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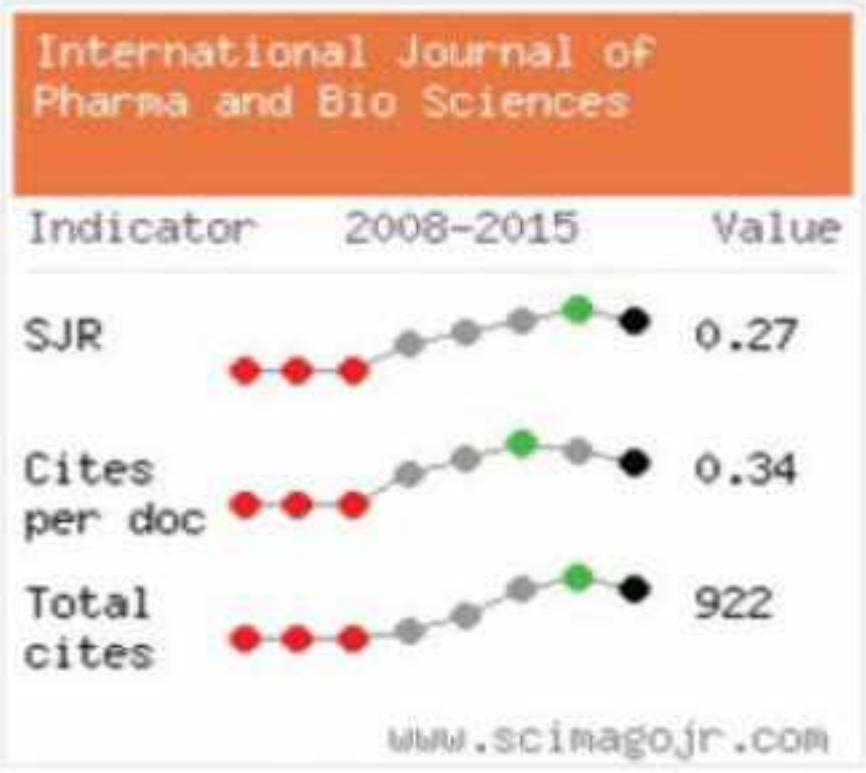

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A BIO-HERBAL MEDICINAL REMEDIES: *M CHARANTIA* LINN. A SCOPE OF CHARACTERIZATION OF MEDICINALLY EVALUATING ANTIDIABETIC COMPOUND.

U. WAHUL[1], A. KADAM[1*], L. KAMBLE[2]

[1]School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded 431 606 (MS), India.
[1]Swami Ramanand Teerth Marathwada University, DSM Arts, Commerce, Science College Jintur, Parbhani - 431 509, India*

ABSTRACT

As we aware diabetes is not only one kind of symptomatic disease but its occurrence spread through the various metabolic channels and hence raises other disorders. The prolonged symptoms of diabetes also cause the complications of eyesight, Night blindness, kidney failure, and other autoimmuno dysfunction including sexual dysfunction. In those symptomatic diseases various drugs are used to treat the diabetes such as biosimilar therapy including use of insulin and insulin analogues, oral hypoglycaemic agents and various other complementary medicines. As herbal remedies i.e. *M charantia* Linn. (Bitter Gourd) are commonly known as fruit vegetables. The Leaves, Seeds, Roots, Fruits and the stem part of the plants are medicinally used in different diseases. It is most effectively used to treat the acidic condition of gastrointestinal tract. *M.charantia* is also called the oxygen radical scavenger, which takes part into metabolic pathway. Due to the oxygen radical scavenging activity of GSH it directly expedites the ROS neutralization and the repair of ROS-induced damage which is important to neutralize the acidic condition of gastrointestinal tract.The present investigation was carried out to study the characterization of present antidiabetic compound having different solvent extract of *M.charantia* in various solvent system. The overall conclusion suggested that the extracted compound shows the antidiabetic and diuretic properties.The total unknown protein concentration was 21.01 µg/mL which is similar with standard antidiabetic drug and the slope consists of 0.0314 with the line of intercept 0.081, which has been elaborated in results and conclusion.

KEYWORDS:*Bio herbal, Biosimilar, (ROS), Diabetes, Insulin, Insulin Analogues etc.*

A. KADAM*

Swami Ramanand Teerth Marathwada University, DSM Arts, Commerce, Science College, Jintur, Parbhani - 431 509, India

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INTRODUCTION

The prevalence of diabetes mellitus in India is predicted to double 2030, posing a significant health care burden.[1]the costs of caring for diabetes and its related complications are also staggering. In India, diabetes care can cost low-income households about one-third of their incomes.[2,3] Providers caring for patients with diabetes recognize that the patients are very interested in alternative supplementation and may choose to supplement their pharmacological regimen with supplementation popularly from natural products, i.e. herbal or botanical sources.[4] From the patient perspective, it is considered very acceptable to include herbal or botanical extracts as part of the medical intervention based on the recognition that the herbal intervention is considered to be natural and that the practice may have been part of the culture for many generations.In spite of the presence of known antidiabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease.[5] Several approaches are presently available to reduced the hyperglycaemia including insulin therapy which suppresses glucose production and augments glucose utilization and several drawbacks like insulin resistance,[6] anorexic nervosa, brain atrophy and fatty liver,[7] after chronic treatment; treatment by sulfonylurea, which stimulates pancreatic islet cell to secrete insulin; metformin, which acts to reduce hepatic glucose production; α-glucosidase inhibitors, which interfere with glucose absorption. Unfortunately, all of these therapies have limited efficacy and various side effects and thus searching for new classes of compounds is essential to overcome these problems.In the current study, we investigated the antidiabetic potential of extracted compound from *M.Charantia* along with one comparative standard dosage forms i.e. Metformin The compound is extracted through their mechanism of action.

Figure 1
The present figure. showing the physical view of Bitter Gourd/Leaves.

MATERIALS AND METHODS

Chemical and Reagent (HiMedia Pvt Ltd, Mumbai)
Solvents required for Extraction, Precipitation and Dialysis:

- Methanol
- Acetone
- Deionized Water (D/W)
- Dulbecco's phosphate-buffered saline (DPBS) - (pH-7.3)
- 75% Ammonium sulfate solution (For the Precipitation Purpose)
- Dialysis Bag (Biotech CE Dialysis Tubing- Part No. 131264; Spectrum Lab)

Chemicals Required for Assay Performance (HiMedia Pvt Ltd, Mumbai)

- Bradford Test:
- Bovine serum albumin
- Coomassie blue staining solution
- Deionized Water (D/W)

Chemicals Required for Purification by Gel Filtration Chromatography (HiMedia Pvt Ltd, Mumbai)

- Sephadex G-100 column
- 0.02 M Sodium acetate buffer (pH.5)
- Dulbecco's phosphate-buffer saline (DPBS) - (pH-7.3)
- Chemical Required for Broth Dilution Assay (MIC-Establishing the level of resistance of a particular bacteria by present investigated Protein)

Chemical Required for Broth Dilution Assay (MIC-Establishing the level of resistance of a particular bacteria by present investigated Protein):

- Nutrient Broth (Mueller-Hinton)
- Antibiotic (Ampicillin)
- Escherichia coli (ATCC 25922)
- Phosphate Buffer Saline (PBS)
- Deionized Water

Chemical Required for the SDS Page (HiMedia Pvt Ltd, Mumbai)

- 15% SDS Gel
- Coomassie blue staining solution
- Methanol
- Acetic Acid
- Distilled Water

Collection of Plant Material and Standard antidiabetic drug from regional Medicine outlets

M.Charantia Linn. was collected from the Private Seed Research Company i.e.Nath Biogene Ltd., Aurangabad, Maharashtra, India. The features of the selected plant are given as follows in the Table No.1. The available *M.Charantia* Linn. (Bitter Gourd)is brought to the laboratory and rinsed with tap water to remove unwanted particle.The standard antidiabetic drugsMetformin were obtained from the Pharmacy Outlet at Nanded, Maharashtra, India to be used for the determination of the antidiabetic effect. Some of the antidiabetic drugs are as follows with their assay value (Table No. 2).

Extraction, Precipitation and Purification of Protein (In House)

Extraction

Protein extraction and their purification were carried out by the soxhlet method (Wahul et al.2016) at the lab with some modification in solvent system. Sequential extraction was carried out to facilitate the simplicity in sample preparation. Different solvents were used based upon their polarity and the list of solvents used are presented in Table No.3.The extracts obtained were filtered with whattman filter paper and evaporated to concentrate to the volume of 10 ml in microwave oven at 30˚±5˚C for 10 hrs. The extracted sample was then mixed with the 50 mL Dulbecco's phosphate-buffered saline, PH 7.2-7.3 and was blended in electric blender to obtain the homogenate. The obtained homogenate then were freeze and centrifuged at 10,000 rpm for 15 min at 4˚C. The supernatants of Leaves and Seeds were then precipitated with Ammonium sulphate precipitation method. The pH of the 10x stock solution of DPBS is ~6.8, but when diluted with water to 1x DPBS; it should get changed to 7.2-7.3.

Precipitation

Precipitation with organic solvents, such as ethanol and acetone, has been in use for over a hundred years, but is probably best known for its use in fractionating human serum. But here we use the Ammonium sulphate (75%) for the concentration, and precipitation purpose.The supernatants were precipitated out with 75% ammonium sulphate solution (Rehman and Khanum; 2011). The precipitated solutions were centrifuged at 10,000 rpm for 30 min at 4˚c. The pellets then were dissolved in 1 mL of deionized water by gentle vortexing. The dissolved ammonium sulphate precipitate and supernatant were dialyzed in dialysis bag (Biotech CE Dialysis Tubing-Part No. 131264; Spectrum Lab) against the distilled water. The protein concentration was estimated in crude extract, dissolved ammonium sulphate precipitate and supernatant.

Assay:

Broth Dilution Assay (MIC - Establishing the level of resistance of particular bacteria by present investigated Protein)

The broth dilution assay aims to determine the concentration of the investigated antimicrobial protein that, under defined test conditions, inhibits the visible growth of the bacteria.

1. 9 sterile test tubes were placed according to the following table.
2. 0.5 ml of sterile N.Broth was added to each tube with 5 ml pipette.
3. 0.5 ml of the ampicillin was added in broth containing 1st test tube, henceforth the concentration of ampicillin in this tube is 64 µg per ml.
4. The fresh micropipette was taken and mixed in the contents,while pipette out of the 0.5 mL of broth from 1st test tube was taken and added to the 2nd test tube (Ampicillin Concentration - 32 µg/mL.)
5. With the fresh micropipette, the content of 2nd test tube was mixed and transferred 0.5 mL of broth to the 3rd test tube (Ampicillin Concentration – 16 µg/mL.)
6. Likewise, the dilution process was continued upto the 7th no. test tube and the ampicillin concentration was maintained as per the table. There was no Antibiotic in the test tube no. 8th and 9^{th} .
7. Suspension of *E.coli* from the plate culture in 5 mL of PBS saline was prepared.
8. With the help of sterile 1 mL pipette, 0.1 mL of *E.coli* suspension was transfered to 9.9 mL of saline containing test tube, the content of tube was mixed well.
9. 0.1 mL of this suspension was added to the Antibiotic containing broth tubes from 1^{st} to 7^{th} and to the Growth control tube, the tubes were shaked well, mixed thoroughly and placed into the shaking incubator for 18 to 24 hrs.

Purification by Gel Filtration Chromatography

The samples of both dissolved ammonium sulphate precipitate and supernatant of leaves and seeds were used for estimation of Broth dilution assay in culture media. As the activity was observed only in dissolved ammonium sulphate precipitate of seeds, dissolved ammonium sulfate precipitate was further purified by gel chromatography using column of Sephadex G-100 with 0.02 M Sodium acetate buffer (pH.5). The column was elution with the DPBS (pH-7.3) with the 1 ml/min flow rate. The absorbance spectrum of collected fractions was monitored at 280 nm[8].

TLC Autography

The eluted sample, dialyzed ammonium sulphate precipitate and crude extracted sample was then applied for the thin layer chromatography, in this instance the samples were applied on the TLC plate or on glass slide which is slurred with the Silica gel in appropriate solvent. By applying the sample on Base line/Origin line, The TLC Plate was kept for development in development chamber containing Chloroform: Methanol: Water in ratio of (65:25:10) (Joseph Sherma et al; 2000). TLC plate was kept in incubator at 36˚C for 1/2 hr, till solvent get evaporated. Finally the Retention factor was calculated according to the spot resulted on TLC plate[9].

SDS page

After the fractionating eluted sample along with crude extract and the dialyzed ammonium sulphate precipitate run on 15 % SDS-PAGE gel (Laemmmli, 1970). As the

sample run at appropriate level to be achieved, the separating gel was placed in coomassie blue Staining solution on a shaker for 1-1.5 hrs. Excess dye was removed by destaining with Methanol: Acetic Acid: Water (30:60:10 v/v). The analysis of absorbance spectrum was plotted against the eluted sample fraction (Fig.1). Finally the protein concentration in sample was estimated by Bradford method using Bovine Serum Albumin (BSA) as a standard, and the remained sample was taken for the animal (Rat) study[10].

Protein Concentration Estimation

Bradford Assay

The Bradford protein assay is a colorimetric assay. The method is based on the proportional binding of the dye Coomassie blue to proteins. The more protein present, the more Coomassie blue stain binds. Furthermore, the colour of the test sample becomes darker where, Coomassie blue stain absorbs at 595 nm.

- A dilution of a 2 mg/mL BSA sample was prepared by adding 50 µL of 2 mg/mL BSA to 150 µL of D/W to make 200 µL of 0.5 mg/mL BSA.
- The test samples were generated for the blank, BSA standards and the protein sample were to be tested as per table no 3.
- Each sample was allowed to incubate at room temperature for 10-30 minutes.
- The absorbance of each sample wasmeasured at 595 nm using a UV-visible spectrophotometer.
- Protein concentration was determined as per the following equation i.e y = mx + b; where y = absorbance at 595 nm and x = Protein concentration.

RESULTS AND DISCUSSION

Collection of Plant Material and Standard antidiabetic drug from regional Medicine outlets

The plant collection was done from private company Nath Biogene India Ltd., Aurangabad. The characteristics for selected plant are as follows;

Table 1
Features of Selected Plant

Sr.No.	Characteristics	Observation
	A. Morphological characteristics of Bitter Gourd Hybrid NBIH-274	
1	Plant Growth Habit	Good Spreading
2	Plant Vigour	Excellent
3	Foliage Color	Dark to Green
4	Leaf Blade: Length	Medium
5	Leaf Blade: Width	Medium
6	Fruit Shape	Spindle Shaped
7	Fruit Size	Medium Long
8	Fruit Color	Uniform Dark Green
9	First Harvest	50-55 Days of Sowing
10	Fruit weight	110-120 gm
11	Prickles	Thick Sharp Dense
12	Disease Resistance	High degree tolerance to Downey & Powdery mildews and mosaic diseases.

The standard drugs chart was given to compare the analysed data

Table 2
Standard Antidiabetic Drugs to Treat Diabetes

Antidiabetic Drug	Company Name	Molecular weight in (Dalton) & (g/mol)	Assay (%)
Mixtard	Novo Nordisk	5808 Dalton	27.5%-More
Glycomet	USV	165.63 g/moL	95.0% -105.0%
Spasmo Proxyvon plus	Wockhardt Ltd	747.96 g/moL	99.0%-102.0%
Lantus	Sanofi, India	6063 g/moL	94.0%-105.0%
Galvus met	Novartis India	303.40 g/moL	99.44%
Liv 52	Himalaya Drug	NA*	NA*
Janumet	MSD Pharma	523.32 g/moL	98.5%-101.5 %
Augmentin (Combination)	GSK	Amoxicillin 419.46/ Clavulanic acid 237.25	90%-120%
Clavamel	Alkem Lab	157.125 g/mol	75.5%-92.0%
Monocef	Aristo Pharma	542.556 g/mol	90%-115%
Novomix	Novo Nordisk, India	5825.6 g/mol	26.5%-More
PAN	Alkem Lab	53.0626 ± 0.0028 g/mol	98.0%-102.0%
Volini	Ranbaxy	NA*	99.0%-101.0%
Synflorix	GSK	Serotype 6A/B 12,259 KDa	NA*
Becosules	Pfizer	476.5 g/mol	NA*

NA*: Not Available

Extraction, Precipitation and Purification of Protein

Extraction

Various solvents were used to extract the *M.charantia* Linn. The parts of the plant i.e. Leaves and seeds were dried in an open environment at temperature (30-43°C). The dried Leaves, seeds were fine grinded by electrical grinder. The defatted material was then extracted in a soxhlet apparatus with different solvent system (Table No.3). The resulted extract was then filtered with whattman filter paper, and concentrated by evaporation in incubator for 10 hrs at 30˚±5˚c. The obtained homogenate by centrifugation i.e. supernatants of Leaves and Seeds were then precipitated with Ammonium sulfate and used for dialysis thereafter.

Figure 2
All the three Present Figs. Showing the Serial Extraction of Plant Seeds and Leaves (M.Charantia Linn.) Extract.

Table 3
Differential solvent system and their properties used for the Extraction

Solvent /Parameter	Density	Boiling Point	Polarity
Ethanol + Water	0.7893 g/cm3	78.24 ± 0.09 °C	Polar as well as Non-Polar
Petroleum Ether	0.653 g/mL	42–62 °C	Non-Polar
Deionized Water	0.998926 g/mL	100 °C	Strongly Polar

Precipitation

Precipitation had to be determined by the optimal Ammonium Sulphate precipitation conditions using centrifugation as we had placed a volume of cell extract supernatant of 10 ml for seed and leaf in two different test tubes. Addition of maximum amount of solid Ammonium Sulphate to give highest amount of saturation was based on highest temperature and the chemical nature of substance. The sample was then allowed to precipitate for 20 Min., and then centrifuged at 10,000 rpm at 4˚c for 30 min. The pellets then were dissolved in deionized water by gentle vortexing, and allowed to put for dialysis in dialysis bag.

Dialysis

On the basis of principle of diffusion the precipitated sample got dialyzed by using the Dialysis Bag **(**Biotech CE Dialysis Tubing- Part No. 131264; Spectrum Lab), hence the separation of smaller molecules from larger molecules in solution by selective diffusion through a semi permeable membrane was carried out. Dialysis bag was used to simulate a cell membrane which is made up of selectively permeable cellulose tubing, perforated with microscopic pores. The pores were used to facilitate the permeability of 100 Da – 100 kDa molecular weight protein.

Figure 3
The Present Fig. Showing the dialysis bag presentation with Plant Seeds and Leaves (M. charantia Linn.) Extract Sample – at Left Side.

Figure 4
The Present Fig. At right corner of Fig. Showing the process of dialysis of Plant Seeds and Leaves (M. charantia Linn.) Extract sample- at Right.

Assay

Broth Dilution Assay (MIC - Establishing the level of resistance of particular bacteria by present investigated Protein)

The further dialyzed sample from dialysis was then used for the establishment of MIC activity i.e. to check the capability of extracted/dialyzed sample to inhibit the growth of *E.coli.* The assay was carried out as per the Table No.3. As per the table, 9 test tubes were arranged with the full volume of Nutrient Broth, Ampicillin, and *E.coli* sample this may with exception of Growth control and Sterility test tube.

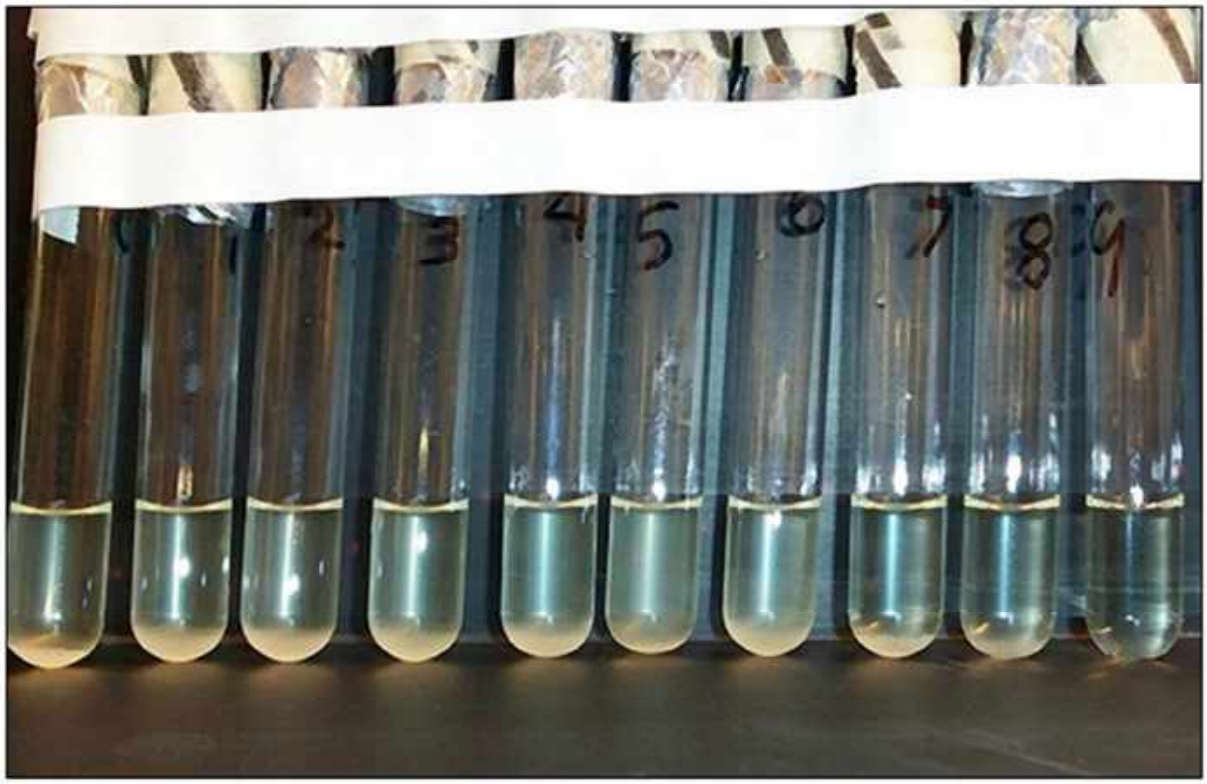


Figure 5
The Present Fig.Showing the Broth Dilution Assay Preparation for MIC Activity.

Table 3
Establishing the level of resistance of particular bacteria by Broth Dilution Assay

Test Sample/Test Tube	1	2	3	4	5	6	7	8	9
N.Broth (mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ampicillin (mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	(No Antibiotic)	(No Antibiotic)
Ampicillin Concentration (µg/mL)	64	32	16	8	4	2	1	Growth Control	Sterility Control
E.coli Suspension (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	NA
Growth obtained (+ or -)	-	-	-	-	-	-	-	+	-

MIC

The present broth dilution assay shows the % inhibition of *E.coli* sample as 64 *µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL & 1 µg/mL respectively*, whereas the Growth control +ve growth of *E.coli*& sterility control shows - ve growth of *E.coli* i.e. the sterility sample is sterile and having none of growth of other microorganism.

Each test tube was examined for the presence or absence of turbidity. The results were recorded in the Table No.3 and indicated the MIC Ampicillin resistance with sample for the *E.coli* bacterial strain.

Protein Characterization

Purification by Column/Gel Filltration Chromatography

Gel filtration (GF) chromatography separates the proteins on the basis of molecular size. It is achieved by using a porous gel to which the molecules, have different degrees of access i.e., smaller molecules have greater access and larger molecules are excluded from the gel column. The stationary phase is composed of a porous gel, and the mobile phase is the buffer that flows in between the gel beads. In this instance of gel chromatography we got separated different fraction of protein in different band pattern by using column of Sephadex G-100 with 0.02 M Sodium acetate buffer (pH.5)[8].

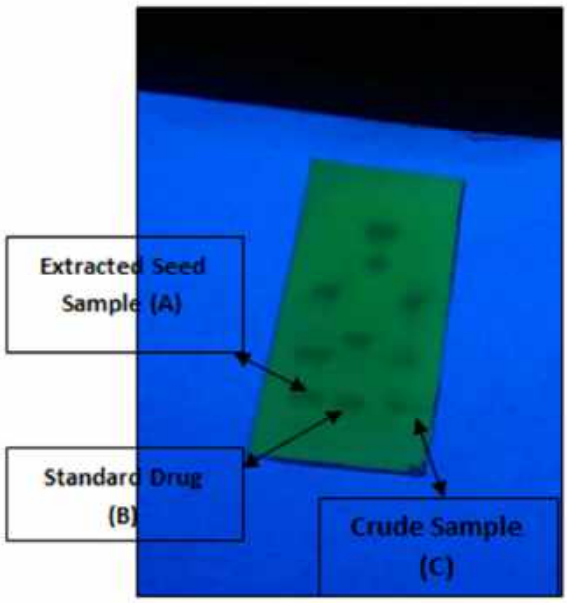
TLC Autography

At the lab level TLC was performed to conform the separation of two or more compounds by the distribution between two phases i.e. one was stationary and other was mobile phase. In these technique we verified the identity of substance & determined the *Rf* value as per given formula;

The *Rf* value was calculated by,

$$Rf = \frac{Distance\ traveled\ by\ Sample}{Distance\ traveled\ by\ Solvent}$$

Distance Travelled by Sample (A)= 6.5 cm
Distance Travelled by Std. Drug (B)= 9 cm
Distance Travelled by Sample (c)= 6 cm
Distance Travelled by Solvent Front (S)=10 cm



$$Rf\ (A,B,C) = \frac{Distance\ traveled\ by\ Sample}{Distance\ traveled\ by\ Solvent}$$

$$Rf\ (A) = \frac{6.5}{10}\quad Rf(A) = 0.65$$

$$Rf(B) = 0.9\quad Rf(B) = \frac{9}{10}$$

$$Rf(C) = \frac{6}{10}\quad Rf(C) = 0.60$$

Comparisons of Rf value for extracted Sample are as follows

Samples/Compound	*Rf* Values
Sample A	0.65
Sample B	0.90
Sample C	0.60

The comparative value stated that the present Sample which is having more *Rf* value ismore polar in nature whereas the sample which is having less *Rf* value is less polar/Non Polar in nature.

SDS page

Once the fractionating band pattern was obtained in gel filtration chromatography, these protein samples i.e. eluted fraction pattern, Crude extract, was dialyzed and precipitated sample was kept for 15% SDS page along with molecular marker ranging from 10.0 kDa to 110 kDa. According to the pattern of molecular marker the separation of unknown protein was found to be almost similar for crude extract, dialyzed and precipitated sample & purified protein sample. Whereas the purified protein obtained from gel filtration chromatography was observed as single band pattern with approx.10.4±0.5kDa.

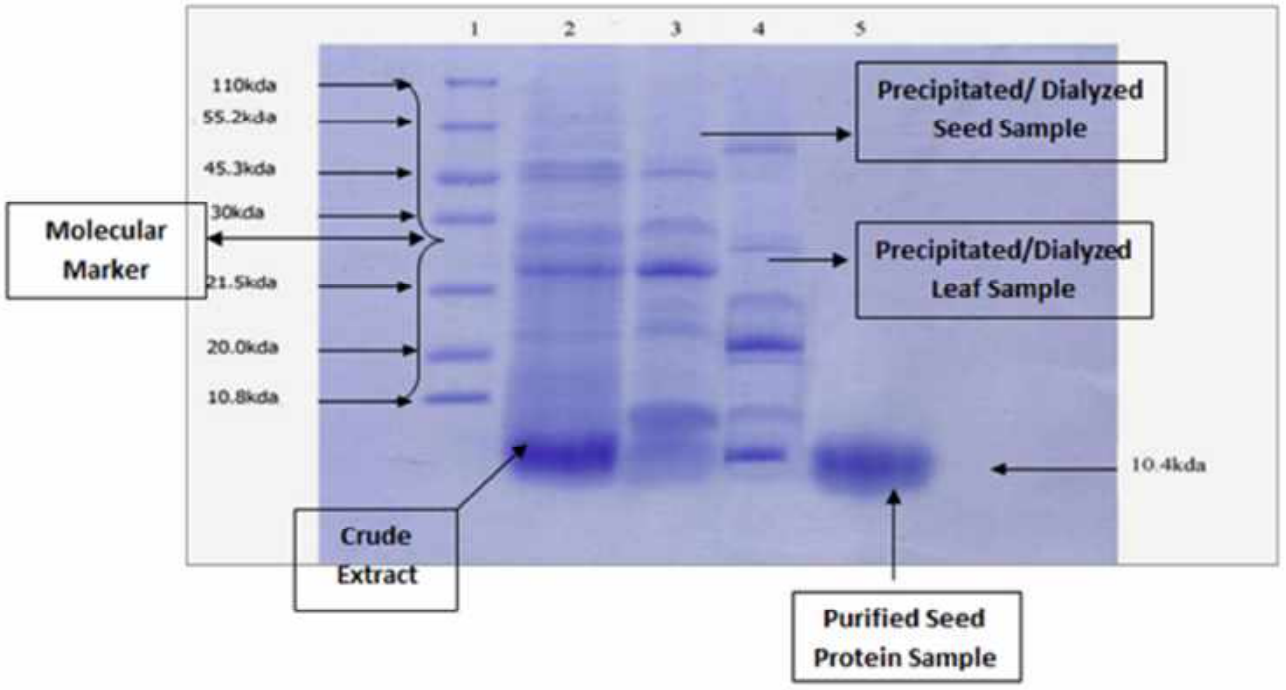


Figure 7
The Resultant View of SDS - PAGE

Protein Concentration Estimation

Bradford Assay

According to the given table, the concentration of unknown protein had been determined as below; The present assay determines the Line of intercept, slope and Unknown protein concentration.

Table 4
Preparation of test samples for the Bradford Protein Assay

Test Sample	Stock Volume, (µL)	Vol. Of Water, (µL)	Vol. of Bradford Reagent, (µL)		U.V Spectra/O.D at 595 nm.
Blank	0	800	200		0.01
BSA Standard (5 µg/mL)	10	790	200		0.224
BSA Standard (10 µg/mL)	20	780	200	**Incubation at R.T For 5 Min.**	0.402
BSA Standard (15 µg/mL)	30	770	200		0.574
BSA Standard (20 µg/mL)	40	760	200		0.730
BSA Standard (25 µg/mL)	50	750	200		0.840
Protein Sample (Unknown)	50	750	200		0.742

****BSA-Bovine Serum Albumin; O.D-Optical Density; µL-Microlitre***

Protein concentration slope of intercept was determined as per the following equation, i.e.

$$Y = mx + b$$

Where,
Y = Absorbance at 595 nm
m= slope, b= origin is 0, and x = Protein Concentration
Determining the line of intercept,
m=Slope

$$m = \frac{X2 - X1}{Y2 - Y1}$$

m= 0.0314

The total concentration of unknown protein (x) is 21.01 µg/mL, Slope is 0.0314and the line of intercept is 0.081.

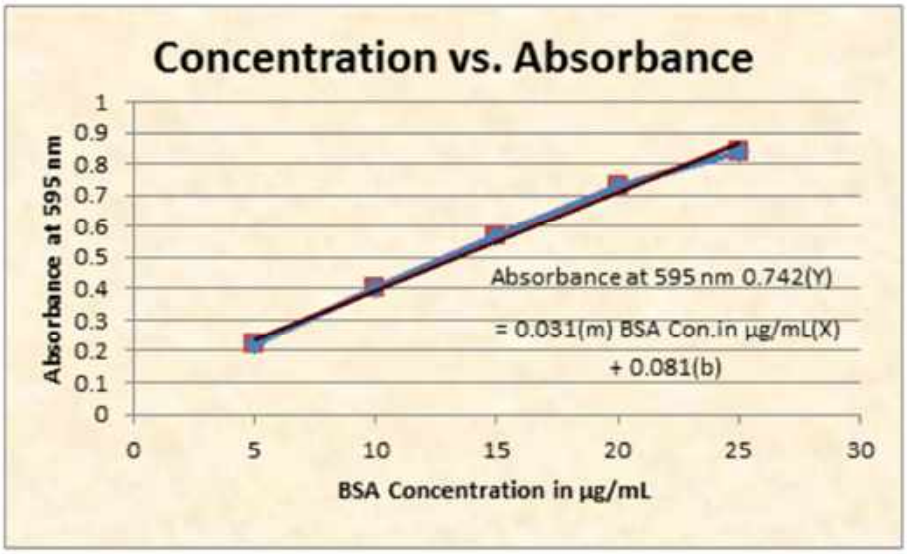


Figure 8
Graphical Representation of Bradford Assay

DISCUSSION

Diabetes mellitus is one of the most common chronic diseases and is associated with hyperglycaemia, hyperlipidemia and comorbidites such as obesity and hypertension.To treat this condition we analyzed the activity of antidiabetic compound, which is discussed as follows

Broth Dilution Assay
Broth microdilution is a reference method used in clinical laboratories. The advantages of the method include considerable savings in media usage requirement of a small quantity of sample, and testing of the susceptibility of multiple microbial at the same time. Moreover, it decreased the intensive labour and time cost compared with agar based method (Luber et al., 2003; Amsler et al., 2010).

TLC Autography
The successful application of the TLC autography technique for separation of solvent extract of *M.charantia* Linn. from complex mixtures makes it promising to further investigations.

SDS page
SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility towards molecular weight as well as higher order protein folding, posttranslational modifications and other factors. The SDS gel electrophoresis of samples having identical charge to mass ratios results in fractionation by the size of the protein.

Protein Concentration Estimation
The data was used for generation of a standard curve and to determine experimental parameters such as limit of linear range, total protein concentration, and line of intercept.Experimentally the standard curve shows line of regression and the equation of standard curve along with the slope obtained i.e. 0.0314. (Fig.8)From all the above mentioned data, it had been discussed that the protein separation, total protein concentration and the protein characterization had been done successfully to achieve the aim of study. Furthermore the animal study would be carried out for the confirmation of antidiabetic and diuretic activity.

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CONCLUSION/APPLICATIONS

1. Side effects of synthetic drugs and inhibitors can be reduced to a certain extent; however, they cannot be completely avoided due to toxic drugs. Therefore the current focus has been shifted towards natural therapeutics.[9] So the use of *M.charantia* as natural therapeutics is more important than synthetic medication.
2. However, the above investigation has concluded that the characterization of antidiabetic protein evaluated by the SDS PAGE, TLC and Gel chromatography shows the comparative relationship between extracted and standard drug compound.
3. The potential of *M.charantia* as natural medicine remains unexplored as the efforts on to explore such medication with an ultimate aim is maintained and restored the vision to use of natural antidiabetic therapy.

CONFLICT OF INTEREST

Conflict of interest declared none.

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