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Investigations of medicinal properties of *Kalanchoe pinnata* under Crassulaceae family

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Abstract

Bangladesh is a good repository of medicinal plants belonging to various families including Crassulaceae. The *Kalanchoe pinnata* under Crassulaceae family contain a wide range of pharmacologically active compounds which has anti-inflammatory, immunomodulator, antimicrobial and antilithic properties. We evaluate the chemical and biological activities of crude ethanolic extract with special emphasis to the brine shrimp lethality bioassay, antimicrobial activity and the free radical scavenging property. The crude ethanolic extract and n-hexane, carbon tetrachloride, dichloromethane, aqueous soluble fractions of ethanolic extract showed the brine shrimp lethality with LC_{50} value 35.48, 2.00, 0.78, 2.20, 12.58 µg/ml, and the free radical scavenging activity with IC_{50} value 41.50, 7.81, 78.21, 6.12, 7.63 µg/ml respectively. The dichloromethane soluble fractions of ethanolic extract exhibited antimicrobial activity against *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Shigella dysenteriae, Candida albicans,* and *Aspergillus niger*. These results provide the valuable medicinal properties of *Kalanchoe pinnata* and can be used for the treatment of different kinds of human diseases.

Keywords: *Kalanchoe pinnata,* Crude ethanolic extract, Brine shrimp lethality, Radical scavenging property, Antimicrobial activity, n-Hexane, Dichloromethane, Aqueous soluble fractions and Carbon tetrachloride.

1. Introduction

Fossil records data shows that human used different kinds of plants as medicines at least 60,000 years ago (Solecki **1975**). The development of traditional medical systems, plants were incorporating as a means of therapy. However, the value of these systems is much more significant than anthropologic or archeologic fact. According to the World Health Organization (WHO), almost 65% of the world's population has incorporated various kinds of plants into their primary modality of healthcare (Farnsworth *et al*. **1985**).

New medicines have been discovered with traditional, empirical and molecular approaches. The traditional approach makes use of drug that has been found by trial and error over many years in different cultures and systems of medicine. With the development of molecular biological techniques and advances in genomics, the majority of drug discovery is currently based on the molecular approach.

Bioactive natural products often occur as a part of a family of related molecules so that it is possible to isolate a number of homologues and obtain structure-activity relationship. Of course, lead compounds found from screening of natural products can be optimized by traditional medicinal chemistry or by application of combinatorial approaches. It can be assumed that natural products will continue to offer novel leads for therapeutic agents. In earlier times, all drugs and medicinal agents were derived from natural substances,

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and most of these remedies were obtained from higher plants. Today, many new chemotherapeutic agents are synthetically derived, based on "rational" drug design. The study of natural products are effective over synthetic drug design, it leads optimally to materials having new structural features with novel biological activity. Not only the plants continue to serve as important sources of new drugs, but also phytochemicals derived from them are extremely useful as lead structures for synthetic modification and optimization of bioactivity. One-half of the starting materials for the medicines which we use today come from natural sources. The future of plants as a sources of medicinal agents for the investigation, prevention, and treatment of diseases is very promising. Natural products are naturally derived metabolites and/or by products from microorganisms, plants, or animals.

In the present study, we used crude ethanolic extract isolated from *Kalanchoe pinnata* and evaluate their pharmacological profiles such as lethality bioassay, antimicrobial and cytotoxic activity.

2. Materials and methods

The investigation of crude ethanolic extract isolated from *Kalanchoe pinnata* plant can be divided into six major steps such as (a) collection and proper identification of the plant sample (b) preparation of the plant sample (c) extraction with ethanol (d) partitioning ethanol crude extract (e) characterization of compounds (f) Chemical and Biological Investigations. The details method were described in our previous paper (Sharmin *et al.* **2016**). Chemical and biological investigations includes as bioassay (brine shrimp lethality test), screening of antimicrobial activity and antioxidant activity (free radical scavenging activity).

2.1. Bioassay (Brine shrimp lethality test)

Two “bench top” bioassays were adopted which do not require higher animals to screen and direct the fractionation of botanical extracts in drug discovery. These are (i) the brine shrimp lethality test (a general bioassay) and (ii) the inhibition of crown gall tumors on discs of potato tubers (an antitumor bioassay). Brine shrimp lethality bioassay (Meyer *et al.* **1982**; McLaughlin *et al.* **1998**) is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin and is considered as a useful tool for preliminary assessment of toxicity. All reagents used in this experiment were purchased from Merck, Germany.
Sea salt (pure NaCl 38 gm) was weighed, dissolved in one litre of distilled water and filtered to get clear solution of seawater and maintained pH 8.5 $\pm$ 0.5. *Artemia salina* leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp at 27 $\pm$ 2 ºC and to be matured as nauplii. With the help of a pasteur pipette, ten living shrimps were added to each of the test tubes containing 5 ml of seawater. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for standard drug vincristine sulphate for ten concentrations and another one test tube is for the control test.

All the test samples such as crude ethanolic extract (ETOH) and n-hexane (n-HEX) , carbon tetrachloride (CCl4) , dichloromethane (DCM) and aqueous soluble fraction (AQSF) partitionates of ethanolic extract of *Kalanchoe pinnata* leaves (4.0 mg) were taken and dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100 µl of stock solution was taken in first test tube containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400 µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. Thus the concentrations of the obtained solution in each test tube were as 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78 µg/ml.

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used as positive control and negative control. Positive control of cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study vincristine sulphate is used as the positive control. Measured amount of the vincristine sulphate is dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions are made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. Then the positive control solutions are added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

DMSO (100 µl) was added to each of three premarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds. After 24 hours, the vials were inspected using a magnifying glass and the number of survivors

were counted. Tests carried out in triplicate and average percentage (%) of mortality was calculated for each dilution. The concentration mortality data were analysed statistically. The effectiveness or the concentration mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

2.2. Microbiological Investigations

The in vitro antimicrobial study was designed to investigate the antibacterial as well as antifungal spectrum of the crude extracts by observing the growth response. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimetre (Barry **1976**; Bauer *et al.* **1966**). In the present study the crude extracts, fractions and some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of all values is required (Bauer *et al.* **1966**). The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram-positive and gram-negative bacteria and fungi were taken for the test and they are listed in the **Table 1**.

The nutrient agar medium, nutrient broth medium, Muller-Hunton medium, tryptic soya broth medium, tryptic soya agar medium, and sabouraud's dextrose agar are used normally to demonstrate the antimicrobial activity and also make subculture of the test organisms. All media used in this experiment were purchased from Oxoid, UK.

The test organisms were transferred from the subculture to the test tubes containing melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the agar media.

Three types of discs were used for antimicrobial screening. We used a positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Kanamycin (30 µg/disc) standard disc was used as the reference. We were also used as negative controls to ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Measured amount of each test sample (Crude ethanolic extract and n-hexane, carbon tetrachloride, dichloromethane and aqueous soluble fraction partitionates of ethanolic extract of *Kalanchoe pinnata* leaves) was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized filter paper discs (BBL, Cocksville, USA) were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried. Measured amount of each test sample (400 µg/disc) was dissolved in specific volume of solvent (chloroform) to obtain the desired concentrations. The sample discs, the standard antibiotic discs and the control discs were placed gently on previously marked place in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator for few hours to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37 ºC for 24 - 48 hours for bacteria and 25 ºC for 3 - 5 days for fungi. The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zone of inhibition in millimeter with a slide calipers.

2.3. Antioxidant Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants (Choi *et al.* **2000**; Desmarchelier *et al.* **1997**). The extract solution (crude ethanolic extract and n-hexane, carbon tetrachloride, dichloromethane and aqueous soluble fraction partitionates of ethanolic extract of *Kalanchoe pinnata* leaves) in chloroform (2.0 ml) of different concentrations (500 to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured against at 517 nm against blank by UV spetrophotometer (Shimadzu, Japan). Inhibition free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \text{ X } 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. tert-Butyl-1-

Hydroxytoluene (tBHT) was used as positive control. Tests carried out in triplicate and average values were taken.

3. Results

3.1. Brine shrimp Lethality Bioassay

Bioactive compounds are always toxic at higher dose. Thus, in vivo lethality in a simple zoological organism can be used as a convenient informant for screening and fractionation in the discovery of new bioactive natural compounds. At present bioactivity study crude ethanolic extract and n-hexane, carbon tetrachloride, dichloromethane and aqueous soluble fraction partitionates of ethanolic extract of *Kalanchoe pinnata* leaves showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. With the help of Meyer procedure (Meyer *et al.* 1982) the lethality of the crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane and aqueous soluble fraction partitionates of ethanolic extract to brine shrimp were determined.

The results of the brine shrimp lethality after 24 hours exposure to all the samples and the positive control, vincristine sulfate compared with the negative control (sea water) was lethal, giving significant mortality to the shrimp. The lethal concentration LC_{50} of the test samples after 24 hour were obtained by a plot. Plotting of log of concentration versus percent of mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples.

The LC_{50} values of crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane, aqueous soluble fraction were found to be 35.48 µg/ml, 2.0 µg/ml, 0.78 µg/ml, 2.2 µg/ml, and 12.58 µg/ml, respectively (**Fig 1 and 2**). However, varying degree of lethality to *Artemia salina* was observed with exposure to different dose levels of the test samples.

3.2. In vitro antimicrobial screening of *Kalanchoe pinnata* (leaf)

The antimicrobial activities of extracts from *Kalanchoe pinnata* were examined in the present study. The zone of inhibition produced by crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane, aqueous soluble fraction partitionates of the ethanolic extract of *Kalanchoe pinnata* leaves were observed at a concentration of 400 µg/disc. The Dichloromethane fraction partitionate of the ethanolic extract exhibited the inhibition against microbial growth of *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Shigella dysenteriae, Candida albicans,* and *Aspergillus niger* of about 7 to 9 mm zone of inhibition (**Table 2**). Out of all the samples, only dichloromethane fraction partitionate of the ethanolic extract was appeared moderate in terms of both zone of inhibition and spectrum of activity while other remained insensitive to the extractives. Bioactivity guided isolation can be carried out to separate their bioactive metabolites.

3.3. Antioxidant activity

The crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane, aqueous soluble fraction partitionates of the ethanolic extract of *Kalanchoe pinnata* leaves were subjected to free radical scavenging activity by the method of Brand-Williams (Brand-Williams *et al.* 1995). Here, tert-butyl-1-hydroxytoluene (tBHT) was used as reference standard. In this investigation, crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane, aqueous soluble fraction partitionate of the ethanolic extract showed the highest free radical scavenging activity with IC_{50} value 41.5 µg/ml, 7.81 µg/ml, 78.21 µg/ml, 6.12 µg/ml, and 7.63 µg/ml respectively (**Fig 3 and 4**). The carbon tetrachloride soluble partitionate of the leaf also exhibited strong antioxidant potential having IC_{50} value 78.21 µg/ml.

4. Discussion

In our previous paper we have shown that two pure compounds (campesterol and 5,6,7,8,4' pentahydroxy flavanone) isolated from the leaves of *Kalanchoe pinnata* (Sharmin *et al.* 2016). These two isolated compounds have some medicinal properties such as campesterol is effective against cholesterol and cancer cell and pentahydroxy flavanone is effective against various pathogenic microorganism (Jung Min Choi *et al.* 2007 and Hazra *et al.* 2007).

The statistical analysis of the brine shrimp lethality bioassay it can be well predicted that the crude ethanolic extract and n-hexane, carbontetrachloride, dichloromethane, aqueous soluble fraction of the ethanolic extract of *Kalanchoe pinnata* leaves possess cytotoxic principles and have considerable cytotoxic potency. The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration (0.78 µg/ml) to highly significant with the highest concentration (400 µg/ml). Maximum mortalities took place at a concentration of 400 µg/ml, whereas least

Table 1 Antimicrobial activity of test samples of *Kalanchoe pinnata* (leaf)

Test microorganisms	Diameter of zone of inhibition (mm)					
	ETOH	n-HEX	CCl_4	DMF	AQSF	Kanamycin
Gram positive bacteria						
Bacillus cereus	-	-	-	-	-	40
Bacillus megaterium	-	-	-	-	-	39
Bacillus subtilis	-	-	-	-	-	38
Staphylococcus aureus	-	-	-	7	-	38
Sarcina lutea	-	-	-	-	-	38
Gram negative bacteria						
Escherichia coli	-	-	-		-	38
Pseudomonas aeruginosa	-	-	-	9	-	41
Salmonella paratyphi	-	-	-	-	-	38
Salmonella typhi	-	-	-	7	-	41
Shigella boydii	-	-	-	-	-	38
Shigella dysenteriae	-	-	-	8	-	42
Vibrio mimicus	-	-	-	-	-	40
Vibrio parahemolyticus	-	-	-	-	-	38
Fungi						
Candida albicans	-	-	-	7	-	36
Aspergillus niger	-	-	-	7	-	38
Sacharomyces cerevacae	-	-	-	-	-	39

n-HEX: n-Hexane, CCl_4: Carbon tetrachloride, DCM: Dichloromethane, ETOH: Crude Ethanol extract, AQSF: Aqueous soluble fraction.

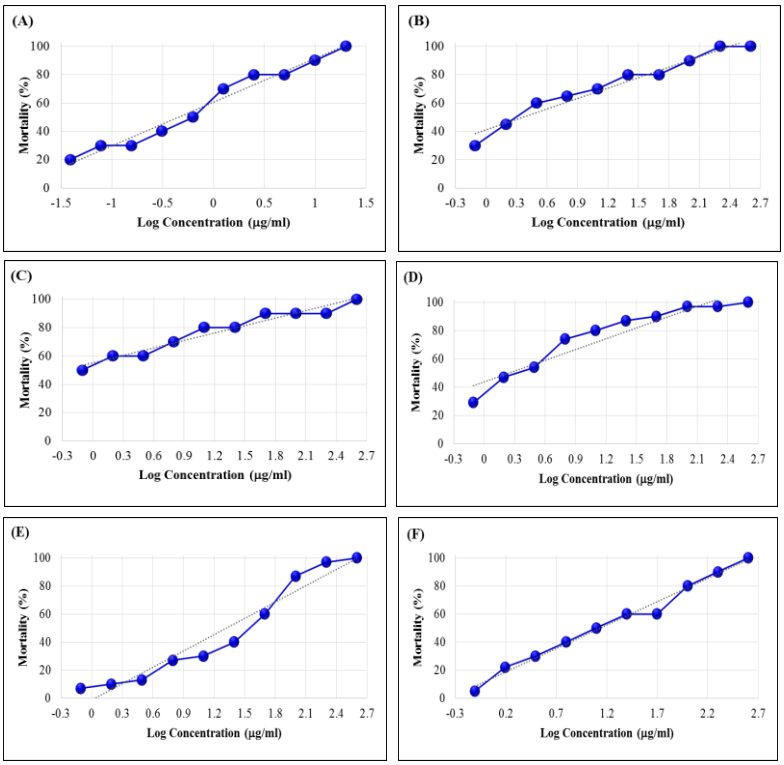

Fig 1 Determination of LC50 value of samples and standards against brine shrimp nauplii. (A) Vincristine sulphate, (B) n-Hexane, (C) Carbon tetrachloride, (D) Dichloromethane, (E) Ethanolic crude extract, and (F) Aqueous soluble fraction.

mortalities were at 0.78 µg/ml concentration. In other words, mortality gradually increased with the increase in concentration of the test samples. Comparison with positive control vincristine signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds. The compound bufadienolides isolated from the leaves of *Kalanchoe pinnata* and these compounds show the anticancer properties (Supratman *et al.* **2001**). However, we need further investigation to prove the anticancer properties of isolated compounds from *Kalanchoe pinnata.*

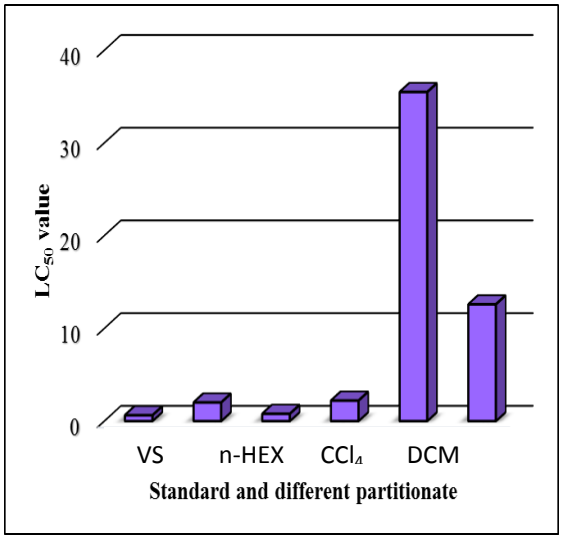

Fig 2 Results of the test samples of Kalanchoe pinnata (Brine Shrimp Lethality Bioassay). VS: Vincristine sulphate, n-HEX: n-Hexane, CCl4: Carbon tetrachloride, DCM: Dichloromethane, ETOH: Crude Ethanol extract, AQSF: Aqueous soluble fraction.

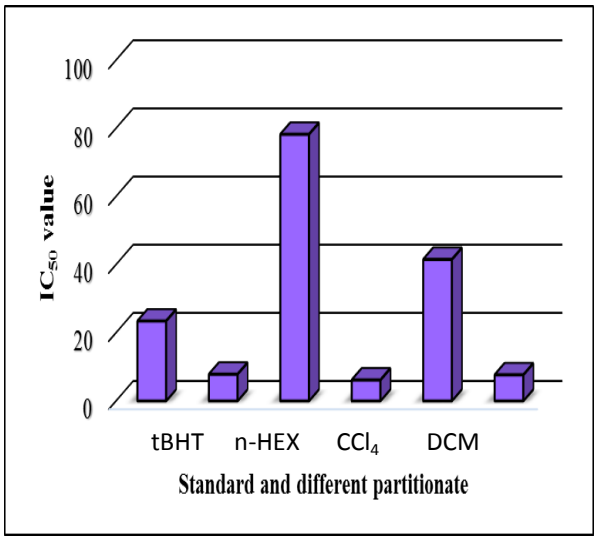

Fig. 4 IC_{50} value of standard and different partitionate of *Kalanchoe pinnata* (tBHT: tert-Butyl-1-Hydroxytoluene, n-HEX: n-Hexane**,** CCl_4: Carbon tetrachloride, DCM: Dichloromethane**,** ETOH: Crude ethanol extract, AQSF: Aqueous soluble fraction.

The dichloromethane soluble partitionates of the ethanolic extract was showed the antimicrobial activity against various kinds of microorganism in terms of zone of inhibition. The compounds in this fraction have strong antimicrobial activity. Joseph *et al.* mentioned that the leaf of *Kalanchoe Pinnata* have significant antimicrobial activity against various kinds of microorganism such as *Staphylococcus spp., E. coli, Shigella spp., Bacillus spp. and Pseudomonas spp. (Joseph et al.* **2011***).*

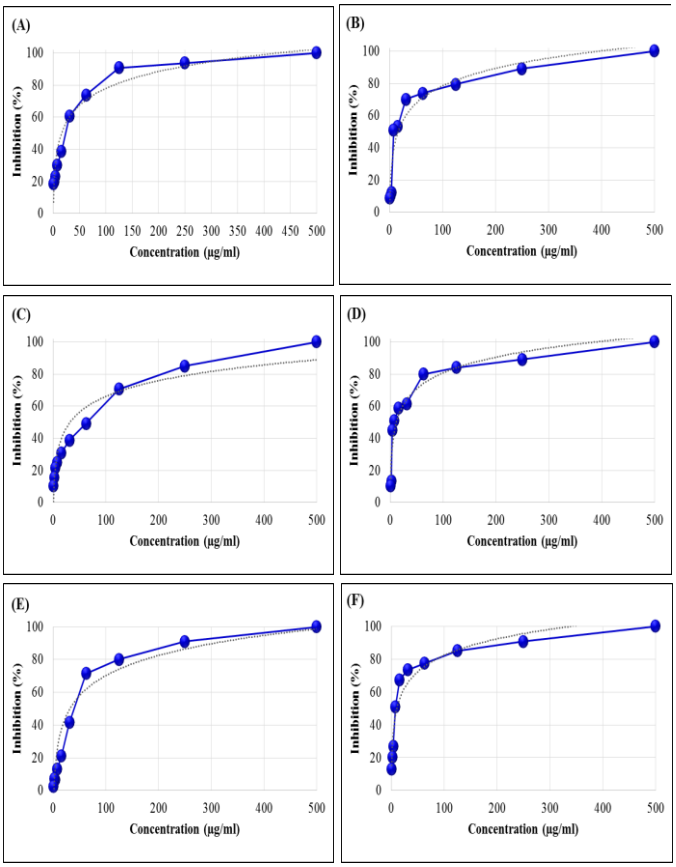

Fig. 3 IC_{50} value of free radical scavenging activity of standard and different partitionate of *Kalanchoe pinnata.* (A) tBHT- tert-Butyl-1-Hydroxytoluene (B) n-HEX- n-Hexane (C) CCl_4-Carbon tetrachloride (D) DCM-Dichloromethane (E) ETOH-Crude Ethanol extract (F) AQSF-Aqueous soluble fraction.

Therefore our results match with their finding. These compounds can be used as a medicinal drug for curing various kinds of microbial diseases in near future.

In the antioxidant activity investigation using Brand-Williams standard method, all samples showed the free radical scavenging activity. Tavares *et al.* found that the aqueous extracts from leaves of *Kalanchoe pinnata* has phenolic compound and its shows antioxidant activity (Tavares *et al.* **2012**). Therefore, we can used our experimental extracts for discovering new antioxidant compounds. However, this cannot be confirmed without further higher and specific tests.

The mechanism and mode of action of isolated compound still unknown. We want to reveal the mechanism and mode of action of these compound on microbial cells as well as cancerous cells in near future.

5. Conclusion

The chemical and biological investigation of leaf of *Kalanchoe pinnata* shows the antioxidant, cytotoxic

activities as well as antimicrobial activity against various kinds of microorganism. Previously we isolated and identified two pure compounds (campesterol and 5,6,7,8,4' pentahydroxy flavanone) from n-hexane fraction of the ethanolic extract of *Kalanchoe pinnata* leaves. There are many other compounds also exist in crude ethanolic extract. All of those compounds are effective against microbial diseases as well as antioxidant, cytotoxic activities. The extract compound showed various medicinal properties, which supports that the traditional use of this plant in various diseases. Therefore *Kalanchoe pinnata* plants play a key role in the development of various kinds of medicines and used for healthcare for the humanity in near future.

6. Acknowledgment

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7. Conflict of interest

The authors declare no competing financial interest.

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