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Isolation and identification of medicinal compounds from *Kalanchoe pinnata* of Crassulaceae family by ^{1}H NMR

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Abstract

The details of phytochemical and biological investigations of *Kalanchoe pinnata*, belong to the Crassulaceae family grown in Bangladesh has not been studied extensively. Here, *Kalanchoe pinnata* has been investigated for the isolation of its secondary metabolites by vacuum liquid chromatography. The powdered leaf of *Kalanchoe pinnata* was extracted with ethanol. The concentrated extract was partitioned with n-hexane, carbon tetrachloride and Dichloromethane. Chromatographic fractionation and purification of n-hexane partitionate provided two compounds. These two isolated compounds were identified by extensive analyses of their high resolution ^{1}H NMR spectral data. The purified compounds were campesterol and 5,6,7,8,4' pentahydroxy flavanone. This is the first report of their occurrence in *Kalanchoe pinnata*.

Keywords: *Kalanchoe pinnata*, ^{1}H NMR, Campesterol and 5,6,7,8,4' pentahydroxy flavanone, n-Hexane, Carbon tetrachloride, Dichloromethane and Ethanol.

1. Introduction

Medicinal plants are highly abundance in the tropics. During recent years, many of these natural sources have been destroyed by over exploitation and deforestation. Apart from their use in the treatment of illness through self-medication, these medicinal plants are valuable for modern medicine in other ways. The success of any healthcare system depends on the availability of suitable drugs on a sustainable basis. With the increasing cost of modern medicines, it becomes more difficult for certain group of people to afford medicine. Thus use of medicinal plants play a key role in the healthcare of rural people.

Isolation and identification of active constituents from traditional medicinal plants can ensure proper medication for the people. In addition, herbal drugs could be scientifically modified for the better pharmacological activity and also it can be established as safe and effective. The rationality of the present study lies for developing herbal medicines, which needs a systematic research on indigenous medicinal plants for the welfare of humanity.

There are several familiar approaches for searching the lead compounds from the plants. The isolated bioactive compounds are utilized in three basic ways (Cox *et al.* **1994**): (a) Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g., digitalis. (b) Unmodified natural products of the therapeutic efficacy which was only remotely suggested by indigenous plant use, e.g., vincristine. (c) Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin. Therefore, this study was undertaken with the following aims and objectives: to assess the bioactive compounds of indigenous medicinal plants; and to highlight the

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values of these bioactive compounds against certain diseases.

Bangladesh is a good repository of medicinal plants belonging to the family Crassulaceae. The Crassulaceae plants contain a wide range of pharmacologically active compound such as anti-inflammatory, immunomodulator, antimicrobial & antilithic activities. Medicinally active compounds are valuable for the treatment of various kinds of diseases (Borchers *et al.* **2000**; Duke **1997**; and Shaheen *et al.* **2003**).

The *Kalanchoe pinnata* belongs to Crassulaceae family possessed medicinal properties. The plant parts (roots, stem, leaves, and flowers) are used for medicinal purposes (Athar and Ahmad **2004**). This medicinal plant have good vegetative growth under various cultivated conditions. Bangladesh is a promising country for growing medicinal plants and sustainable utilization of these medicinal plants. Although a large number of plants under Crassulaceae have been investigated all over the world. In Bangladesh, Crassulaceae plants have been investigated for searching the wide range of secondary metabolites.

Therefore, an attempt has been taken to investigate the chemical constituents of Crassulaceae plants i.e. *Kalanchoe pinnata* and evaluate their pharmacological profiles. This investigation may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained from this investigation and it can be used as lead compounds in the discovery of new and effective drug molecule. So, the objective is to explore the possibility of developing new drug candidates from these plants for the treatment of various diseases.

2. Materials and methods

2.1. Collection and Preparation of the plant material

Plant sample of *Kalanchoe pinnata* was collected. One voucher specimen has been deposited in Dhaka University Herbarium. The leaves (after cutting into small pieces) were sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried plant was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka.

2.2. Crude extract preparation

About 200 gm of leaf powdered material was taken in clean, round bottomed flask (5 liters) and add 2.5 liters of ethanol. The container was sealed by cotton plug and aluminum foil and kept for 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered and concentrated by a rotary evaporator at low temperature (40-50°C) under reduced pressure. The concentrated extract thus obtained is termed as crude extract. The concentrated extract was air dried to solid residue.

2.3. Partitioning with n-Hexane, Carbon tetrachloride, and Dichloromethane

Here we used modified Kupchan Partition (Beckett and Stenlake **1986**) method for the solvent-solvent partition of crude extract. Total 5 gm of ethanol crude extract was triturated with 90 ml of methanol and 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned successively by three solvents of different polarity (n-hexane, carbon tetrachloride (CCl_4), and dichloromethane (DCM)). The mother solution was taken in a separating funnel. The n-hexane (100 ml) was added to it and the funnel was shaken and then kept in a plane surface. The organic as well as the aqueous phase were collected separately. This process was repeated three times and n-hexane fractions were collected together. Distilled water (12.5 ml) added to the mother solution left after washing with n-hexane and mixed well. This solution was taken in a separating funnel and extracted with CCl_4 (100 ml × 3). The CCl_4 fractions were collected together. The aqueous fraction was preserved for the next step. To the mother solution that left after washing CCl_4, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with DCM (100 ml × 3). The DCM soluble fractions were collected together. The aqueous methanolic fraction was preserved as aqueous fraction. Finally, all the fractions (organic phases as well as the aqueous phase) were collected separately and evaporated by Rota evaporator to dryness. Each of the fraction was analyzed separately for the detection and identification of pure compounds.

2.4. Isolation of compounds

The crude extract and different partitioned fractions were subjected to Thin Layer Chromatography (TLC) for screening the different types of compounds. The n-hexane fraction (400 mg) was subjected to Vacuum Liquid Chromatography (VLC) for separation. VLC is a relatively recent separation technique which involves short column chromatography under reduced pressure. This technique is used for the initial rapid fractionation of crude extracts.

The column was packed with fine TLC grade silica (Kiesel gel 60H). Details of the method have been published by Pelletier et al (Pelletier *et al.* **1986**) and by

Coll and Bowden (Coll and Bowden **1986**). The column is packed with silica gel (kiesel gel 60H) under vacuum. The size of the column and the height of the adsorbent layer are dependent upon the amount of extract to be analyzed. A column having 50 cm length & 5 cm in diameter was packed with silica gel up to a height of 30 cm under reduced pressure. The column was initially washed with a non-polar solvent (petroleum ether) to facilitate compact packing. The sample to be separated was adsorbed onto silica gel (kiesel gel 60, mesh 70-230), allowed to dry. The column was eluted with a number of organic solvents of increasing polarity (**Table 1**) and the fractions were collected in 50 & 100 ml beaker. The VLC fractions were analyzed by TLC. The fractions with satisfactory resolution of components were subjected to Preparative Thin Layer Chromatography (PTLC) to obtain the pure compound.

2.5. Visualization/detection of compounds

Detection of compounds in TLC plates is very important method for analyzing extractives and isolate the pure compounds. The following techniques were used for detecting the compounds in TLC/PTLC plates. (a) The developed chromatogram is viewed visually to detect the presence of colored compounds. (b) The developed and dried plates were observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light. (c) The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots were marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing. The pure compound was subjected to chemical characterization.

2.6. Identification by 1H NMR

Spectral data was taken for chemical characterization of compounds. The ^{1}H NMR spectra were recorded using a Broker AMX-400 (400 MHz) instruments. For NMR studies deuterated chloroform was used as solvent and the NMR instrument was locked at the solvent peak and the t5 values for ^{1}H were referenced relative to the residual undeuterated solvent signal.

3. Results

3.1. Preliminary investigation of the plant material

A species of the Crassulaceae family, *Kalanchoe pinnata*, has been investigated in this research. The plant part used was the leaves. Fresh leaves of *Kalanchoe pinnata* were collected and grinded. The coarse powder sample (200 gm) was subjected to cold extraction with ethanol. The crude extract was partitioned successively by n-hexane, carbon tetrachloride, and dichloromethane. The crude extract and different partitioned fractions were subjected for screening the compounds. Among different VLC fractions 6A, 6B and 9A, 9B fractions had given positive result, i.e. only these fractions showed the effective color spot in TLC plate. So, these fractions were undergone for PTLC to get pure compound. The two pure compounds were isolated applying various chromatographic techniques. The isolated pure compounds (KP-6 and KP-9) were then characterized using spectroscopic techniques. The physical properties of the isolated compounds and their reactions to vanilline-sulfuric acid are summarized in **Table 2**.

3.2. Characterization of isolated compounds from *Kalanchoe pinnata*

3.2.1. Characterization of KP-6 as campesterol

KP-6 was isolated from the n-hexane as slight yellow color crystals. It was evident as a purple spot on TLC (Silica gel PF_{254}) when the developed plate was sprayed with vanillin- sulfuric acid followed by heating at 110°C for 5-10 minutes. The compound KP-6 was solubilized in n-hexane, ethyl acetate and chloroform (**Table 2**). The ^{1}H NMR spectral features were in close agreement to those observed for campesterol (Jung Min Choi *et al.* **2007**) (**Table 3** and **Fig 1 - 3**). On this basis, the identity KP-6 was confirmed as campesterol.

3.2.2. Characterization of KP-9 as 5,6,7,8,4' pentahydroxy flavanone

KP-9 was isolated from the n-hexane as colorless crystals. It was evident as a purple spot on TLC (Silica gel PF_{254}) when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110°C for 5-10 minutes. The compound KP-9 was solubilized in n-hexane, ethyl acetate and chloroform (**Table 2**). All of these spectral features were found compatible with 5,6,7,8,4' pentahydroxy flavanone (**Table 4** and **Fig 4 - 6**). On this basis compound KP-9 was identified as 5,6,7,8,4' pentahydroxy flavanone.

4. Discussion

The compound KP-6 is isolated from 6A and 6B fraction. The 6A and 6B fraction were mixed together and then subjected to PTLC. The compound KP-9 is isolated from 9A and 9B fraction. Also the 9A and 9B fraction were mixed together and then subjected to

Table 1 Different solvent system used for VLC

Solvent system	Volume collected (ml)	Fraction No.
n-hexane - Ethyl acetate (99:1)	100	-
n-hexane - Ethyl acetate (98:2)	100	1
n-hexane - Ethyl acetate (97:3)	100	2
n-hexane - Ethyl acetate (95:5)	100(50+50)	3
n-hexane - Ethyl acetate (92.5:7.5)	100(50+50)	4A+4B
n-hexane - Ethyl acetate (90:10)	100(50+50)	5A+5B
n-hexane - Ethyl acetate (85:15)	100(50+50)	6A+6B
n-hexane - Ethyl acetate (80:20)	100(50+50)	7A+7B
n-hexane - Ethyl acetate (70:30)	100(50+50)	8A+8B
n-hexane - Ethyl acetate (50:50)	100(50+50)	9A+9B
Ethyl acetate 100%	100(50+50)	10A+10B
Ethyl acetate - Methanol (99:1)	100	11
Ethyl acetate - Methanol (98:2)	100	12

Table 3 ^{1}H NMR spectral data of KP-6

Position	δ_H in ppm in $CDCl_3$
H-3	3.51 (m)
H-6	5.35 (m)
H_3-18	0.68(s)
H_3-19	1.00(s)
H_3-21	0.91(s)
H_3-26	0.83
H_3-27	0.81
H_3-29	0.97

Table 4 ^{1}H NMR spectral data of KP-9

Position	δ_H in ppm in $CDCl_3$
1'	7.31 (J= 8.4 Hz)
5'	6.86
5	12.08 (J=6.0 Hz)

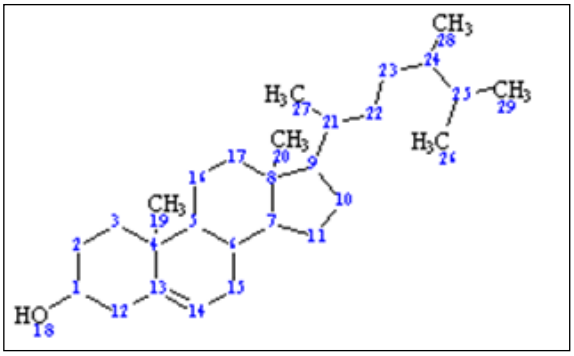

Fig 1 Structure of KP-6

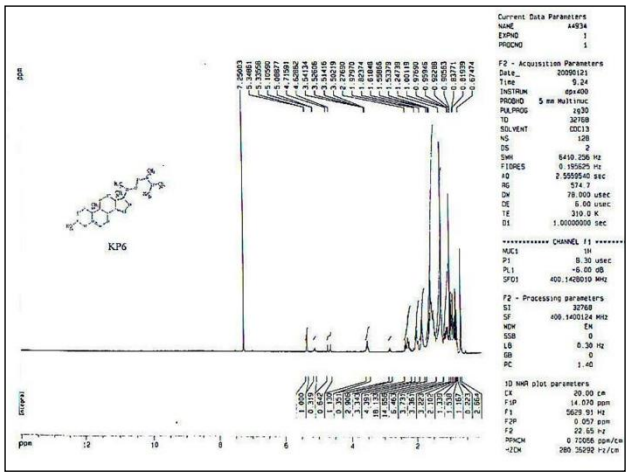

Fig 2 ^{1}H NMR spectral data of KP-6 in $CDCl_3$

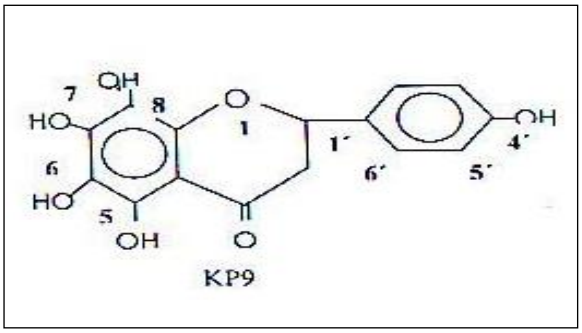

Fig. 4 Structure of KP-9

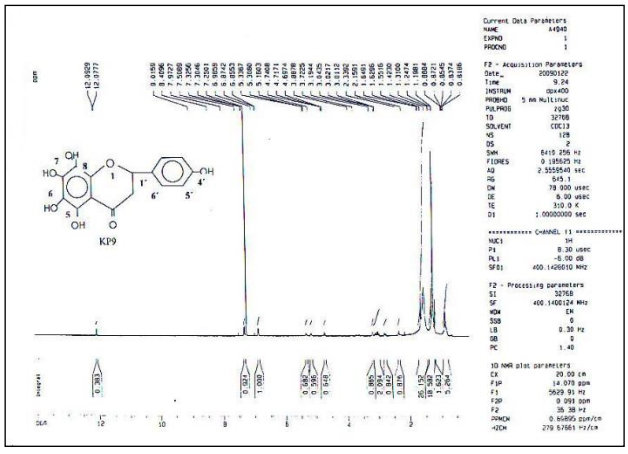

Fig 5 ^{1}H NMR spectral data of KP-9 in $CDCl_3$

Table 2 Physical properties of the isolated compounds

Compound	Physical form	Color	Solubility					Color with vanilline-H_2SO_4
			Ethyl-acetate	n-Hexane	$CHCl_3$	MEOH	DMSO	
KP-6	Slight yellow color crystal	Slight yellow	+	+	+	-	+	Purple
KP-9	Colorless crystal	Colorless	+	+	+	-	+	Purple

(+) indicated completely soluble and (–) indicated not soluble

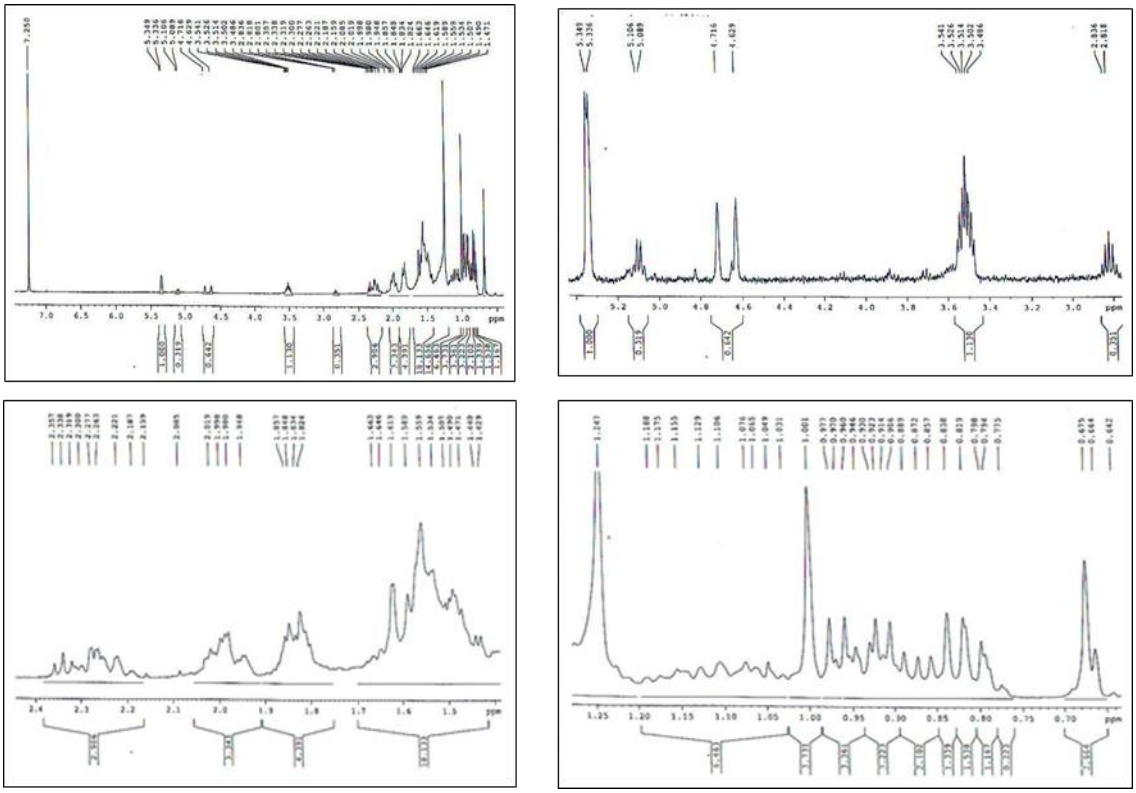

Fig 3 Partially expanded ^{1}H NMR spectral data of KP-6 in $CDCl_3$

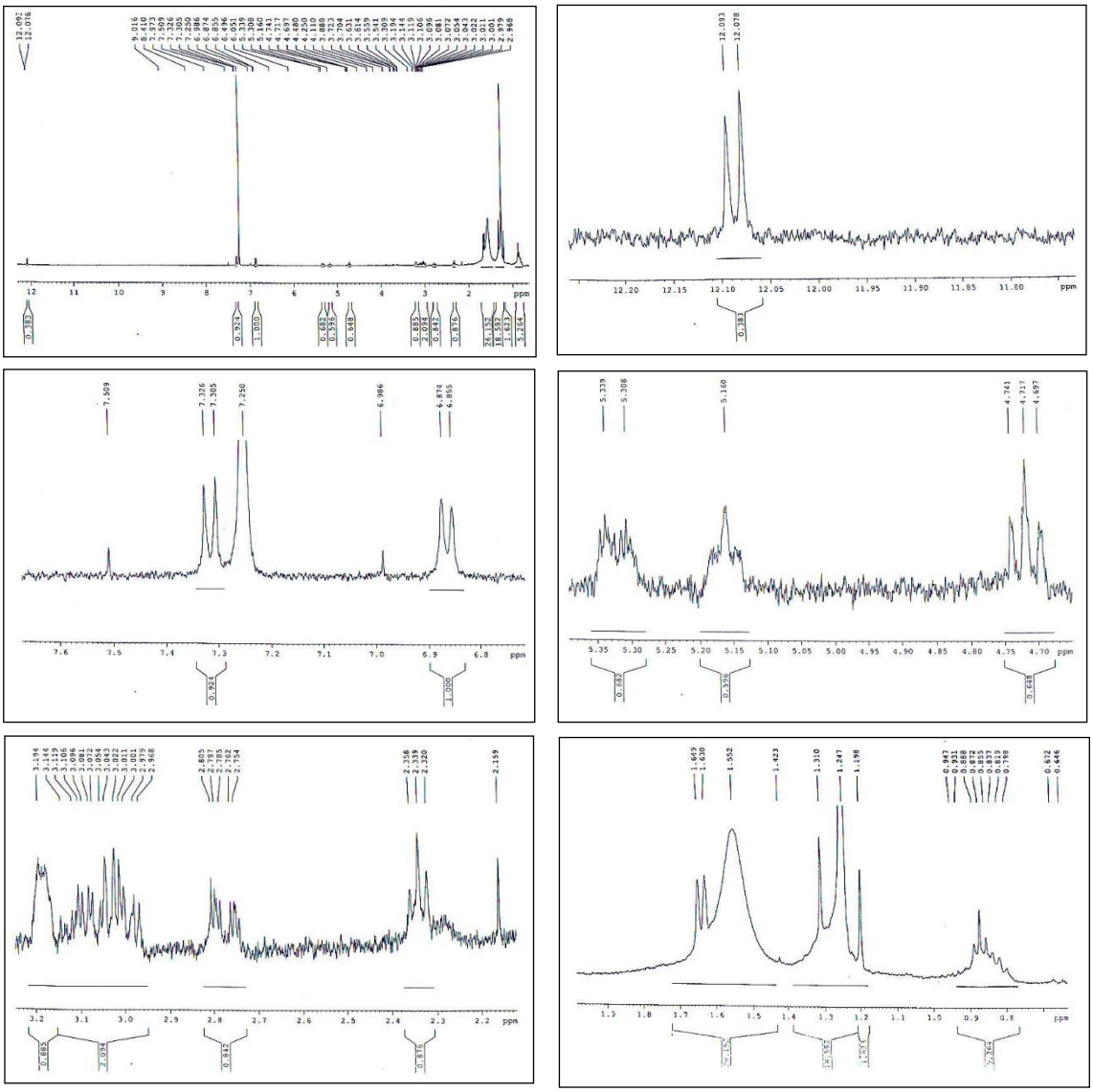

Fig 6 Partially expanded ^{1}H NMR spectral data of KP-9 in $CDCl_3$

PTLC. After PTLC screening, we found that two compounds were chemically pure.

In case of first compound, the ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of KP-6 (**Table 3 and Fig 1 - 3**) revealed a one proton multiplate at δ 3.51 the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a multiplate at δ 5.35 integrating one proton. The spectrum further revealed signals at 1.00, 0.91, 0.97, 0.83,0.81 and 0.68 (3H each) assignable to six tertiary methyl groups. The above spectral features were in close agreement to those observed for campesterol (Jung-Min *et al.* **2007**). On this basis compound KP-6 was identified as campesterol.

On the other compound, the ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of KP-9 (**Table 4 and Fig 4 - 6**) displayed a doublet δ 7.31 (J 8.4 Hz) integrated for two protons intensity. The spectra also showed another doublet for two protons at δ 6.86 having coupling constants 7.6 Hz. All these splitting pattern and coupling constant demonstrated the presence of a distributed benzene ring. Again ^{1}H NMR spectra also displayed a doublet at δ 12.08 (J 6.0 Hz) demonstrating the presence of a aromatic hydroxyl proton of a flavonoid nucleus. The downfield signal is due to chelating effect of hydroxyl group with neighboring carbonyl oxygen at C-3. All of these spectral features were found compatible with 5,6,7,8,4' pentahydroxy

flavanone. On this basis compound KP-9 was identified as 5,6,7,8,4' pentahydroxy flavanone.

These two isolated compounds have some medicinal properties. Campesterol, a plant sterol in nature, is effective against cholesterol and cancer cell. Campesterol decreased the cholesterol concentration in blood and also inhibit the proliferation of cancer's cells (Jung Min Choi *et al.* **2007**). Pentahydroxy flavanone also naturally available compound with various pharmacological properties. Pentahydroxy flavone is effective against various pathogenic microorganism (Hazra *et al.* **2007**). Campesterol and 5,6,7,8,4' pentahydroxy flavanone isolated from *Kalanchoe pinnata* plant have interesting pharmacological properties. To establish the pharmacological properties of these compounds, further studies are indispensable.

5. Conclusion

These investigations provide two interesting compounds, which may be pharmacologically active. The ethanolic crude extract, n-hexane, carbon tetrachloride, dichloromethane and aqueous soluble fractions will be used as a medicine for various diseases. After successive VLC separation of n-hexane soluble fraction of the ethanolic extract of *Kalanchoe pinnata* yielded two compounds (1) Campesterol and (2) 5,6,7,8,4' pentahydroxy flavanone. Isolated compounds from *Kalanchoe pinnata* plant play a key role in the healthcare of the people in near future. These new compounds in this report will be used to reveal antioxidant, antimicrobial and cytotoxic activities and to design new kinds of drugs for different kinds of diseases.

6. Acknowledgment

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7. Conflict of interest

The authors declare no competing financial interest.

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