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ISOLATION AND CHARACTERIZATION OF ANTI-HYPERGLYCEMIC COMPOUND FROM FRACTIONATION OF ETHANOLIC EXTRACT OF *KALANCHOE PINNATA* LEAF.

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ABSTRACT

The present study was aimed to evaluate the effect of active compounds from *Kalachoe pinnata* leaves on blood glucose level in normal and diabetic rats. Diabetes was induced by Streptozotocin (STZ) in male wistar rats. An isolated fraction of EEKP (600 mg/kg b. wt.) and Glibenclamide (10 mg/kg b.wt.) was administered orally. An isolated fractions showing for higher antihyperglycemic activity was subjected to column chromatography that led to isolation of a pure compound, which was given trivial name KP-1. The fraction H from EEKP was found to lower the blood glucose level significantly (P<0.05) in diabetic rats. To ensure the compounds responsible for anti-hyperglycemic activities associated with H respectively. Further column chromatographic analysis was carried out using various solvent systems and which was given trivial name KP-1. KP-1 was phenolic compound nature (Flavonoids) confirmed by spectral analysis.

KEYWORD: *K.pinnata,* Antihypergycemic, Flavanoid.

INTRODUCTION

Phytochemistry deals with various organic substances accumulated in plants. Chemical compounds such as carbohydrate, protein, alkaloid and flavonoids etc are used as food and medicines by people in various ways. The qualitative and quantitative estimation of phytochemical constituents of a medicinal plant is considered to be an important step in medicinal plant research. Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals. The plants are rich in secondary metabolite. The chemistry of natural products helps the scientists to find out the structure of the secondary metabolites by using various separation techniques such as colum chromatography, thin layer chromatography (TLC) and sophisticated analytical techniques such as UV, IR, NMR and Mass spectroscopy. The results of our preliminary studies with the fraction of ethanolic extracts of *K. pinnata* have provoked to isolate anti-diabetic active compounds from the leaves of KP for the management of hypoglycemic potential and antioxidant activities on streptozotocin induced diabetic rats.[1-3]

MATERIALS AND METHODS

Plant Material

The plant of *K. pinnata* has been collected from Sikar district, Rajasthan, with the help of field botanist. The plant of *K. pinnata* has been authenticated by Dr. P.M. Padhye, Scientist, 'F'& Head of Office, Botanical Survey of India, Arid John Circle, Jodhpur, Rajasthan, India. (Ref. BSI/AZC/01/2011-12/Tech 408). The leaves of *K. pinnata* were dried initially under shade. Then 200 g coarse leaves powdered were extracted with solvents of increasing polarity such as petroleum ether, chloroform and ethanol using soxhlet apparatus. The extracts were collected and preserved in desiccators until used for further studies.[4-5]

Fractionation, isolation and characterization of compounds from the acetone insoluble fraction of ethanolic extract

Chromatographic techniques were used for the isolation of compounds. The column chromatographic technique most commonly used for the separation of compounds into several fractions according to the affinity of the solvent used. The study involves in fractionation and isolation of compounds from pharmacologically active in ethanolic extract. The structure of the compound were tried to establish by spectroscopic methods.[7]

Study design
In column chromatography, a solvent system was established by developing TLC technique. The silica gel (100-200 mesh size) slurry was made with the solvent system established earlier. The slurry was poured into the column very carefully and the silica gel was allowed to settle down to from a uniform packing. Then the stop-cock of the column was opened and the excess of solvent over the column head was allowed to run. The dry crude acetone insoluble fraction of ethanol extract (4 g) was mixed with small amount of silica gel in a mortar to get a free flowing powder. The powdered sample was then applied carefully on the top of the prepared column and successfully eluted with solvent system using various solvent systems such as chloroform, chlorofom: methanol to separate the elute. The eluate with same Rf value are pooled together and evaporated to dryness. When the mixture of solvent system used, the ratio of mixtures are prepared as 100, 90:10, 80:20, 70:30, 60:40, 50:50, 10:90, 20:80, 30:70 and 40:60 respectively. Elutes were collected in a number of conical flasks marked from fractions 1-226. Elutes were spotted on TLC plate and the flasks having similar spots were combined together.[8-10]

Animals
Adult male wister rats (150-180 g) were procured and housed in the animal house of Goenka College of Pharmacy, with 12 hrs light and 12 hrs dark cycles. Standard pellets obtained from Hafed, Rohatak, India, were used as a basal diet during the experimental period. The control and experimental animals were provided food and drinking water ad *libitum*. After randomization into various groups, the rats were acclimatized for a period of seven days under standard environmental conditions of temperature, relative humidity, and dark/light cycle. All the animal experiments were conducted according to the ethical norms approved by CPCSEA, held on 17th Dec 2012 at Goenka College of Pharmacy, Lacchmangarh, and Sikar. (Ethical committee IAEC reg.no.1224/ac/08/ CPCSEA).

Effect of acetone insoluble fraction of ethanolic extract of *K. pinnata* on blood glucose level in diabetic rats
Various isolated fractions of EEKP (600 mg/kg) were evaluated for their hypoglycemic effect. STZ was dissolved in freshly prepared 0.1 M cold citrate buffer (pH 4.5) and administered by intraperitoneal route (45 mg/kg) to the overnight fasted rats. Diabetes was confirmed 72h after induction by measurement of tail vein blood glucose levels using glucometer by glucose oxidase-peroxidase method using strips. Diabetic rats were kept 7days under standard laboratory condition for the stabilization of blood glucose levels. After 7 days induction of diabetes, blood glucose was again determined and animals with a blood glucose level greater than 250 mg/dl were selected for the study. The hyperglycemic rats were divided into 11 groups containing 6 rats each. Distilled water, glibenclamide and various isolated fractions of EEKP (600 mg/kg b.wt.) daily administered orally to normal control, diabetic control and the treatment groups respectively.

Purification of isolated fraction
Nearly 1gm of the fraction was weighed and mixed with silica gel and poured into the column. The column was eluted with different solvents by polarity basis. Fractions were separated by the solvent petroleum ether. In this solvent system, some distinct bands were formed. The polar fraction present in the extract is eluted by using chloroform. The second polar fraction of the extract is eluted by using methanol. The column fraction which is collected and evaporated to dryness is used for further studies.[12-16]

Analysis of fraction
The fraction was characterized by spectroscopic techniques like UV, IR, NMR and Mass spectroscopy.

Statistical analysis
The results of the study were subjected to one way analysis of variance followed by student's t-test for multiple comparisons. Values with $P < 0.05$ were considered significant.

RESULTS
Column chromatography study
The column chromatography study was carried out with EEKP to separate the eluates namely, F 1–32 using chloroform as a solvent system, F 33–65, 66–99, 100–115, 116–149 , 150– 187, 188-210 and 210-226 using chlorofom: methanol as a solvent system with different ratio. The pooled fraction of EEKP such as F 1–32, F 33–65, F 66–99, F 100–115, F 116–149, F 150–187, F 188–210, and F 211-226 are named as A, B, C, D, E, F , G and H respectively.The pooled eluates of A, B, C, D, E, F, G were tested for blood glucose level in streptozotocin induced diabetic rats.

Effects of 28 days studies of various isolated fractions EEKP (600 mg/kg b.wt.), and Glibenclamide (10 mg/kg b.wt) on blood glucose level in streptozotocin (STZ) induced diabetic rats

On the basis of above study, it was observed that the fraction 'H' at dose of 600 mg/kg b. wt after 28 days, produced more significant antidiabetic activity as compare to other fractions. Results were determined by one-way analysis of variance (ANOVA non parametric) with student's t-test with $P<0.05$ considered significant. Results are shown in Table 1.

Purification of pooled column fraction of EEKP by column chromatography
From the results of anti-diabetic effect, the fraction "H" from EEKP showed promising results. Hence this fraction was subjected to further purify using column chromatography and followed by TLC. The natures of fractions obtained are listed in Table 2.

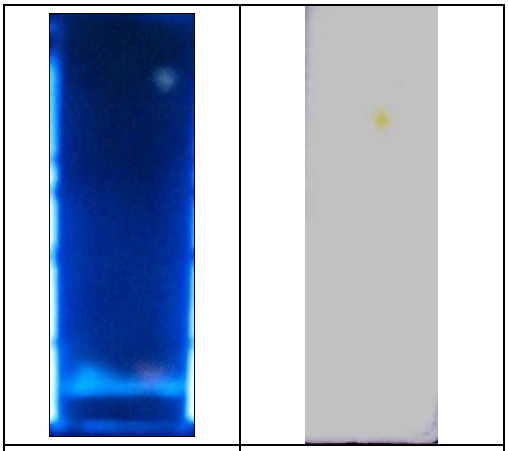
Group	Treatment	Blood glucose level mg/dl				
		0 Day	7 Days	14 Days	21 Days	28 Days
I	NC	68.26 ±3.44	68.96 ±3.62	70.16 ±3.74	71.83 ±4.44	72.13 ±3.44
II	DC	379.83 ±8.04	384.66 ±9.61	389.66 ±11.52	393.16 ±10.87	402.83 ±10.30
III	Standard 10	395.00 ±8.63	266.16 ±6.40**	194.66 ±8.95**	183.83 ±6.94**	145.00 ±5.65**
IV	AIEE (1-32) (A)	401.50 ±9.33	320.00 ±11.89*	311.33 ±10.51*	293.00 ±7.22*	233.33 ±7.45**
V	AIEE (33-65) (B)	392.00 ±10.68	300.66 ±9.89*	298.00 ±7.94*	273.50 ±8.08*	222.16 ±5.67**
VI	AIEE (66-99) (C)	401.50 ±9.33	309.00 ±11.89*	312.33 ±10.51*	280.00 ±7.22*	243.12 ±7.45**
VII	AIEE (100-115) (D)	402.15 ±8.22	310.00 ±10.79*	313.33 ±10.51*	301.00 ±6.25*	243.33 ±8.43**
VIII	AIEE (116-149) (E)	399.17 ±7.12	315.00 ±9.89*	324.33 ±10.61*	298.00 ±7.35*	256.33 ±9.43**
IX	AIEE (150-187) (F)	403.15 ±8.22	318.00 ±7.19*	330.33 ±9.41*	290.00 ±7.05*	261.33 ±9.13**
X	AIEE (188-210) (G)	400.50 ±9.33	312.00 ±11.89*	315.33 ±10.51*	293.00 ±7.22*	239.33 ±7.45**
XI	AIEE (211-226) (H)	392.16 ±11.16	295.33 ±8.36**	224.50 ±5.96**	218.33 ±5.39**	165.66 ±5.31**

n=6, *****p<0.05- significant, ******p<0.01-more significant v/s diabetic control, SEM= standard error mean, SD = standard deviation, n= number of animals

NC - Normal control , DC - Diabetic control , Standard 10- Glibenclamide10 mg/kg b.wt , AIEE (A) - Acetone insoluble fraction of ethanolic extract 600 mg/kg b.wt , AIEE (B) - Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt , AIEE (C)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt, AIEE (D)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt, AIEE (E)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt, AIEE (F)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt, AIEE (G)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt, AIEE (H)- Acetone insoluble fraction of ethanolic extract 600mg/kg b.wt

Table 2. The column chromatographic fractions from EEKP and their TLC analysis.

S. no	Fraction number	Solvent system	Ratio	Number of spots		R_f value
				UV light	I_2 chamber	
1	1-32	Chloroform	100	-	-	-
2	33-65	Chloroform : Methanol	90:10	2	2	0.22, 0.27
3	66-99	Chloroform : Methanol	80:20	2	2	0.29,0.38
4	100-115	Chloroform : Methanol	70:30	2	2	0.24, 0.33
5	116-149	Chloroform : Methanol	60:40	3	3	0.26, 0.42, 0.44
6	150-187	Chloroform :Methanol	10:90	2	2	0.18, 0.52
7	188-210	Chloroform : Methanol	20:80	3	3	0.22, 0.25,0.41
8	211-226	Chloroform : Methanol	30:70	1	1	0.73



Under UV light	Under visible light

Figure 1: TLC of isolated compound.

Characterization of compounds using various analytical techniques

UV studies with KP–1: The UV-visible spectrum exhibit two peaks at 371 and 256 nm. UV-visible spectra shown the absorbance at 371 nm might be due to presence of coloured compound because isolated compound produced absorbance under visible light wavelength range (200-400 nm). The spectrum of the compound is given in Figure 1.

IR studies with KP–1: IR spectrum revealed the presence of aromatic hydroxyl of flavonoid showing wide peak at 3411 cm^{-1}. The presence of stretching vibrations at a wave length of 1663 cm^{-1} depiciated the presence of C=O group. The presence of vibration at 1608, 1523 and 1496 cm-1 indicated the presence of C-C group in aromatic ring. The spectrum of the compound is given in Figure 2.

H^1-NMR Studies with KP–1: The H^1-NMR spectrum of isolated compound KP-01 exhibited a series of signals in the range of δ 4-8. Numbers of signals were observed in the NMR spectrum of isolated compound KP-01 but some of signals on the basis of interpretation gave specific δ value which played important role to understand the presence of aromatic ring structure and type of proton present in isolated compound. Signals were observed δ 4.47-5.24 revealed the presence of OH group of flavanol and flavonoids. Signals observed at δ 6.18 – 7.14 indicated the presence of arryl proton. The spectrum of the compound is given in Figure 3.

C^{13}-NMR studies with KP–1: The C^{13}-NMR spectrum of isolated compound KP-01 exhibited a series of signals in the range of δ 97-170. Numbers of signals were observed in the C^{13}-NMR spectrum at δ 97, 106, 117, 127 and 136 ppm due to the presence of one carbon of benzene ring at 15, 14, 11, 1 and 2 positions. The presence of other signals were observed due to the two carbon of benzene ring at δ 149 (at 6C and 8C) , 161 (at 12C and 13C) ,166 (at 5C and 7C) and δ 170 ppm owing to C of C=O in chroman ring. The spectrum of the compound is given in Figure 4.

Mass spectrum studies with KP–1: Mass spectrum of isolated compound KP-01, mass/charge ration (m/z) of base peak and molecular ion peak was obtained at 151.1 and 301.0 respectively. It indicated that most stable fragment has molecular weight of 203.1 and molecular weight of the isolated compound KP-01 is 301.0. The m/z ratio of the fragments at 151.1, 179.1 and 257.1 indicated the presence of ring A, B and aromatic structure, which can be related to flavonoid. Others m/z ratio of fragments also provide the information about different types of fragment such as 301.1 (C_6H_9 or C_5H_5O) and 151.1(aromatic ring). The spectrum of the compound is given in Figure 5.

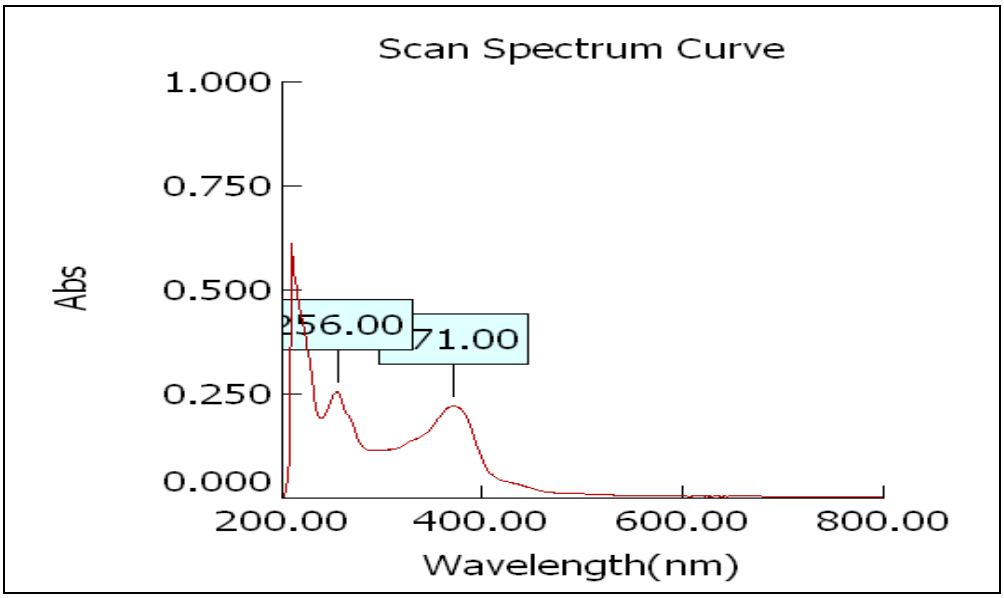


Figure 1: UV Spectra of Compound KP-01.

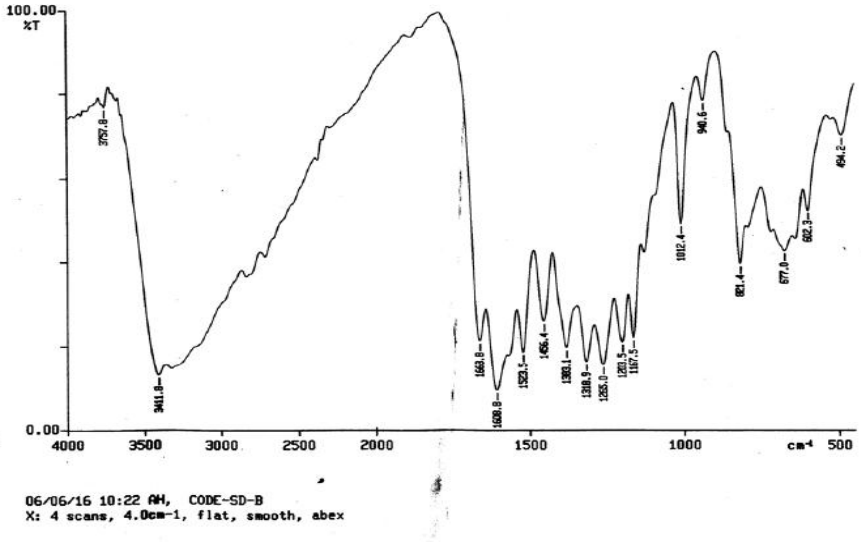


Figure 2: IR spectra of compound KP-01.

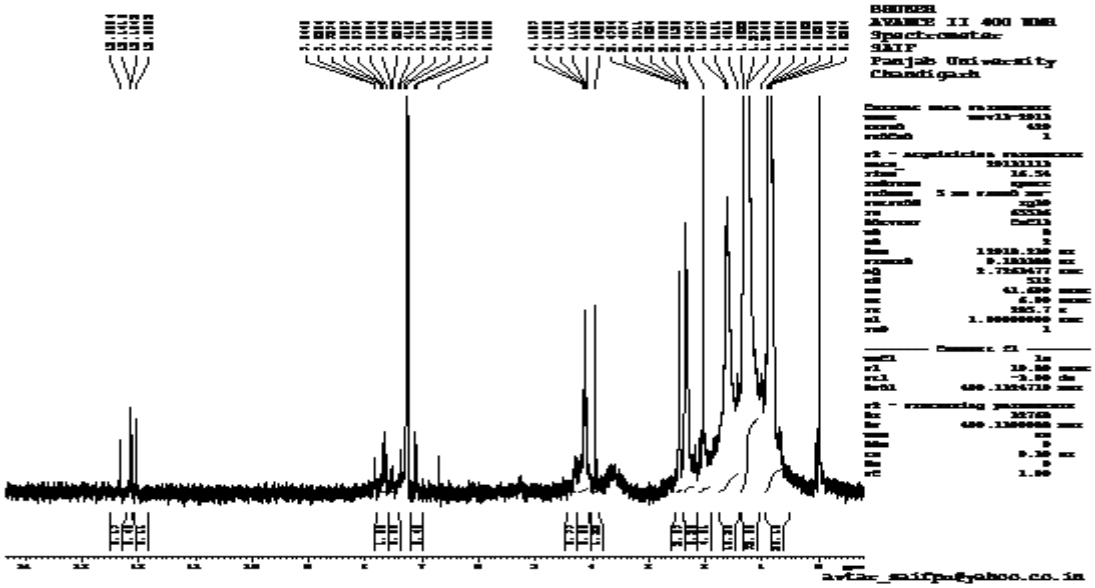


Figure 3: NMR spectroscopy of isolated compound KP-01.

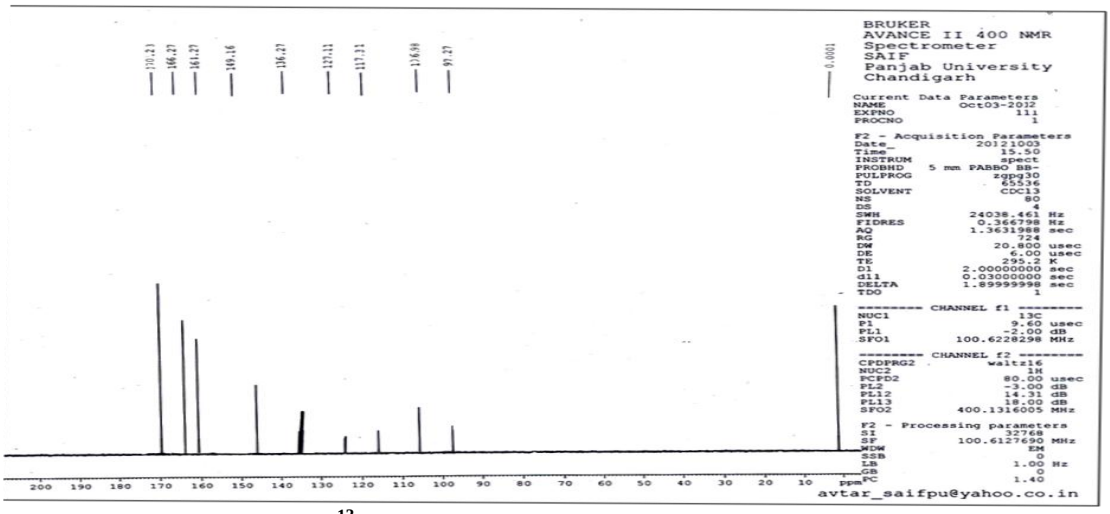


Figure 4: ^{13}CNMR spectrum of isolated compound KP-01.

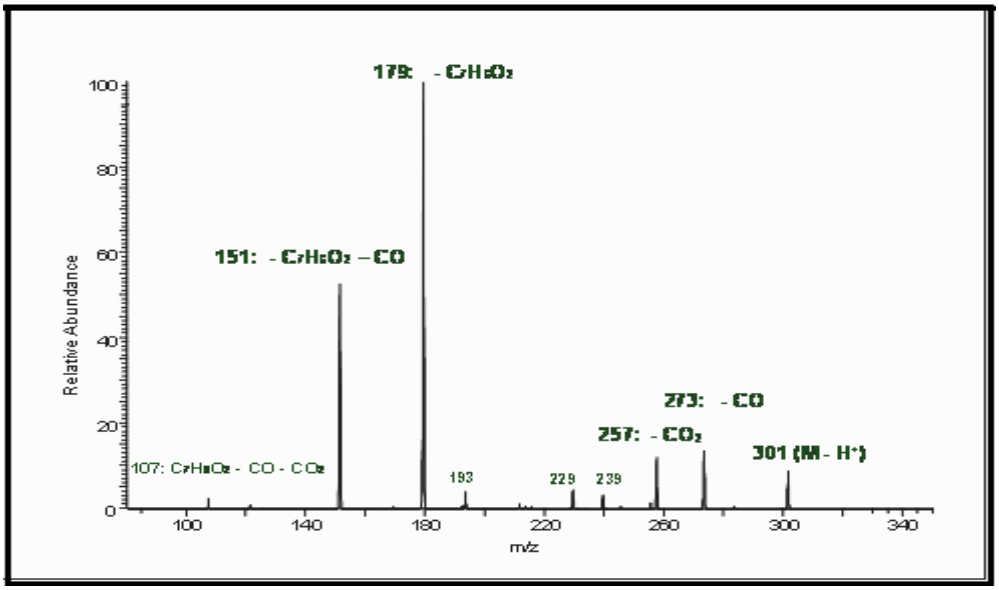


Figure 5: Mass spectrum of isolated compound KP-01.

On the basis of above spectral interpretations (UV, IR, NMR and LC-MS) of isolated compound KP-01, it can be concluded that KP-01 might be related to phenolic group category such as flavonoids. It may be quercetin. The literature review also indicates that quercetin may play important role to produce hypoglycemic activity.

DISCUSSION

Now a day, the interest in the study of natural product is growing rapidly, especially as a part of drug discovery programs. In continuation to the previous study, we have shown interest to isolate the pure constituents responsible for the above mentioned pharmacological action. An attempt was made to isolate the purified compounds responsible for anti-diabetic activity using column chromatography technique with EEKP. The fraction "D" from EEKP showed strong anti-diabetic activity on a par with the standard drug metformin. To ensure the compounds responsible for anti-diabetic activities associated with "D" respectively, In addition a column chromatographic analysis was carried out with "D" using various solvent systems. We isolated one compound named as KP-1 from the column which were amorphous powders with decomposition point; however KP-1 was phenolic compound nature (Flavonoids) confirmed by spectral analysis. At present, the exact Mechanism of action of the isolated fraction of KP-1 is not yet known and will be the subject of further studies.

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