Section A-Research paper

ANTI-DIABETIC PROPERTIES OF *KALANCHOE PINNATA* (LAM.) PERS. IN ALLOXAN INDUCED DIABETIC MICE

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Abstract
The main purpose of the present study was to analyze the antidiabetic activity of methanolic and aqueous extracts of the stem of Kalanchoe pinnata in alloxan-induced diabetic rats. Diabetes mellitus (DM) is a chronic metabolic and endocrine disease regarded as a serious global public health problem. Albino Wistar rats were divided into four groups. Group I (normal) received drinking water throughout the course till 20 days. Groups II-IV received alloxan (120 mg/kg b.w) i.p on the 1st day of the study period. Group III animals received Glibenclamide (10 mg/kg p.o) and Group IV & V received K. pinnata methanolic and aqueous extract (200 mg/kg b.wp.o) for 20 days. The body weight, blood glucose level, serum urea), creatinine, cholesterol, total protein (TP), uric acid, and tissue (liver) antioxidant parameters: Malondialdehyde (MDA) and glutathione (GSH) were measured. K. pinnata treated rats showed the percentage increase in the body weight, decrease in the blood sugar level, creatinine, TP level, urea, uric acid, and MDA, and GSH when compared to alloxan-induced diabetic mellitus control rats. Thus, K. pinnata could be possibly employed to treat DM.This preliminary analysis tested the most promising profile. It seems that methanolic extract of the of K. pinnata stem improved general, blood serum, and liver antidiabetic agent. However, further studies confirming its potential is certainly warranted.

Keywords: Diabetes mellitus, *Kalanchoe pinnata*, Glibenclamide, antioxidant parameters.

Introduction

The Crassulaceae family comprises approximately 33 genera and 1500 species distributed worldwide, except for Australia and the Pacific Islands [1]. This family presents xeromorphic characteristics that allows its species to adapt to bright light and water scarcity [2]. The *Kalanchoe* species are mainly abundant in Madagascar and eastern continental Africa and perform in situ diverse modes of crassulacean acid metabolism (CAM), an ecophysiologically relevant adaptation of photosynthesis [1], [3]. Even though these plants are naturalized in Brazil, they are not endemic[4].Frequently, Kalanchoe spp. occur as exotic or invasive species. Many members of the genus are able to self-propagate from plantlets produced on the leaf margin, making established populations hard to eradicate [5], [6]. The plant is rich in both macro and micro elements, vitamins, calcium, phosphorus, ascorbic acid, inulin [7] and other compounds

like saponin, flavonoids, anthraquinones, xanthones, bryophyllin A and B [8]. Anti-inflammatory, hypoglycaemic, anti-diabetic and anticancer properties have been reported [9].Inthepresentstudy,KalanchoepinnataLam.(Crassulaceae), cultivated as a garden plant, was evaluatedfor its antihyperglycemic action.Kalanchoe pinnatais used astraditional medicine worldwide to treat several ailments. Themajor reported pharmacological activities ofKalanchoepinnataare immunosuppressive action [10], hepatoprotective activity [11], andantitumor activity [12].Kalanchoepinnatais also reported to lower blood glucose [13], [14]. The local people of southernMaharashtra, India are known to consume the leaves ofKalanchoe pinnataas they are believed to possess anti-hyperglycemic activity. The use of traditional medicines in the treatment of diabetes iswidespread. With escalation in the number of diabeticsworldwide, the production of drugs for treatment of diabetesis also on the rise [15]. Most of the currently usedtherapy options suffer from multiple side effects. Theseinclude weightgain and hypoglycemia for insulin,b-cellburnout for secretagogues [16], lacticacidoisis for sensitizers such as metformin [17] and gastric disturbances fora-glucosidase inhibitors[18]. However, in the absenceof better alternatives, they continue to be used. Due to these sideeffects of synthetic remedies, there is a growing interest in useof natural remedies in recent years (WHO, 1980)[19]. More than400 plants worldwide have been documented for the treatmentof diabetes and a majority of them await proper scientific andmedical evaluation [20]. Many times,the exact mechanism of action of the plant remedy used is notknown. Toxicity outcomes are not duly addressed and thecomponents responsible for the bioactivity are not identified.It is, therefore, essential to work on traditional therapies toisolate and identify the exact component, determine themechanism of antidiabetic action. In this study we will emphasis on the antidiabetic activity of different parts of kalanchoe pinnata.

Material and Method

Material

Collection and Authentication of Plant material

The plant part utilised in the study were gathered in the months of March and April from a local market in Gurgaon, Haryana, India. The CSIR-NIScPR, New Delhi, recognized and authenticated the obtained plant material (the entire plant) based on macroscopic and microscopic features. To serve as supplementary references, the voucher specimens have been deposited at our institute's department of pharmacognosy museum.

Pharmacognostic study:

The macroscopic and microscopic study of collected plant parts i.e., flower, stem and roots were done.

(A) Macroscopic study

Organoleptic examination was used to determine the macroscopic characteristics such as colour, odour, taste, form, size, and surface. By preserving samples of flowers and stems in a formalin-acetic acid solution for 48 hours, anatomical examinations were carried out. This substance was then transferred to acetic-alcohol, a 75 percent ethanol and 25 percent acetic acid solution used for long-term storage. The part was cut using a freehand sectioning technique. The permanent

slides were prepared by successive dehydration in ethanol using fast green and double stained safranine methods. Using a binocular microscope, the slides were examined [21]. Similar to this, research was done on the macroscopic properties of *Kalanchoe pinnata* roots.

(B) Microscopic study

Small amounts of fine powder were placed separately on glass slides for powder microscopy stem and root, and two drops of chloral hydrate solution were added to each slide. The powdered substance was then thoroughly blended with chloral hydrate using a tiny needle. Once the glass cover slip was on the slide, the slides were cleaned using the fine blotting paper. The tissues' structures were examined using a binocular microscope [21], [22].

Physicochemical study:

Determination of moisture content/ Loss on drying

10gm of leaf powder was dried at 105^0C in a hot air oven using a pre-weighed porcelain dish to measure the moisture content. The percentage was determined using the initial leaf powder weight.

Preparation of Extracts

Different types of extracts were prepared from the flower, leaves, stem and roots of *Kalanchoe pinnata.*The flowers (100.0 g), the stem (200.0 g) and roots (200.0 g) of *Kalanchoepinnata* (Lam.) were separated, washed with tap water and air dried. The separated components were macerated in 70 percent methanol at room temperature for 7 days with intermittent shaking after drying. Pet ether, ethyl acetate, benzene, chloroform, alcohol, and water were also used in the extraction process. Following that, Whatmann's filter paper No. 1 was used to filter each extract. In a rotary evaporator, the solvent was evaporated at a controlled temperature and reduced pressure (BUCHI Rotavapour R-200, Switzerland) [21], [23].

Extractive Values

The following techniques were used to determine the extractive values of Kalanchoe pinnata's flower, stem, and roots:

Determination of water-soluble extractive value:

100 ml of water was added to 3gm of powdered dried leaf, flower, stem, and root, which was then thoroughly combined. The mixture was heated to a boil on a water bath (100 0C), and then filtered. In a pre-weighed porcelain dish, the filtrate was evaporated and dried at 1050C. The extractive value of water was computed.

Determination of alcohol soluble extractive value:

100 ml of alcohol was mixed with 4 gm of powdered material while it was being shaken, and the mixture was allowed to stand for 16 hours before being filtered. The filtrate was then evaporated in a porcelain plate that had been previously weighed and dried at 105^0C. The extractive value of alcohol was determined [24].

Qualitative phytochemical screening of plant extracts

The presence of different phytochemicals in the plant's extracts, such as Carbohydrates, alkaloids, flavonoids, phenolic compounds, steroids, terpenoids, glycosides, tannins, proteins and saponinsetc.[25], [26].

Quantitative phytochemical screening of plant extract:

The resulting plant extracts were additionally analysed for the quantitative presence of phytochemicals. Each sample's total phenolic and flavonoid content was measured.

Determination of Total Flavanoids

The aluminium chloride method was used to quantify flavonoids in triplicates. In a test tube, 0.5 ml of each quercetin standard (Merck, Germany) (100, 200, 400, 600, 800, and 1000 µg/ml) and plant extract were diluted in 4.5 ml of 70% ethanol before being mixed with 0.3 ml of NaNO2. 0.3 ml of 10% AlCl3 was added and incubated for an additional 5 minutes after the initial 5 min. This was followed by the addition of 2 ml of 1M NaOH and the addition of distilled water to bring the volume up to 10 ml. A spectrophotometer was used to measure the absorbance at 510 nm after 15 minutes of incubation. The standard calibration curve was drawn using the standards' absorbance. In terms of mg of quercetin equivalent (QE)/100 g of dry mass, the total flavonoid content was calculated.

Determination of Total Phenolics

Using the Folin-Ciocalteu reagent (Merck, Germany) method, the total phenolic compounds were quantified in triplicates. As a standard, gallic acid (Merck, Germany) was used to prepare various concentrations, including 10, 20, 40, 60, 80, and 100 µg/ml. After adding 5 ml of distilled water and 0.5 ml of Folin-reagent, Ciocalteu's a test tube was filled with one millilitre of each plant extract and Gallic acid standard. This was combined and left to stand for 5 minutes. Then, 1.5 ml of 20 percent sodium carbonate was added, and the volume was brought up to 10 ml using distilled water. The absorbance of the test and standard was measured at 750 nm in comparison to a reagent blank after two hours of incubation. The standard calibration curve was created using the standards' optical densities. Gallic acid equivalent (GAE)/100 g of dry mass was used to express the total phenolic components in the plant extracts [25], [27], [28].

In Vitro antioxidant study:

Reduction of 1, 1- diphenyl- 2- picryl hydrazyl (DPPH) free radical

This assay was done by the method of Chandel d. et al. (2022) [29]. Ascorbic acid standard solutions at various concentrations (ranging from 50 to 350 µg/ml) were developed. 50 µl of the extract were added to 1 ml of DPPH (0.3 mM in 100 percent ethanol) solution. As a control, a comparable amount of regular phosphate buffer was added to the reaction mixture in place of the extract sample. The reaction mixtures' absorbance was measured at 517 nm after the mixture was agitated and let to stand at room temperature for 30 minutes. Different concentrations' percentage scavenging activities were calculated, and the formula is as follows:

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance}_{control} - \text{Absorbance}_{test} \times 100}{\text{Absorbance}_{control}}$$

ABTS scavenging activity

The scavenging assay by ABTS assay was done according to the method of Miller and Rice-Evans (1997). ABTS radical cation was prepared by mixing 200 µl of potassium per sulfate (70 mM) and 50 ml of ABTS (2 mM). 50 µl of the extract were combined with 1.7 ml of phosphate buffer (pH 7.4) and 0.3 ml of ABTS radical cation. The standard (Ascorbic acid) underwent the

same procedure with varying concentrations (50-500 µg/ml), with water serving as the control. At 734 nm, the absorbance was measured [29] and calculated according to following formula:

Nitric oxide scavenging activity

The ability of the extracts to scavenge nitric oxide radical was determined spectrophotometrically in a Multiskan Ascent plate reader according to a described procedure (Bogucka-kocka A. et al., 2018), with some modifications. The extract and SNP were dissolved in a saline phosphate buffer with a pH of 7.4 to create the reaction mixes in the sample wells. The plates were incubated for 60 minutes at 25°C in the light. The absorbance of the chromophore created during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was measured at 540 nm after the addition of Griess reagent (1 percent sulphanilamide and 0.1 percent naphthylethylenediamine dihydrochloride in 2 percent H_3PO_4) [30].

Superoxide Dismutase scavenging activity

Using a Multiskan Ascent plate reader, antiradical activity was measured spectrophotometrically by observing how the extracts affected the reduction of NBT to the blue chromogen formazan caused by an O_2 free radical at 560 nm. The NADH/PMS system created superoxide radicals. Every component was dissolved in 19 mM of phosphate buffer at a pH of 7.4 [30].

Pharmacological study for antidiabetic activity:

Additionally, the prepared extracts had their anti-diabetic. First, the plant extract's acute oral toxicity was identified.GivenProtocol ID: SIP/IAEC/PCOL/10/2022 was approved by animal and ethical committee.

Acute toxicity study

According to OECD recommendations, Kalanchoe pinnata's acute oral toxicity was assessed using the technique described by Saravanan V. et al., 2021 [31].

Animals

Wistar strain young adult animals (8 to 12 weeks old) in good health were employed in the investigation. The females were declared nulliparous and incapable of becoming pregnant. The vendor disclosed the animals' state of health. The animals were all confirmed to be alive and in good health. The animals were individually kept in polypropylene cages with stainless steel top grills for a six-day acclimatisation period. The animals were given sterile rice husk bedding. Except when fasting was required, purified drinking water and typical laboratory foods (Rodent pellets) were offered ad libitum throughout the experiment. The animals were maintained under controlled environmental conditions at a temperature of 22°C (± 3°C) and relative humidity of 36-64% with 12 h light and 12 h dark cycle. Individual animals were identified by tail marking with unique numbers.

Acute oral toxicity

The Organization for Economic Cooperation and Development's methods were followed in order to evaluate acute toxicity in a limit dose test (OECD 423). 6 female mice were used in a limit test at one dose level (2000 mg/kg body weight) (3 animals per step). Rats who had fasted the night before received a single oral dosage of the ethanolic leaf extract of K. pinnata. 3 hours after the

dose, food was discontinued. Each animal was watched closely after dosing at least once in the first 30 minutes, several times in the first 24 hours, with extra focus on the first four hours, and then every day for the following 14 days. Animals' individual weights were measured both before and after each weekly administration of the test drug. Gross necropsies were performed on all test animals. Gross pathological alterations were noted for each animal, if any [31].

Anti-diabetic activity in rats

In the experimental study, albino Wistar rats of any sex weighing around 200± 10 g were employed. Standard laboratory setups were used to care for the animals. All of the animals had unlimited access to free rat food and water. All experiments were conducted in accordance with the Institutional Animal Ethical Committee's regulations (registration number 233/CPCSEA).

Induction of diabetes in rats

Normal albino male for this investigation, 200 ± 10 g Wistar rats served as the subject. After 18 hours of fasting, alloxan (120 mg/kg ip) dissolved in normal saline was administered intraperitoneally to Groups II–V of the rats, while Group I (the vehicle control) received only the normal saline. The regular diet (20% glucose solution for 12 hours to prevent first drug-induced hypoglycemia mortality) and unlimited water were given to the rats. Fasted blood glucose levels were calculated using a glucometer and the tail-tip method 72 hours after an alloxan injection. Only diabetic rats were utilised in the experiment, defined as those with blood glucose levels greater than 150 mg/dL [32].

Anti-diabetic activity

There were five kinds of animals used for anti-diabetic activities. Up to day 20, the regular group (Group I) got only normal saline. On the first day of the trial period, Groups II–IV received alloxan (120 mg/kg b.w.) intravenously. Glibenclamide (10 mg/kg p.o.) was administered to group III animals starting on the fourth day for a total of 20 days. Group IV received K. pinnata extracts beginning on day 4 (200 mg/kg b.w. p.o.) for 20 days. For the one touch glucometer blood glucose estimation of the 0th, 10th, and 20th days, fasted blood samples were taken from the tail vein. Throughout the 20-day experimental period, each rat's daily food and water intake as well as any irregular variations in the individual animals' body weight were tracked [32]. The study plan was depicted in table 1.

Table 1: Anti-diabetic study plan

Groups	Treatments
Group I	Normal control (Normal saline)
Group II	Diabetic control (Alloxan, 120 mg/kg b.w)
Group III	Diabetic+ glibenclamide (10 mg/kg b.w)
Group IV	Diabetic+ Methanolic Extract (200 mg/kg b.wp.o)
Group V	Diabetic+ Aqueous Extract (200 mg/kg b.wp.o)

Twice daily oral administration of Glibenclamide or its fractions was given to the animals, whilst control rats were given a vehicle to eat. 3.65 mg/kg body weight was determined to be the extract's ED50.

Estimation of Parameters

Fasting Blood Sugar

Rats receiving treatment had their fasting blood glucose levels measured using ACCU-CHECK. From the tail tip, blood was collected.

Biochemical Analysis

Following 20 days, the rats were given light ketamine anaesthesia at a dose of 40 mg/kg body weight intravenously 24 hours after the last dosing regimen. The blood was then removed from the retro-orbital plexus using a capillary tube. In Eppendorf tubes, 1 ml of blood was drawn from each animal in all the groups. The collected blood was centrifuged at a low speed for 20 minutes at 2000 rpm after being incubated upright for 30 to 45 minutes to aid in coagulation. Another Eppendorf tube was used to collect the supernatant serum, which was then tested for turbidity. For the estimation of various biochemical parameters, clear serum was employed. Total triglycerides were determined using a liquid gold test, glycated haemoglobin, serum cholesterol, and high-density lipoprotein cholesterol were all assessed using biochemical kits from Crest Biosystems, and serum insulin was determined using an insulin ELISA kit from CalBiotech. ThermoScientific'sMultiskan EX ELISA microplate reader was used to measure the amount of insulin released at 450 nm. An alkali technique was used to assess liver glycogen levels. Using the Erba Diagnostic kit, the serum creatinine level was calculated. Using an autoanalyzer, the absorbance of the standard and test were read against a blank at 505 nm or 505-670 nm, and the concentration displayed on the autoanalyzer was noted down. Using the Erba Diagnostic kit, the determination of serum total protein uric acid was also performed [32], [33].

Euthanasia and liver homogenate preparation

According to CPCEA guidelines, all test animals were killed by thiopental sodium anaesthetic overdose (150 mg/kg b.w.i.p.). Only the liver was taken out right away, and it was washed with ice-cold physiological saline. The liver was divided into small pieces and homogenised with an RMS tissue homogenizer in a 0.1M tris-HCl buffer at pH 7.4 to produce a 10 percent homogenate. This homogenate was utilised to estimate the antioxidant properties (malondialdehyde [MDA] and GSH).

Estimation of lipid peroxidation

The amount of lipid peroxidation was calculated using the assay mixture method. 0.2 ml of 8.1 percent sodium dodecyl sulphate, 1.5 ml of 20 percent acetic acid solution, 1.5 ml of 0.8 percent thiobarbituric acid aqueous solution, and 0.2 ml of post-mitochondrial supernatant (10 percent weight/volume) of homogenate made up the reaction mixture. After being diluted to 1 ml with distilled water, 5 ml of the 15:1 v/v combination of n-butanol and pyridine was added, and the mixture was centrifuged, the mixture was brought up to 4 ml with distilled water and heated at 95°C for 60 minutes. The organic layer was removed, and using a semi-autoanalyzer, its absorbance at 532 nm was evaluated in comparison to a blank.

Estimation of GSH

The Ellman's method was applied to the assay combination. 2.4 ml of a 0.002 M EDTA solution was added to 0.1 ml of tissue homogenate and maintained on an ice bath for 10 minutes. Then,

0.5 ml of 50% trichloroacetic acid and 2 ml of distilled water were added. The mixture was kept in an ice bath for 10 to 15 minutes, followed by a 15-minute centrifugation at 3000 rpm. After adding 2 ml of tris-HCl buffer and 0.05 ml of DTNB solution (Ellman's reagent) to 1 ml of supernatant, the reaction was completed. Using a semi-autoanalyzer, an immediate yellow hue was created and read against a blank. Extension coefficient $13.6 \times 103\ M^{1}\ cm^{-1}$ was used to calculate GSH content [32]. The values are expressed as units/mg protein.

$$GSH = \frac{\text{Absorption at 412 nm}}{\text{Extinction coefficient} \times TP \times \text{total volume}}$$

Histopathology

The portion of the liver was treated for the histopathological observation.

Results

In the present work, different parts of Kalanchoe pinnata plant viz., flower, stem and roots were investigated as very few studies have been conducted on these plant parts. The plant material was collected and authenticated byCSIR-NIScPR, New Delhi.Initially, macroscopic and microscopic investigation of different parts of Kalanchoe pinnata plant was done.

Macroscopic study

Macroscopic analysis showed that flowers were pinkish red, soft and tubular; leaves were green, non-aromatic, bitter, simple, pinnate and fleshy. These are hanging in large panicles and opposite stout branches. Further, the corolla is usually swollen and shaped octagonal at the base. The calyx of the flower can be observed to be tubular and brownish or purplish in color, which can be 3.5 to 4 cm long. The base of the flower is usually colored pale green and has triangular teeth. The stem was greenish purple nonaromatic, astringent, round and glabrous. The stem changes color, depending on its age. Older stems are characterized as light colored, while younger stems are usually reddish with white speckles. Also, the stems are usuallybranched and smooth, which are also tall and hollow. Meanwhile, the leaves have an ovate or elliptical shape, and a crenated or serrated leaf margin. The leaves also contain anasymmetric base, a glabrous leaf surface, and a long petiole. In addition, the color of the leaves depends on the side of the epidermis,i.e., upper epidermis is usually coloured dark green while lower epidermis is usually lighter. The leaves are also characterized witha distinct odor and having a bitter taste.The fruit of the Kalanchoe pinnata plant are usually enclosed by the calyx and corolla that are characterized as papery.Moreover, the seeds of the species are distinguished as smooth and oblong-shaped. The root has dark brown in colour, odorless, bitter in test,varying in size.

Results for histological study of flowers, stem and roots are shown in Figure 1. Histological study of the flowers revealed the presence of upper and lower epidermis, spongy cells and color pigmentswhich determines the color of the plant’s flower.Transverse section of stem showed that there isan outer layer of thick-walled epidermis coveredwith cuticle. Beneath the epidermis, there was athree-layered hypodermis made up ofsclerenchymatous cells. The inner cortexconsisted of thin-walled parenchymatous cells,which were loosely arranged with deposition ofstarch grains. In the middle region, vascularbundles were arranged in a ring. Xylem elementswere mainly in the form of tracheids with fewvessels of xylem parenchyma fibers. Pith regionconsists of

parenchymatous cell in the centershowed the deposition of starch grains andcalcium oxalate crystal.The xylem of the stem is like the characteristics of the herbaceous dicots, i.e., usually in the form of tracheids withsome parenchymal fibers. Meanwhile, the pith of the stem of the species is characterized by thedeposition of starch grains andcrystals made from calcium oxalate (CaC_2O_4)and is composed of parenchymatous cells.The transverse section of root shows Vascular bundles, Epidermis, Endodermis Cortex, cork etc.

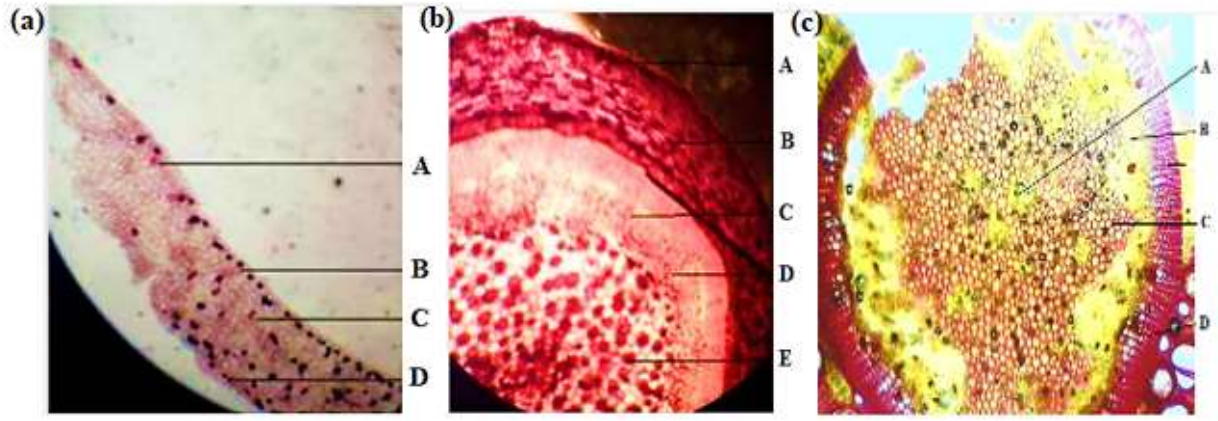

Figure 1:(a) Histology of Flower. A: Upper epidermis, B:Color pigment C: Spongy cell, D: Lower epidermis; (b) Histology of Stem. A: Epidermis, B: Cortical region, C: Phloem. D: Xylem, E: Pith;(c)Transverse section of Kalanchoe pinnata root. A: Phloem, B: Cortex, C: Xylem, D: Cork

Microscopic study

The powder microscopy of stem indicates the presence of spongy parenchyma with chlorophyll, annual vessel, xylem vessel, a fibre attached to parenchyma cell, epidermal cell, cork cell with fibres, epidermal cells with fibre, patches of fibre, cork cell, pitted vessel and spiral vessels (Figure 2).

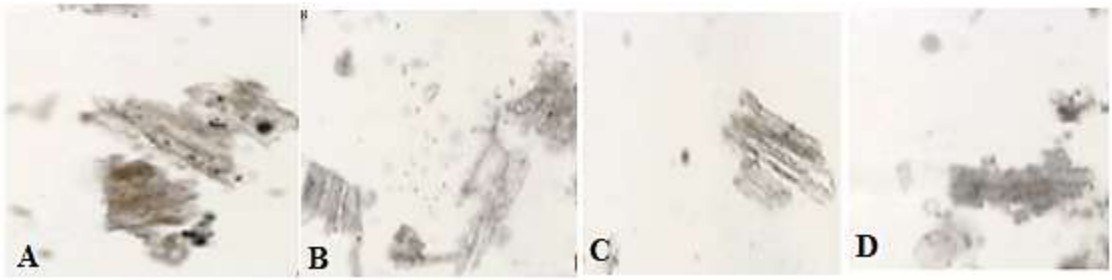

Figure 2: Powder microscopy of *Kalanchoe pinnata* stem. A: Cork cell with fibre, B: Epidermal cells with fibre, C: Patches of fibre, D:annular and spiral vessel

The powder microscopic study of *Kalanchoe pinnata* roots indicates the presence of Calcium oxalate crystals, fibres, cork cells and xylem vessels (Figure 3).

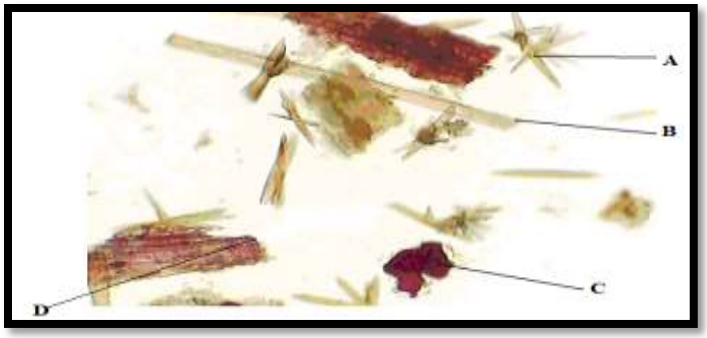

Figure 3: Powder characteristic of *Kalanchoe pinnata* root. A: Ca oxalate crystal, B: Fibres, C: Cork cells, D: Xylem vessel

Physico-chemical study of Kalanchoe pinnata plant parts

Ash Value: Total ash, acid insoluble ash and water-soluble ash values

For the proper identification of plant, physicochemical parameters (moisture content (LOD), extractive values and ash values) provide useful information. Moisture content of drugs could be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. These can serve as a valuable basis of information and provide suitable standards to establish the quality of this plant material as future prospects. In case of stem, moisture content was found to be 1.25% in *Kalanchoe pinnata*which was the lowest one. An ash values are used to decide quality and purity of crude drug, it indicates presence of various impurities like, silicate, oxalate and carbonate. The water-soluble ash is used to determine the quantity of inorganic compounds present in drugs. The acid insoluble ash helps to estimate the amount of silica present in the material. The total water-soluble portion of the ash is considered as water soluble ash. Total ash obtained with stem sample was 8.5% in which 0.3% of ash was acid insoluble whereas 2.15% of ash was water soluble. The physical parameters of flower and root sample showed LOD-5.6% & 11%, Total ash-10.3%&11.8%, Acid insoluble ash-1.5%&2.5%, Water soluble ash-3.7%&4.2%, respectively. Physical parameters were presented in table 1. Less amount of these parameters in stem indicated that the inorganic matter and silica were less in Kalanchoe pinnata stem.

Table 1: Physical Parameters of *Kalanchoe pinnata*plant parts

Parameters	Flower	Stem	Root
LOD	5.6%	1.25%	11%
Total ash	10.3%	8.5%	11.8%
Acid insoluble ash	1.5%	0.3%	2.5%
Water soluble ash	3.7%	2.15%	4.2%

Preparation of Extracts

Different types of extracts were prepared from the flower, stem and roots of *Kalanchoe pinnata*. Reddish brown (flowers), greenish black (stem) and dark brown (roots) extracts were obtained.

Extractive Values

Extractive values of flower, stem and roots of Kalanchoe pinnata were determined. Alcohol soluble extractive value of stem was found greater (31.8%) than alcohol soluble extractive value

(25.5%) which means alcohol soluble extract contains morecomponents. Water soluble extractives obtained from flower & roots were 7.6%& 9.5% while alcohol soluble extractive value was about 10.7%& 12.5% from flower & roots, respectively. The results were summarized in table 2.

Table 2: Extractive values of different parts of *Kalanchoe pinnata*

Parameters	Flower	Stem	Root
Water soluble extractive value	7.6%	25.5%	9.5%
Alcohol soluble extractive value	10.7%	31.8%	12.5%

The different plant parts were further treated with different solvents and their extractive values were determined. The successive solvent extractive values were shown in table 3a-3c.

Table 3a: Successive solvent extractive values and nature of Kalanchoe pinnata Flower extracts

Solvents	Colour	Consistency	Extractive value (%w/w)
Petroleum ether	Reddish brown	Sticky mass	0.48%
Ethyl acetate	Reddish brown	Sticky mass	0.28%
n-hexane	Brown	Semisolid mass	1.05%
Chloroform	Brown	Sticky mass	0.99%
Methanol	Brown	Sticky mass	4.13%
Aqueous	Reddish brown	Sticky mass	3.81%

Table 3b: Successive solvent extractive values and nature of Kalanchoe pinnata Stem extracts

Solvents	Colour	Consistency	Extractive value (%w/w)
Petroleum ether	Greenish black	Sticky mass	0.59%
Ethyl acetate	Greenish	Sticky mass	0.31%
n-hexane	Greenish	Sticky mass	1.12%
Chloroform	Greenish black	Sticky mass	1.08%
Methanol	Greenish black	Sticky mass	4.27%
Aqueous	Brown	Sticky mass	3.57%

Table 3c: Successive solvent extractive values and nature of Kalanchoe pinnata Root extracts

Solvents	Colour	Consistency	Extractive value (%w/w)
Petroleum ether	Blackish brown	Semisolid sticky	0.68%
Ethyl acetate	Brown	Sticky mass	0.35%
Benzene	Brown	Sticky mass	1.41%

Chloroform	Chocolate brown	Sticky mass	1.18%
Methanol	Dark brown	Sticky mass	4.62%
Aqueous	Yellowish brown	Sticky mass	3.91%

The highest extractive value was obtained with stem sample (methanolic extract) of Kalanchoe pinnata among the all three parts used for the study.

Qualitative phytochemical screening of plant extracts

The presence of various phytochemicals viz., alkaloids, flavonoids, phenolic compounds, steroids, terpenoids, glycosides, tannins, saponins, proteins, gum & mucilage and volatile oil etc. in the extracts from the plant. The results of preliminary phytochemical analysis were shown in table 4a-4c.

Table 4a: Preliminary phytochemical analysis of Kalanchoe pinnata flower extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	+	-	-	-	+	+
Carbohydrates and glycosides	-	-	+	-	+	+
Phytosterols	-	+	+	+	+	-
Fixed oils and fats	+	+	+	-	+	-
Phenolic compounds and tannins	-	-	+	+	+	+
Saponins	-	-	-	-	+	+
Flavonoids	+	-	-	-	+	+
Gums and mucilage	+	-	+	+	+	+
Proteins	-	-	+	+	+	+
Volatile oils	-	-	-	+	+	+

+, present, -, absent

Table 4b: Preliminary phytochemical analysis of Kalanchoe pinnata stem extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	-	-	-	-	+++	++
Carbohydrates and glycosides	-	+	+	-	+++	+++

Phytosterols	-	+	+	+	+	-
Fixed oils and fats	+	+	+	-	-	-
Phenolic compounds and tannins	-	-	+	+	+	+
Saponins	-	+	-	-	+	+++
Flavonoids	+	-	-	-	+++	++
Gums and mucilage	+	-	+	+	-	+
Proteins	-	-	+	+	+++	+++
Volatile oils	-	-	-	-	+	-

+, present, -, absent

Table 4c: Preliminary phytochemical analysis of Kalanchoe pinnata root extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	-	-	-	-	+	+
Carbohydrates and glycosides	-	+	+	-	+++	++
Phytosterols	+	+	+	+	++	-
Fixed oils and fats	-	+	+	-	++	++
Phenolic compounds and tannins	-	-	-	+	++	+
Saponins	-	-	-	-	-	+
Flavonoids	-	-	-	-	+++	++
Gums and mucilage	-	-	-	-	-	+
Proteins	-	-	+	+	+	+
Volatile oils	-	-	-	-	+	-

+, present, -, absent

From the phytochemical screening of the different extracts obtained from different plant parts, it was observed that the methanolicand aqueous extracts obtained from stem sample showed

presence of phytoconstituents in appreciable quantity. Hence, these two extracts were evaluated for quantitative phytochemical parameters.

Quantitative phytochemical screening of plant extract:

The resulting plant extracts were additionally analysed for the quantitative presence of phytochemicals. Each sample's total phenolic and flavonoid content was measured.The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. TPC of methanolic and aqueous extract of Kalanchoe pinnata stem showed the content values of 4.5473 and 2.5292, respectively. The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. The total flavonoids estimation of methanolicand aqueous extracts of Kalanchoe pinnata stem showed the content values of 3.519 and 1.2863, respectively (Table 5).

Table 5: Estimation of total phenolics and total flavonoids content in Kalanchoe pinnata stem

S. No.	Kalanchoe pinnata Extracts	Total phenolic content (mg/100mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	**Methanol**	4.5473	3.519
2.	**Aqueous**	2.5292	1.2863

The above results showed that aqueous extract contain less phenolic and flavonoids content than the methanolic extract. It may due to the solubility of principle contents presence be higher in case of alcoholic solvent, thus it has been accepted that it is a universal solvent for the extraction of plant constituents. These extracts were further evaluated for anti-oxidant and anti-diabetic activity.

Anti-oxidant activity of *Kalanchoe pinnata* extracts

An agent that prevents the consumption of oxygen is referred to as an antioxidant. Antioxidants act as metal chelating agents, enzyme inhibitors, electron givers, hydrogen donors, peroxide decomposers, radical scavengers, and hydrogen donors. Antioxidants also act as protectors of our body against a polluted environment or free radicals.For all selected fractions, IC50 (µg/mL) values were determined, indicating the concentration of each fraction required to reduce 50% of the radicals under the test conditions or causing mortality of half of the cells in culture. Based on the obtained values, both tested fractions possessed antioxidant activity(Table 6). Protective capacity against superoxide was established for both extracts, but the most pronounced effect was observed for the stem methanolic extract (IC_{50} = 61.7 ± 0.50 µg/mL).The study also revealed that K. pinnata stem extract possesses significant antioxidant and oxidative radical scavenging activities. In the DPPH method, IC_{50} value for stem extract was found to be 101.5±0.11 µg/mL and 275±0.20 µg/ mL for nitric oxide radical–scavenging activity. The IC50 value in ABTS study was found to be 49.97±0.23 for stem methanolic extract. Ascorbic acid was taken as standard drug and it showed best anti-scavenging activity against free radicals.

Table 6: Comparison of inhibitory concentration (IC_{50}) values of methanolic extracts of different parts of *Kalanchoe pinnata*; expressed in µg/mL

IC_{50} (µg/mL)				
Compound	**DPPH**	**Superoxide**	**Nitric oxide**	**ABTS**
Ascorbic acid	15.85±0.28	48.5±0.19	110±0.15	32.16±0.38
Kalanchoe pinnata (Stem methanolic extract)	101.5±0.11	61.7 ± 0.50	275±0.20	49.97±0.23
Kalanchoe pinnata (Stem Aqueous extract)	125.8±0.15	74.3± 0.50	329±0.13	57.11±0.15

Pharmacological study for antidiabetic activity:

From the results obtained from phytochemical and anti-oxidant study, it was revealed that methanolic extract showed significant results and thus, selected for further study. Further, the prepared stem extracts were evaluated for anti-diabeticactivity. Before proceeding further, the acute oral toxicity of the plant extract was determined.

Acute toxicity study

Methanol and aqueous extracts of K. pinnata was checked for lethal dose 50 (LD_{50}) by using OECD guidelines (423 guidelines). The LD_{50} of methanol and aqueous extracts was found to be 2500 mg/kg.

Anti-diabetic activity in rats

Anti-diabetic activity of methanolic and aqueous extracts of Kalanchoe pinnata stem was assessed as these two fractions have represented satisfactory in-vitro results. Albino Wistar rats of any sex weighing about 200±10 g was used in the experimental study. Alloxan injection was given to the animals to induce the diabetes. After 72 h of alloxan injection, fastedblood glucose levels were estimated by the tail-tip method usingglucometer.

Estimation of Parameters

Fasting Blood Sugar and body weight

The antihyperglycemic activity of solvent fractions of the stem was evaluated using the oral glucose tolerance test (OGTT).In Table 7, the alloxan-induced DM rats have shown remarkable decrease in the body weight on the 20th day when compared to normal vehicle group rats (Group I). Groups III, IV& Vdiabetic rats treated with glibenclamide, Methanolic and aqueous extracts showed an increase in the body weight level when compared to Group II DM control rats. The efficacy of methanolic extract in increasing the body weight wascomparable to the standard.

The alloxan-induced DM rats (Group II) have shown significant increase in the blood glucose level on the 20th day when compared to normal vehicle group rats (Group I). Groups III, IV and V DM rats treated with glibenclamide, methanolic and aqueous extracts have shown significant decrease in the blood glucose levels when compared to Group II DM control rats.The remaining

fractions, viz., pet ether and aqueous fractions, were not able to reduce blood glucose levels as compared to glibenclamide control and the methanol fraction group. Hence, further studies were carried out using the methanol fraction.Kalanchoe pinnata stem methanol fraction showed very good antihyperglycemic activity in a dose-dependent manner.

Table 7: Effect of solvent fractions isolated from Kalanchoe pinnata on blood glucose levels

Groups	Blood glucose (mg/dl)		
	0 min.	60 min.	120 min.
Group I (Normal control, Normal saline)	78.5±1.7*	105.9±2.2**	95.6±3.5**
Group II (Diabetic control, Alloxan, 120 mg/kg b.w)	248.7±1.2	558.3±1.3	465.2±2.7
Group III (Diabetic+ Glibenclamide-10 mg/kg b.w)	241.3±2.1*	366.5±3.8*	281.4±1.9**
Group IV (Diabetic+ Methanolic Extract-(200 mg/kg b.wp.o)	251.7±3.7*	327.1±2.4**	266.5±4.3**
Group V (Diabetic+ Aqueous Extract-200 mg/kg b.wp.o)	233.8±3.5*	536.5±4.6	419.2±2.5

Values are mean±SD; n=6. *p<0.05 and **p<0.001 with respect to diabetic control

Biochemical Analysis

The changes in blood glucose, glycated hemoglobin, serum insulin, liver glycogen and lipid profile of long term treated rats are shown in Table 8. After treatment for 45 days, it wasobserved that Kalanchoe pinnata stem methanolic fraction significantly reduced the fasting blood glucose level as compared to diabetic control. At a low dose of 200 mg methanol fraction/kg body weight, there was a reduction in fasting blood glucose levels than aqueous extract in the similar concentration. There was improvement in serum insulin level and liver glycogen level and decrease in glycated Hb in treated rats as compared to the diabetic controls. After a 45 days prolonged treatment, the levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were significantly reduced, whereas the level of high-density lipoprotein (HDL) was significantly increased in rats treated with methanol extract/kg body weight. The improvement in HDL and decrease in LDL, which is very pronounced as compared to diabetic, would have important significance in prevention of cardiovascular diseases (CVD).

Table 8: Effect of *Kalanchoe pinnata*stem methanol& aqueous fraction on biochemical parameters

Parameters	Normal control	Diabetic control	Glibenclamide control	Kalanchoe pinnata stem methanol extract	Kalanchoe pinnata stem aqueous extract
0day FBG (mg/dl)	75 ± 3.6**	206 ±1.4	217 ±4.2*	239 ±2.5*	248 ± 4,5*
45th day FBG (mg/dl)	83 ±5.4**	346 ±3.1	112 ± 2.5**	105 ±2.7**	139 ± 10**
GHb (%)	5.11 ± 4.9**	13.65 ±0.29	7.5 ± 1.8**	9.47 ± 1.15	11.61 ±2.6*
Serum insulin (**µ**IU)	14.2±2.6**	5.09±1.52	12.3±1.5**	12.5±1.9**	9.4±4.8**
Liver glycogen (mg/g of tissue)	4.31±3.1*	2.26±5.6	3.99±4.52*	4.48±3.3*	3.53±4.3*
TC (mg/dl)	74.9±2.4*	127.5 ±3.1	98.2±3.9**	95.4± 4.8*	115.6±5.5**
TG (mg/dl)	71.5± 4.9**	122.3± 5.9	87.5±5.3**	97.1±3.8*	99.0 ± 5.9**
HDL-C (mg/dl)	42.5±3.6**	25.7±5.1	38.3±3.0	35.0 ± 2.5	29.2 ± 4.2*
LDL-C (mg/dl)	14.8±3.5*	76.5±2.9	39.7± 1.2**	36.2±4.4**	65.5 ±3.2**
VLDL (mg/dl)	12.8± 2.0**	23.7± 2.6	16.8± 1.7**	18.2± 4.7*	24.5± 3.9**

Fasting blood glucose (FBG), glycated hemoglobin (GHb), triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). Values are mean ± SD; n ¼ 6. *p<0.05 and **p<0.001with respect to diabetic control.

Tissue (liver) parameters - lipid peroxidation (MDA) and reduced GSH

In Table 9, the MDA and GSH values have shown that the alloxan-induced DM rats (Group II) have shown significant rise in the liver tissue lipid peroxidation and decrease in the reduced GSH levels on the 20th day when compared to normal vehicle group rats (Group I). Groups III, IV& V DM rats treated with glibenclamide and methanolic and aqueous extracts have shown significant decrease in the liver tissue lipid peroxidation levels and increase in the reduced GSH levels when compared to Group II DM control rats.

Table 9: Effect of MDA and GSH in normal, DM and treated DM

Group number	Group name	MDA nm/mg Protein	GSH units/mg protein
I	Normal control (Normal saline)	648.42±5.78	14.7±1.83
II	Diabetic control (Alloxan, 120 mg/kg b.w)	1258.72±4.6	12.54±3.8
III	Diabetic+ glibenclamide (10 mg/kg b.w)	973.65±3.9	18.51±2.5
IV	Diabetic+ Methanolic Extract (200 mg/kg b.wp.o)	985.83±4.1	35.37±3.3
V	Diabetic+ Aqueous Extract (200 mg/kg b.wp.o)	127.26±2.8	44.64±5.1

Histopathology study

Normal vehicle group rats liver histology illustrated in figure4A and expressed prominent nuclei of the hepatocytes, central artery, sinusoids, Kupffer cells, canaliculi, hepatic ductules, etc. Inflammatory cells infiltration was not observed. Alloxan administrated rats in figure 4Bresulted in the disruption of hepatocytes, steatosis to steatohepatitis, and liver fibrosis; morphological, ultrastructural lesions were observed in the lobules of the liver.

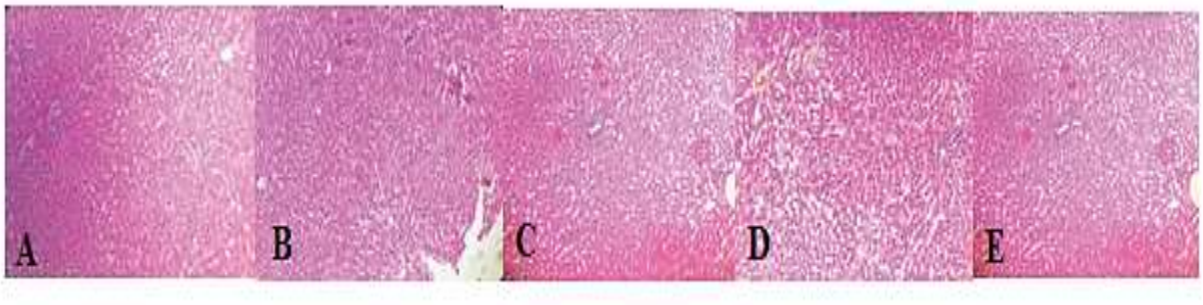

Figure 4: A-Normal liver; B-Diseased liver; C-Glibenclamide treated liver; D-Methanol extract; E-Aqueous extract

DM rats administered with glibenclamide (Group III) (figure 4C) and Methanolic and aqueous extracts (Group IV & V) in Figs. 4D & 4E have shown significant protection to the hepatocyte and other structural components of the liver. Only mild disruptions were observed in Groups III, IV and V rats.

Discussion

DM is characterized by hyperglycemia and abnormal increase in the lipid profile including TC, triglycerides, low-density lipoproteins (LDL), very LDL, and other lipoproteins. The alloxan-induced DM rats were used in the present study. DM rats (Group II) have shown significant decrease in the body weight when compared to the normal vehicle group (Group I). Alloxan-induced DM caused a significant loss in body weight while treatment with stem methanolic extract restored the body weight. This effect of methanolic extract may be due to its prevention effects on glycogenolysis, lipolysis, and gluconeogenesis. This leads to the prevention of muscle wasting and loss of tissue protein. These results confirm the efficacy of methanolic extract in restoring body weight in alloxan-induced DM rats. The important phytoconstituents (including flavonoids) present in MLEKP are believed to lower the blood glucose level by promoting the glucose into the tissue cells, by stimulating the enzymes needed for the glycogenesis, and by increasing the expression of insulin receptors. Oral administration of methanolic extract produced significant beneficial effects in the lipid profile (serum TC and triglyceride) of the alloxan-induced DM rats significantly reducing serum cholesterol and triglyceride levels. These results suggest that stem methanolic extract might be considered as a substitute for drugs to reduce complications associated with DM. An increase in blood creatinine may indicate kidney dysfunction. The alloxan-induced DM rats have showed elevated levels of serum creatinine. Methanolic extract administered rats have shown significant decreased levels, suggesting protection to the kidney in alloxan-induced DM rats. The alloxan-induced DM rats have shown significant increase in the lipid peroxidation (MDA) due to oxidative stress. DM rats treated with

methanol extract have shown significant decrease in the level of lipid peroxidation, indicating the effect of stem methanolic extract in preventing the oxidative stress caused by alloxan in rats. The alloxan-induced DM rats have shown significant increase in the GSH due to oxidative stress. DM rats treated with stem methanolic extract have shown significant increase in GSH level, indicating the effect of the extract in preventing the oxidative stress caused by alloxan in rats.DM rats administered with glibenclamide (Group III) and methanolic and aqueous extract (Group IV &V) have shown significant protection to the hepatocyte and other structural components of the liver. Only mild disruptions were observed both in Groups III and IV rats.

Conclusion

We conclude that the methanolic extract of the stem of K. pinnata has potent antioxidant and antidiabetic effect in alloxan-induced diabetic mellitus rats. The flavonoid and tannins isolated from other antidiabetic medicinal plants have been found to stimulate secretion of insulin-like effect. Further, pharmacological investigations are needed to elucidate the mechanism of the observed antidiabetic activity. The present investigation has also opened avenues for further research, especially with reference to the development of potent formulation for DM from methanolic extract of the stem of *K. pinnata*.

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