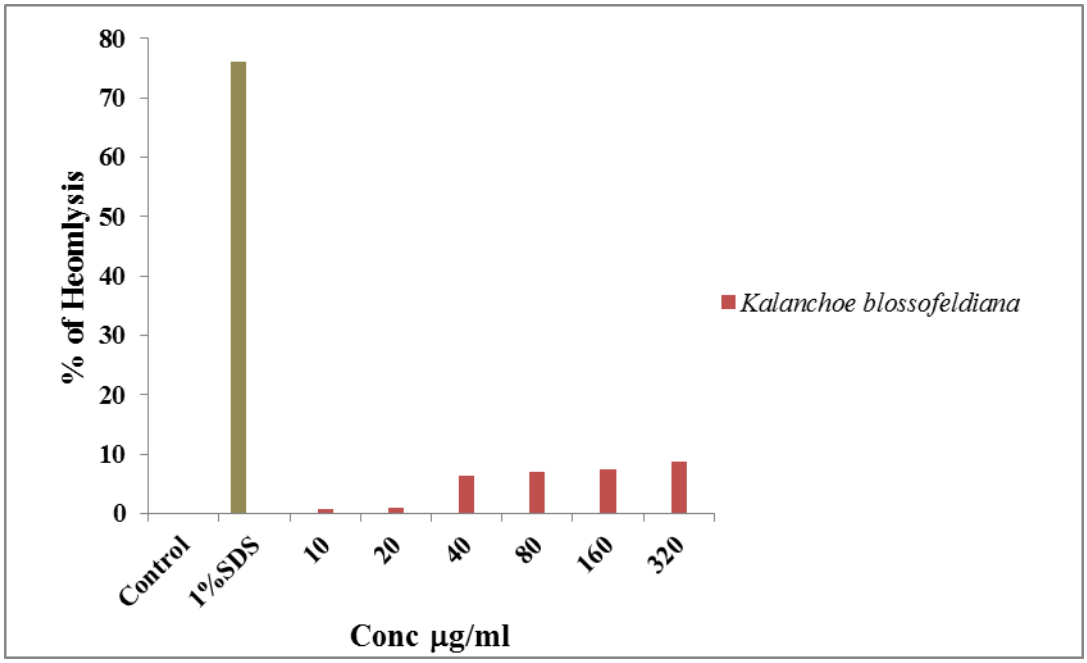


Figure II Effect of *k.blossfeldiana* on hemolysis of human erythrocytes.

Human erythrocytes were incubated with various concentrations (0, 10, 20, 40, 80, 160 and 320 µg/ml) of above mentioned plant extracts at 37^0C water bath for 60 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer (JENWAY 6305 UV/Vis.) to determine the extent of hemolysis. The percentage of hemolysis was calculated by the equation *% Hemolysis = [(^{A}Control– ^{A}Sample) / ^{A}Control] x 100*. All analyses are the Mean of three replicates

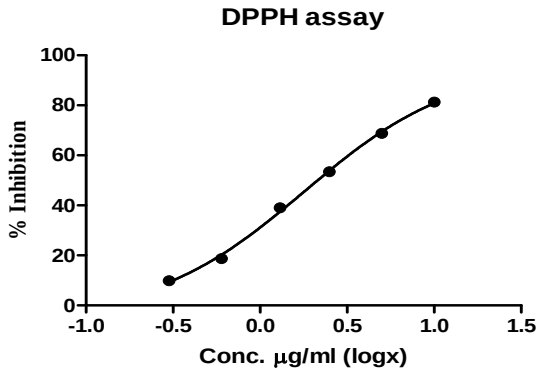
The effects of methanolic extract of *k.blossfeldiana* has antioxidant.

DPPH radical scavenging activity and ABTS assay of the plant showed IC_{50} value of 54.32 µg/ml and 72.67 µg/ml respectively against standard Gallic acid as shown in Table 4, 5 & Figure III, IV.

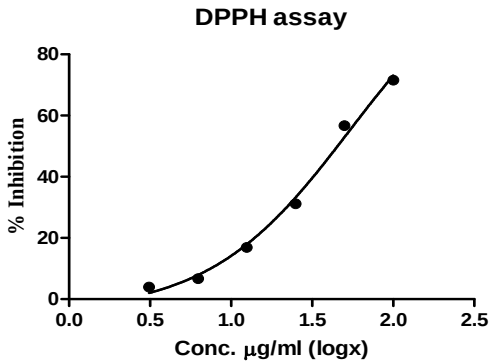
Table 3: DPPH assay of plant extract.

Plants Name	Concentration (µg/ml)	Absorbance 590nm	% Inhibition	IC_{50} µg/ml
Control	0.0	0.583	0.00	
Standard (Gallic acid)	0.3	0.525	9.91	
	0.6	0.474	18.73	
	1.3	0.355	39.05	1.793
	2.5	0.271	53.46	
	5.0	0.182	68.78	
	10.0	0.109	81.25	
Kalanchoe blossflediana	0.0	0.583	0.00	
	3.1	0.560	3.97	54.32
	6.3	0.544	6.72	

	12.5	0.485	16.89	
	25.0	0.401	31.15	
	50.0	0.252	56.73	
	100.0	0.166	71.57	



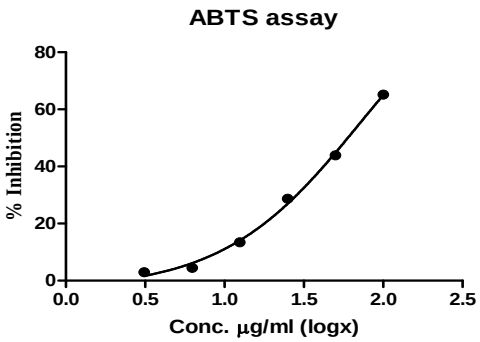
	Gallic acid
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	96.24
TOP	-5.198
LOGIC50	0.2535
IC50	1.793



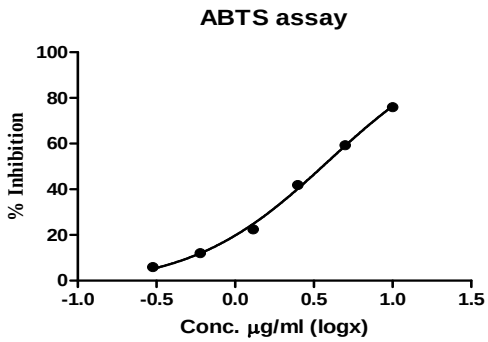
	K. blossflediana
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	115.2
TOP	-4.471
LOGIC50	1.735
IC50	54.32

Table 4: ABTS assay of plant extract

Plants Name	Concentration (µg/ml)	Absorbance 590nm	% Inhibition	IC_{50} µg/ml
Control	0.0	0.491	0.00	
Standard (Gallic acid)	0.3	0.462	5.92	
	0.6	0.432	12.05	
	1.3	0.381	22.48	3.903
	2.5	0.285	41.89	
	5.0	0.200	59.27	
	10.0	0.118	75.99	
Kalanchoe blossflediana	0.0	0.491	0.00	
	3.1	0.476	2.97	
	6.3	0.469	4.51	
	12.5	0.425	13.43	72.67
	25.0	0.350	28.68	
	50.0	0.276	43.87	
	100.0	0.171	65.19	



	K. blossflediana
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	114.5
TOP	-3.185
LOGIC50	1.861
IC50	72.67



	Gallic acid
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	107.2
TOP	-2.644
LOGIC50	0.5914
IC50	3.903

The effects of methanolic extract of k.blossfeldiana on microorganisms.

Antibacterial activity of methanolic extract of leaves of *K.blossfeldiana* was tested at 25%, 50%, 75%, 100% against six bacterial species which showed a moderate activity. *B. subtilis* showed 09 to 16mm inhibition, *K. pneumonia* showed 09 to 11mm inhibition and *S. typhi* showed 10 to 13 mm inhibition **.**The *S. aureus, E. coli, P. aeruginosa*, did not show any zone of inhibition at different concentration when treated with *K.blossfeldiana***.** Compared to synthetic antibiotic tetracycline at 25mg concentration, the inhibition zone was in the range of 28mm to 33mm.

Table 5: Effect of *K.blossfeldiana* on different microorganisms.

Test Organisms	**Blank**	**Methanolic extract of *K. blossfeldiana***				**Standard drug tetracycline**
Gram positive						
B.subtilis	-	16 ± 0.22	15 ± 0.13	11 ± 0.26	9 ± 0.14	32.0 ± 0.0
S. aureus	-	-	-	-	-	30.0 ± 0.2
Gram negative					-	
E. coli	-	-	-	-	-	32.0 ± 0.0
P.aeruginosa	-	-	-	-	-	30.0 ± 0.0
K.pneumonia	-	11 ± 0.13	11 ± 0.22	10 ± 0.19	9 ± 0.11	33.0 ±0.16
S. typhi	-	13 ± 0.16	13 ± 0.15	12 ± 0.21	10 ± 0.13	28.0 ±0.12

Zone of Inhibition (mm) ± S.E.M, n=3

DISCUSSION

The present study demonstrated the promising anticancer, antimicrobial, phytochemical activities of the crude methanolic extract of *K.blossfeldiana.* Preliminary phytochemical screening of various extracts revealed the presence of different primary and secondary

metabolites. Our investigation has found that the plant to be rich in biomolecules like Carbohydrates, Alkaloids, Cardiac glycosides, Carbohydrates, Coumarins, Anthocyanin whereas, Proteins & amino acids were found in negligible amount. This indicates that, the presence of secondary metabolites may have suppressed the activity of proteins. In addition, the solvent might have also denatured the proteins because of which it is only observed as very less quantity in a methanolic extracts. Saponins was found to be absent in all the above mentioned extract. Hence it may not posses hemolytic activity which is considered as one of the main biological activity of saponins. When compare to other extract, methanol showed high degree of precipitation of phytochemicals.

Plant derived compounds are gaining increasing interest as potential cancer therapeutics. The present study demonstrated the anticancer activities of the crude methanolic extract of *K.blossfeldiana* against A549 cell lines, which inhibited cell proliferation with IC_{50} 21.9 µg/ml. Hemocompatibility of biomaterial was evaluated using hemolytic assay. It was determined that compared to cancer cells, the toxicity of *K. blossfeldiana* was less in normal erythrocytes isolated from human blood, which might be because plant does not contain saponins which is proved in phytochemical analysis.

Based on the literature survey, there is no report on anticancer activity of *K. blossfeldiana* but *Kalanchoe* genus showed anti cancer activity. It was found that extracts from *Kalanchoe* genus, exhibited significant anti-proliferative effects against a variety of human cancer cell lines. It was found that *Kalanchoe tubiflora* has antiproliferative activity, which is due to the induction of multi-polar spindles and chromosomal misalignment of mitotic cells. These abnormal mitotic events lead to mitotic catastrophe, a desirable effect of a cancer therapeutic drug. Targeting of mitotic cells is one of the bases of therapy for patients with multiple types of solid tumors.[2] The phytochemical investigation of the most potent cytotoxic fraction; dichloromethane fraction of *Kalanchoe thyrsiflora* Harv, had led to isolation of two pure compounds 3-oxo-olean-12-ene and β-sitosterol. Three new compounds, kalanchosides, were isolated from the aerial parts of *Kalanchoe gracilis.* All isolated compounds showed significant cytotoxic activity against a panel of human tumour cell lines. Although the isolation and purification of compounds with promising cytotoxic activities from active plant extracts has been extensively researched, testing on *in-vivo* systems remains a significant step.[1, 16] Therefore, it was proved that *Kalanchoe* genus may be a highly effective candidate as a future anti-cancer drug.

The antioxidant assay (ABTS and DPPH) has proved *K. blossfeldiana posses antioxidant* property. Plant showed IC_{50} value of 54.32µg/ml for ABTS assay and 72.67µg/ml DPPH assay against standard Gallic acid. Plants possess antioxidant principles. Various classes of phytochemicals have been shown to have antioxidant property which is due to the presence of substituted groups such as carbonyl, phenolic, phytyl side chain, electron withdrawing group, electron donating group etc.[12] The emergence of microbial resistance and the decreases in effectiveness of currently available antimicrobial agents have spurred an increased effort to search for new and alternative antimicrobial substance with novel inhibitory. In this study, *K. blossfeldiana leaf* was evaluated for antimicrobial activity against gram positive (*B. subtilis, S. aureus,)* and gram negative (*E. coli, P. aeruginosa, K. pneumonia, S. typhi,).* Among them 100% and 75% concentration of methanol extract displayed strong activity against *B. subtilis* (16 mm and 15mm), followed by *S. typhi* (13 mm), *K. pneumonia* (11 mm). It was observed that, there was decline in the zone of inhibition as the concentration is decreased at 25% and 50% when tested against *B. subtilis, K.pneumonia, and S. typhi.* No zone of inhibition was observed for *S. aureus, E. coli, and P. aeruginosa.*

CONCLUSION

Finally, it could be suggested that the methanolic extract of *K.blossfeldiana* leaves possess cytotoxic, antioxidant and antibacterial activities. . Further research on the underlying mechanism of *K.blossfeldiana* in inhibiting cell proliferation and research involving the identification and isolation of pure compounds will be necessary to determine the cellular targets. Further in vivo research will be needed to determine the effectiveness of *K. blossfeldiana* as an actual anti-cancer compound. 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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