Original Article

In vivo and *in vitro* Hyperglycaemic Screening of *Adenocalymma alliaceum* Miers, A Promising Herb

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ABSTRACT

Background: *Ajos sacha* is a shrubby herb and has attracted scientists' attention due to its antioxidative, antineoplastic, larvicidal, and antibacterial properties. Hence, Ajos sacha assessed *in vitro* and *in vivo* diabetes protective properties. **Materials and Methods:** The methodology started with the quantitative phytochemical estimation of ethanolic extract of *Adenocalymma alliaceum* (EEAA). Then, the extract was studied for cytotoxicity, glucose utilization, and GLUT-4 redistribution. *In vivo* study, comprising six groups of Wistar rats. Different parameters of diabetes, antioxidant properties, and lipid profile were performed. **Results:** Phytochemical screening demonstrated positive results in phenolic compounds, tannins, and flavonoids. The EEAA found a minimal level of toxicity, with no dosages tested causing 50% cell death. EEAA had shown GLUT-4 redistribution assay in a dose-dependent manner on cell line procedure. After NOAEL on acute toxicity, glucose level, metabolic symptoms, serum creatinine, serum urea, and lipid profile were normal under EEAA treatment, and bodyweight, high-density lipoprotein, and protein levels increased. The liver homogenate included more antioxidative enzymes such as glutathione, catalase, and superoxide dismutase. The histopathological analysis also reveals EEAA's ability to protect pancreatic β-cell from damage. **Conclusion:** The current investigation found the blossoms of Ajos sacha to restore glucose abnormalities in the body.

Keywords: β-cell, Alloxan monohydrate, *Adenocalymma alliaceum*, Glucose utilization, GLUT-4 redistribution, Diabetes Mellitus.

INTRODUCTION

Diabetes mellitus (DM), an endocrine disease affected by glucose imbalances in human beings, is linked with insulin regulation.[1] DM is the principal cause of death and an influential risk factor for various diseases like COVID-19 and autoimmune diseases. 90% of the world's population has Type 2 diabetes mellitus (T2DM), with the remaining 10% having Type 1 diabetes mellitus (T1DM). Besides this, a woman during pregnancy also develops the gestational type of DM but recovers after successful delivery.[2] Women who had gestational diabetes were more liable to develop T2DM than women who did not have gestational diabetes. People with type 1 and type 2 diabetes, both known and untreated, now number 463 million, up from 151 million in 2010. Furthermore, by 2045, this population will increase by 700 million.[3] Rising diabetes globally has multiple reasons, including overweight, obesity, diet, lifestyle factors, physical activity, smoking, alcohol intake, genomics, and gene environmental interactions. Furthermore, patients with long-term diabetes are more likely to develop atherosclerosis and peripheral and cerebrovascular disorders.[4] ocular manifestations, nervous system abnormalities, kidney issues, and peripheral neuropathy. Many therapies were developed to care for diabetes, but few were found to be efficient. These include gene therapy, therapy targeting β-cells, their regeneration, stem cells of bone marrow, enhanced self-replication of β-cells, and pharmacological

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approaches.[5] Many clinical trials are also conducted for antidiabetic drugs. However, the utility of synthetic antidiabetic medicines is challenging due to cost, sub-maximal efficacy, and several related adverse effects such as Vit-B_{12} deficiency and heart failure. In undeveloped and emerging countries, it is also challenging to afford hospitalization. However, we need to find a long-term solution to many disorders. A rigorous scientific search with essential data to help health professionals eliminate flaws linked with therapies is necessary to achieve this goal. Hence, researchers shifted to utilizing plants as a permanent solution to take care of diabetes economically with fewer adverse effects. Data shows that developing countries treat nearly 90% of their diabetic patients with plant and plant-derived products. India has an Ayurvedic traditional medicine system that dates back thousands of years. The WHO also reported that there are about 21,000 medicinal plants worldwide. India is the richest, with over 2,500 medicinal plant species.[6] *Adenocalymma alliaceum* Miers plant is reported for its analgesic, antipyretic, antiallergic, antimalarial, and antipneumatic properties.[7] So plant also has a principal function on free radicals. So the plant is expected to counteract and improve glucokinase enzyme, free radicals formation, and disruptions in intracellular calcium homeostasis coordination in pancreatic functions. Henceforth, we aimed to study *Adenocalymma alliaceum* Miers for *in vitro* and *in vivo* study of DM.

MATERIALS AND METHODS

Chemicals, Reagents

NCCS in Pune, India, provided the INS-1 cells. Sigma Aldrich, Bangalore, provided the Eagle's minimal essential medium (EMEM), MTT (3-(4, 5-dimethylthiazol-2yl)-2, and 5-diphenyl tetrazolium bromide. Bangalore-based Sigma Aldrich (PBS) gave the fetal calf serum (FCS) and phosphate-buffered saline (PBS). Each chemical used in the studies was analytical grade and came from the Kolkata-based Sigma Aldrich. Each chemical employed in the alloxan-induced diabetes model was from reliable suppliers and laboratory quality.

Plant Material

Flowers of Ajos sacha were collected in March and April. Prof. K. Prabhu identified the plant, Department of Pharmacognosy, given the herbarium number SCSCOP. Ph.Col Herb. 008/2017-18.

Experimental animals

Rat weighing 150-200 g was chosen for the study. The rats were housed at a temperature of 25±5°C in the animal house at SSUTMS in Sehore on a 12-hr light and dark cycle. The experiment was done with the IAEC's official permission 1587/P.O./Re/S/11/CPSEA.

Preparation of Extract

Extraction of Ajos sacha was carried out based on solvent's polarity. The solvents used were petroleum ether, chloroform, alcohol, and water. A Buchner funnel and Whatman No. 1 filter paper were used to filter the extracts. To yield, the extracts were frozen at -40°C and dried for 48 hr in a freeze drier. The dried extracts were kept at -4°C before reconstituting in DMSO for bioactivity tests.[8]

Phytochemical Screening

The EEAA was screened using a standard procedure to identify phytoconstituents such as alkaloids, carbohydrates, flavonoids, glycosides, tannin, protein, steroids, and phenols.[9-10]

Quantitative Analysis

Total Phenol Content

In a 10 ml glass tube, aliquots of the EEAA were diluted to a volume of 3 ml. Then, 2 ml Na_2CO_3 (20%) and 0.5 ml Folin ciocalteau reagent (1:1) were added to each tube. The blue color's intensity produced in each tube was directly proportional to the phenol level. The blue color in the tube is caused by a complicated redox reaction between phenols and phosphomolybdic acid in the Folin ciocalteau reagent in an alkaline media, which results in the creation of molybdenum blue. The test solutions were warmed for 1 min, then cooled before measuring absorbance at 650 nm. The calibration curve was made with catechol. The plant's phenol content was expressed as an mg equal to phenol per gram of extract.[11]

Total tannin content

Prior to quantifying tannin content, the sample was tested for tannins using the traditional $FeCl_3$ and Gelatin assays. In a 100 mL flask, 0.1 g of dry plant material was placed, 50 mL of water was added, and the mixture was boiled for 30 min. After filtration, the solution was transferred to a 500 ml flask using a cotton filter, and water was added to the 500 ml mark. Finally, 10 mL of water was added to 0.5 mL of aliquots, 1 mL of 1% $K_3Fe\ (CN)_6$ and 1 mL of 1% $FeCl_3$, and 0.5 mL of aliquots were transferred to vials. After five minutes, the solutions were spectrophotometrically examined at 720 nm. The exact tannin concentration was measured using the optical absorbance values obtained for the standard solutions in 5-30 g/10 ml.[12]

In vitro study of EEAA

Maintenance of cell culture

All cell cultures were incubated at 37°C in a humidified % CO_2 environment. Every 2-3 days, The INS-1 cells were grown in RPMI medium with 5% fetal calf serum. After reaching 90% confluence, all cell lines were sub-cultured.

Cytotoxicity assay

Cytotoxicity assessment was tested based on the procedure given by Sagbo.[8] with minor modifications. The INS-1 cells were plated onto 96-well plates with an approximate count of 8000 cells per well dissolved in a growth medium, with the final volume being made up to 100 µl. The cells were left on the plate overnight to allow them to adhere to it. Following overnight incubation, the plated cells were treated with 100 µl of plant extract at various concentrations (50 µg/ml, 100 µg/ml, and 200 µg/ml). 10% DMSO was taken as the positive control. Following treatment with plant extracts, the 96 well plates were incubated at 37°C for roughly 2 days. After 48 hr, the spent medium was aspirated out of the wells containing the cells, and 100 l of EMEM media with roughly 10% Fetal calf serum and 0.5 g/ml MTT solution was added. The MTT solution was prepared by dissolving 25 mg MTT, with the final volume being made up of a 50 ml complete culture medium. Following the addition of MTT, the 96 well plates were incubated for approximately 3 hr at 37°C. The spent media was removed after 3 hr, and MTT crystals formed (purple formazan) were dissolved in DMSO (200 µl/well) to solubilize the crystalized formazan particles. The absorbance was recorded at 540 nm using a microplate reader. The cytotoxicity was reported as a percentage of control (medium only), considered zero.

Glucose utilization assay

The test was performed in INS-1 cells using van de Venter's standard technique with minimal changes. Nineteen cells were subjected to light exposure of 0.25% Trypsin in phosphate-buffered saline to break their adhesion. The adhered cells were counted and suspended in a growth medium after dislodged. The freshly suspended cells were plated into a 96-well culture plate at a density of 6000 cells per well. The newly planted cells were then allowed to adhere for three days before being grown at 37°C in a humidified incubator with 5% CO_2. Two rows of the 96 well plates were left cell-free to act as a negative control. After three days of seeding, 10 µl of extract at 12.5 g/ml, 25 g/ml, and 50 g/ml were applied to the medium without aspirating it. Metformin, a standard antidiabetic medicine, was used as a positive control at a 0.1 g/ml concentration. The 96 plate wells were cultured for two days after treatment. After 48 hr, the media was sucked, and a 25-liter incubation buffer (RPMI medium was further diluted with phosphate buffer saline, 0.1 percent bovine serum albumin, and eight millimolar glucose). The 96 well plates were incubated for 3 hr at 37°C. After 3 hr, 10 µl of the incubation material was transferred to a new 96-well plate. Finally, 200 µl of glucose oxidase reagent (SERAPAK Plus, Bayer) was added to a new 96 well plate with 10 µl of added incubation solution to measure glucose concentration. This phase was incubated for 15 min at 37°C, after which the absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Lab Systems). The mean difference between the cell-free and cell-containing wells of the 96 well plate was used to calculate the amount of glucose the cells used. In addition, the proportion of glucose utilization in the untreated controls was determined.

GLUT-4 redistribution assay

The GLUT-4 redistribution assay was carried out in the agonist mode to assess the efficacy of the plant extracts in facilitating glucose uptake. The experiment was performed based on published protocol with minor modifications.[13] The entire experiment was carried out on a 96-well plate. Before the beginning of the experiment, the assay buffer Was pre-warmed to 37°C control, and test samples were diluted to a final concentration of 4X with the DMSO component of the preparation not going over 0.5% to prevent toxicity. The assay buffer was prepared using Ham's F12 with L-Glutamine, 10 mM HEPES, and 1% Penicillin-Streptomycin The control was prepared according to the supplier's protocol based on the values mentioned in the table below. 50 µl of control or compound solution diluted to 4X in Assay Buffer were added to appropriate wells of the 96 well cell-seeded plate. The plate was incubated at 37°C, in a 5% CO_2 environment, with a 95% humidity incubator. The incubation was carried out for 5 min. Following the incubation, the cells were fixed gently by removing the buffer and adding 150 µl Fixing Solution per well. The fixing solution was prepared using 10% formalin, neutral-buffered solution (approximately 4% formaldehyde) diluted with an equal volume of 96% ethanol. The fixing process was carried out for 20 min, following which the cells were washed four times with 200 µl of phosphate buffer saline to remove any trace of fixing solution. The phosphate buffer saline was decanted, and 100 µl. 1 µM Hoechst Staining Solution was added. The plate was then sealed with a black plate sealer and incubated at 37°C for 30 min before imaging (Table 1).

Table 1: Assay Procedure.

Group	Assay Buffer	Control Stock	DMSO	4X Concentration	Final assay concentration	Final DMSO concentration
Negative control	12 ml	----	60 µl	0.5% DMSO	----	0.31%
Positive control	12 ml	12 µl insulin	60 µl	200 nm insulin	50 nM insulin	0.31%

µl: microliter; µm: micrometre; ml: millilitre; nm: nanometre; DMSO: Dimethyl sulfoxide.

In vivo studies

Acute toxicity study

Dose selection was made using OECD TG-423. The method enabled a judgment for dividing the test substance into a series of toxicity classes defined by fixed LD_{50} cut-off values.[14]

Alloxan-induced diabetic model

To test the hypothesis, a single i.p. dose of 120 mg/kg alloxan monohydrate in sterile saline. After five days of alloxan administration, the rats with blood glucose levels greater than 250 mg/dl were classified as hyperglycaemic and segregated. A total of six animals were added to each of the six groups, which consisted of normal control, diabetic control, standard group, low dose (100 mg/kg), medium dose (250 mg/kg), and high dose (500 mg/kg) animal groups, the subcutaneous method of administration was chosen for induction of diabetes. On the other hand, the extract was administered via sterile oral gavage. Blood glucose levels were determined 12 hr after overnight fasting on the 0th, 1st, 7th, 14th, and 21st days using the tail blood withdrawal technique. On the 21st day, Animals were sacrificed, followed by cardiac puncture under mild ether anesthesia, and then blood samples were used for biochemical estimations.[15]

Effect of EEAA on body weight in diabetic rats

From the commencement of the trial through the 21st day, the animal's body weight was measured and reported, followed by the technique stated.[16]

Effect of EEAA on Polyphagia, Polydipsia, and Polyuria

The urine output of the animals was measured by keeping them in their metabolic cage for 24 hr a day to collect urine excretion.[17] The daily food intake rate was computed for each animal category. Every day, the animals were fed the same amount of food. Regular water consumption was measured using a calibrated feeding bottle with nozzles. The daily water information was confirmed by deducting the amount of water left in the water bottle at the end of 24 hr of provisioning from the starting amount at the beginning of the day. Then the amount of food consumed was calculated.[18]

Effect of EEAA on biochemical markers

Biochemical parameters such as creatinine, urea, serum protein, and lipid profile were measured.[19,15]

Effect of EEAA on antioxidants properties

By extracting the liver and homogenizing it, researchers were able to assess free radical scavenging properties such as Glutathione (GSH), Catalase (CAT), and Superoxide dismutase (SOD).[20]

Histopathological studies

The pancreases were removed on the 21st day from six groups of animals. The isolated organs were kept in a 10% formalin preparation. The organ investigation utilized eosin, hematoxylin staining, and the Acmas technology microtome to slide 5m thick tissue sections. At 400 magnifications, histopathology examinations were performed using an Olympus binocular.[21]

Statistical analysis

The average standard error (n=6) was used to obtain the results. Graph Pad InStat Software was utilized, including one-way analysis of variance (ANOVA) and the Tukey test comparison for significance. Compared to the normal control and diabetes control groups, a P-value of less than 0.05 was judged statistically significant.[22]

RESULTS

Preparation of extract and Properties of the Flower of the Plant

The successive Soxhlet extraction process of the *Adenocalymma alliaceum* Miers flowers yielded 1.18% brown and sticky petroleum ether extract, 1.89% yellowish-black, and non-sticky chloroform extract, 22.60% of the blackish-brown sticky ethanolic section, and 23.10% of brown and non-sticky aqueous extract. The results are expressed in Table 2.

Table 2: The percentage yield of successive soxhlet extraction.

Sl. No.	Solvent	Colour and Consistency	Percentage yield
1.	Pet. Ether	Brown and sticky	1.18%
2.	Chloroform	Yellowish-black and non-sticky	1.89%
3.	Ethanol	Blackish-brown and sticky	22.60%
4.	Aqueous extract	Brown and non-sticky	23.10%

Pet. Ether: Petroleum Ether; **%:** Percentage.

Table 3: Pharmacognostic screening of EEAA.

Types of phytochemical constituents	Petroleum ether extract	Chloroform extract	Alcoholic extract	Aqueous extract
Alkaloids	–	–	–	–
Carbohydrates	–	+	+	++
Flavonoids	–	–	+++	+++
Glycosides Cardiac Anthraquinone Coumarine	 + – –	 + – –	 + – +	 + – +
Tannins	–	–	+++	+
Proteins	–	+	+	+
Steroids	+	+++	–	–
Phenolic compounds	–	–	+++	+

– Absent ++ More clarity + Indies presence response +++ Better.
EEAA: Ethanolic extract of *Adenocalymma alliaceum*.

Preliminary phytochemical screening of the *Adenocalymma alliaceum* Miers

Pharmacognostic screening of *Adenocalymma alliaceum* Miers flowers showed that steroids are present in petroleum ether and chloroform extracts but absent in the other two extracts. Carbohydrates are present in chloroform, ethanol, and aqueous extracts. Cardiac glycoside was present in all sections. In ethanolic and aqueous extracts, coumarin glycosides, tannins, and flavonoids. The ethanolic part of the extract includes a larger concentration of polyphenol components, including flavonoids and tannin, which was quantitatively observed, so this extract was designated for future study. The results are compiled in Table 3.

Quantitative Analysis

Quantitative determination of total polyphenolic and tannins content

The total phenol and tannin content of a 70% ethanolic and aqueous extract of *Adenocalymma alliaceum* flowers

Table 4: Total phenol and tannin contents of alcoholic extract and Aqueous extract.

Sl. No	Name of secondary metabolite	Absorbance at	Mg equivalent	
			70% ethanolic Extract	Aqueous Extract
1.	Phenol	650nm	268.64mg/g	156.32mg/g
2.	Tannin	700nm	223.20mg/g	156.30mg/g

nm: nanometer; **mg/g:** milligram/gram.

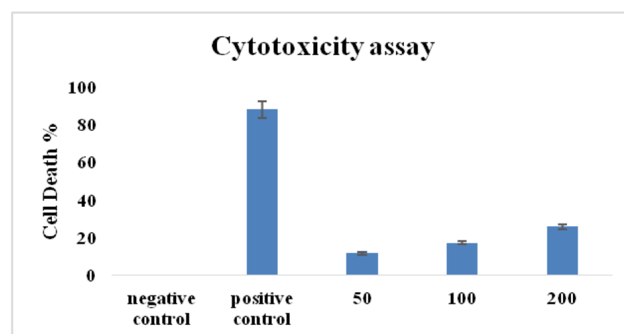

Figure 1: Cytotoxicity assay.

was 268.64 mg/g, and 156.32 mg/g expressed as catechol, 223.20 mg/g, and 156.30 mg/g expressed as tannic acid, respectively, as shown in Table 4.

In vitro study of EEAA

Cytotoxicity results

It was observed that the plant extracts have no significant cytotoxicity. While the positive control exhibited almost 88% cell death, the highest concentration of the plant extract (200 μg/ml) exhibited approximately 26% cell death. While the two other concentrations of plant extracts, 50 μg/ml, and 100 μg/ml, exhibited cell death of approximately 11% and 17%, respectively (Figure 1).

Glucose Uptake study

The plant extracts exhibited significant enhancement in glucose utilization at the investigated concentrations. While the glucose utilization was not as significant as metformin which is widely prescribed as an antidiabetic drug, it was significant. The two lower concentrations of the extract, -12.5 μg/ml and 25 μg/ml exhibited similar enhancement in glucose utilization of 54% and 53%, respectively. There was no significant difference between these two concentrations. However, the extract with a 50 μg/ml concentration demonstrated a glucose utilization of 68% (Figure 2).

Glut 4 redistribution assays

Metformin restored the transporter activity to almost 94% efficacy. Even though not as effective as metformin,

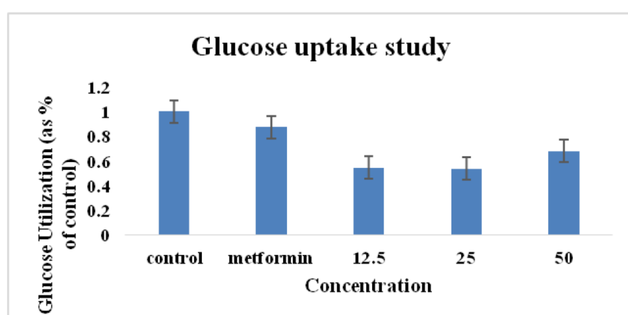

Figure 2: Glucose uptake study.

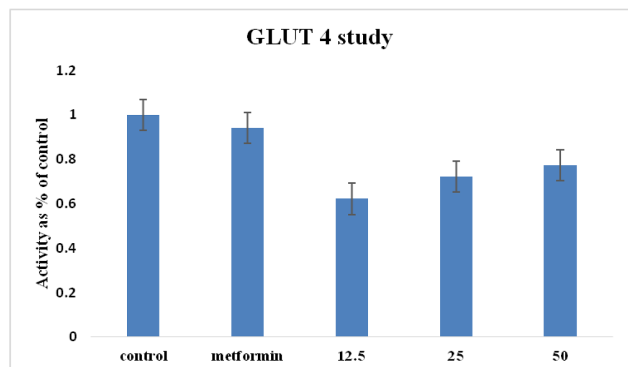

Figure 3: GLUT 4 study.

the plant extract follows a somewhat dose-dependent pattern in restoring transporter activity. The 12.5 μg/ml extract had glut4 restoration of 62%, while 25 μg/ml and 50 μg/ml had glut4 restoration activity of 72 and 77, respectively. This indicates that the extracts exhibited pharmacological effect by the GLUT4 restoration pathway (Figure 3).

In vivo study of EEAA

Acute toxicity

Mice were taken to carry out an acute toxicity study. EEAA was administered orally using oral gavage. No mortality or fatal was found during acute toxicity up to 2000 mg/kg per OECD TG-423. As per the limit test, 1/10, 1/25, and 1/50 of maximum tolerated doses were selected for EEAA. Hence our therapeutical dose has chosen 100 mg/kg, 250 mg/kg, and 500 mg/kg.

Effect of EEAA on fasting blood glucose levels

All the animals survived the treatment period. Alloxan monohydrate treated DC animal's serum glucose level was raised. On the other hand, the standard group experienced a significant decrease in glucose levels. The extract reduced elevated glucose levels at the trio's doses from the 1st day to the 21st day than diabetic control. The high dose group shows the effective result till 21st day than the diabetic control, low dose group, and median dose group. However, EEAA was found to be less significant than the Standard group. The results are shown graphically in (Figure 4).

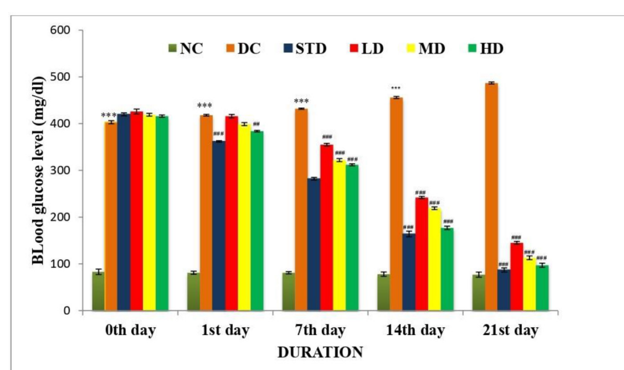

Figure 4: Effect of EEAA on fasting blood glucose level.

Effect of EEAA on Body weight

Marked falls in the mass of animals were more observed in the diabetic control group than in the normal control group. Glibenclamide-treated animals gained weight from the beginning to the end of the experiment. At low dose, animals earned their weight was moderately significant ($^{\#\#}P<0.01$) than the DC group on the 7th, 14th, and 21st day. However, it was less effective than the standard. At the median dose, animals gained significantly higher weight on the 1st, 7th, 14^{th,} and 21st day ($^{\#\#\#}P<0.001$) than the diabetic control group. At a high dose, EEAA showed high significance ($^{\#\#\#}P<0.001$) on the 1st, 7th, 14th, and 21st day and moderate effectiveness ($^{\#\#}P<0.01$) on the 0th day. The body weights earned at the high dose were closed to the Standard group. The results are listed in Table 5.

Effect of EEAA on Polyphagia, Polydipsia, and Polyuria

Throughout the study, there was high consumption of food observed in the diabetic control group (21.55±0.39) than the normal control group (14.3±0.70). High dose (15.00±0.36) showed emerged increased consumption of food than ($^{***}P<0.001$) diabetic, median dose (16.90±0.37) and low dose (16.60±0.40).

There were differences amongst groups based on water intake, but Glibenclamide (13.50±0.47) succeeded in restoring normal conditions. At the same time, EEAA shows dose-dependent results. At a low dose, median dose, and a high dose gives (18.20±0.61), (16.60±0.60), and (14.90±0.60), respectively. The high dose of *Adenocalymma alliaceum* Miers showed high significance ($^{\#\#\#}P<0.001$) than diabetic control and was close to the Standard group.

The values found in normal control were in a range of (10-15 ml/d). There was a distinct increase in urine

Table 5: EEAA on Body weight.

Groups	Bodyweight of the animal (gm)				
	0th day	1st day	7th day	14th day	21st day
Normal Control	180.16 ± 5.83	195.50 ± 3.35	200.83 ±2.38	210.66 ± 4.66	212.50 ± 5.50
Diabetic Control	170.50 ±3.35	162.00±1.82***	142.16 ±1.30	131.00±2.25***	95.50 ± 2.14***
Standard (Glibenclamide 10 mg/kg) + Alloxan	165.66±3.07###	173.83±1.53###	179.83±2.89###	193.33±6.00###	196.16±4.40###
EEAA (100 mg/kg) + Alloxan	155.66 ± 5.11	156.66 ±3.67#	161.00±2.70###	164.16 ±2.38##	169.00 ±3.00##
EEAA (250 mg/kg) + Alloxan	160.83 ± 3.00#	162.16±3.20###	168.33±3.33###	170.00±2.88###	176.66±4.01###
EEAA (500 mg/kg) + Alloxan	165.50 ±2.50##	168.83±1.53###	172.50±2.14###	178.33±3.67###	183.33±4.41###

All the values are mean ±SEM (*n*=6). **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to Normal control. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 as comparing to Diabetic control; **EEAA:** Ethanolic extract of *Adenocalymma alliaceum*.

Table 6: Effect of EEAA on Polyphagia, Polydipsia, and Polyuria.

Groups	Polyuria (ml/d)	Polyphagia (gm/d)	Polydipsia (ml/d)
Normal Control	15.00 ± 0.49	14.3 ± 0.70	11.00 ± 0.53
Diabetic Control	21.10 ± 0.80***	21.55 ± 0.39***	20.30 ± 0.49***
Alloxan + Standard (10 mg/kg)	14.80 ± 0.53###	15.20 ± 0.48###	13.50 ± 0.47###
Alloxan + EEAA (100 mg/kg)	18.40 ± 0.47#	16.60 ± 0.40	18.20 ± 0.61
Alloxan + EEAA (250 mg/kg)	17.60 ± 0.40###	16.90 ± 0.37	16.60 ± 0.60###
Alloxan + EEAA (500 mg/kg)	16.50 ± 0.40###	15.00 ± 0.36###	14.90 ± 0.60###

All the values are mean ± SEM (*n*=6). **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to Normal control. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 as comparing to Diabetic control; EEAA: Ethanolic extract of *Adenocalymma alliaceum*.

Table 7: Effect of EEAA on biochemical markers in alloxan-induced Diabetes in rats.

Groups	Serum creatinine (mg/dl)	Serum urea (mg/dl)	Serum protein (mg/dl)	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Normal control	0.46±0.01	30.50±2.30	14.16±0.94	0.52±0.01	102.50±5.86	15.33±0.66	47.16±1.47
Diabetic control (Alloxan 120 mg/kg)	1.22±0.05***	82.00±3.05***	4.83±0.60***	1.49±0.05	173.66±5.94	4.16±0.47***	77.33±2.80***
Standard group (Glibenclamide 10 mg/kg)	0.59±0.02###	53.00±2.12###	11.00±0.57###	0.64±0.05###	125.16±5.75###	13.66±0.71###	50.16±2.92###
EEAA (100 mg/kg)	0.85±0.04###	66.66±1.82###	7.50±0.42	0.97±0.03###	141.33±6.11##	8.83±0.65##	68.16±1.24
EEAA (250 mg/kg)	0.74±0.02###	57.16±2.44###	8.50±0.67##	0.88±0.02###	136.16±5.14###	10.50±0.42###	63.83±2.63##
EEAA (500 mg/kg)	0.63±0.02###	52.83±1.64###	10.83±0.60###	0.66±0.03###	132.83±4.33###	12.66±1.22###	56.16±2.452###

All the values are mean ± SEM (*n*=6). **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to normal control. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 as comparing to diabetic control; **EEAA:** Ethanolic extract of *Adenocalymma alliaceum*; **TG:** Triglyceride; **TC:** Total cholesterol; **HDL:** High density lipoprotein; **LDL:** Low density lipoprotein.

drainage in diabetic control (21.10±0.80), which is highly significant (****P*<0.001) in comparison to normal control (15.00±0.49), Standard group (14.80±0.53), low dose (18.40±0.47) and high dose (16.50±0.40). After standard, high dose reduced urine output (###*P*<0.001) compared to diabetic control. Results are expressed in Table 6.

Effect of EEAA on Biochemical markers

The diabetic control group showed marked elevation of serum urea, serum creatinine, serum cholesterol, total triglyceride and fall of HDL, and serum protein than the normal control group, standard, high dose, medium dose, and low dose. Whereas high dose, median dose, and low dose were able to restore elevated serum urea, serum creatinine, total cholesterol, and triglyceride to (52.83±1.64), (0.63±0.02), (132.83±4.33), (0.66±0.03), and HDL and serum protein to (12.66±1.22),

Table 8: EEAA on tissue GSH, CAT, and SOD level.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GSH (nm/mg protein)
Normal control	51.56±2.36	2.35 ± 0.13	345.33 ± 5.35
Diabetic control	26.83±2.48***	0.45 ± 0.02*	227.66 ± 7.35***
Alloxan + Standard (10 mg/kg)	45.16±3.29###	1.98 ± 0.09*	333.16 ± 8.31###
Alloxan + EEAA (100 mg/kg)	36.50±2.18	0.61 ± 0.03	263.50 ± 4.49##
Alloxan + EEAA (250 mg/kg)	42.66±1.97###	0.84 ± 0.05#	274.83 ± 3.34###
Alloxan+ EEAA (500 mg/kg)	44.83±1.51###	1.55 ± 0.07###	321.16 ± 6.37###

All the values are mean ± SEM (*n*=6). **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to normal control #*P*<0.05, ##*P*<0.01, ###*P*<0.001 as comparing to diabetic control; **EEAA:** Ethanolic extract of *Adenocalymma alliaceum*; **SOD:** superoxide dismutase; **GSH:** Glutathione; **CAT:** Catalase.

(10.83±0.60), respectively as than the diabetic control group. The high dose of extract possessed a highly significant result (###*P*<0.001) for diabetic control and was close to the standard group. Results are expressed in Table 7.

Effect of EEAA on antioxidants properties

During the treatment period, glutathione, catalase, and superoxide dismutase were lowered in the DC. GSH, CAT, and SOD levels were increased in a graded manner from a high to a low dose of extract. The result of the high dose was close to that of the standard group. The high dose group was highly significant (###*P*<0.001), then the alloxan treatment group alone. The results are expressed in Table 8.

Histopathological studies

The normal control (Figure 5 A) photomicrograph shows normal acini and cellular population. Diabetic control implies extensive damage to Langerhans's islets, reduced dimensions of islets, distinct interstitial cell separation, and neutrophil infiltration (Figure 5. B). Standard suggests restoring the normal cellular size with hyperplasia, reduced interstitial cell separation, and less neutrophil infiltration (Figure 5. C) EEAA at 100 mg/kg, 250 mg/kg, and 500 mg/kg, trios restored pancreatic damaged β-cells in islets of Langerhans, interstitial cell separation, and inflammatory infiltration graded way (Figure 5. D, E, and F).

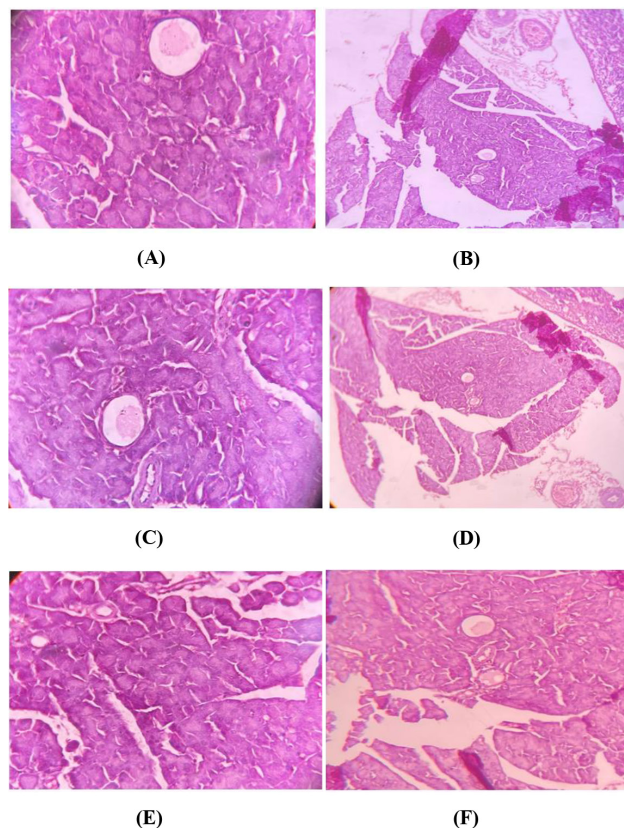

Figure 5: Histopathology of (A) Normal control, (B) Diabetic control, (C) Standard, (D) Low dose, (E) Median dose, and (F) High dose. The experimental parameters of histopathological findings were based on β-cell damages and their count.

DISCUSSION

Our study aimed to investigate the antidiabetic property of *Adenocalymma alliaceum* Miers against hyperglycaemia. Diabetes becomes a household disease due to abnormal lifestyles and food habits. However, developed, developing, and underdeveloped countries face the problem of DM. It was proclaimed that rehospitalization is due to the impersonal indication of the diabetic patient.[23]

Adenocalymma alliaceum Miers was selected based on a literature survey and availability. After collection and drying, flowers of *Adenocalymma alliaceum* Miers were subjected to extraction under laboratory conditions. Based on the polarity of the plant, petroleum ether, chloroform, ethyl alcohol, and distilled water were selected to extract the phytoconstituents of *Adenocalymma alliaceum* flowers. India has been the richest source of medicinal plants for a long decade.[24] *Adenocalymma alliaceum* Miers flower contains a variety of essential phytochemicals. The ethanolic extract has a higher qualitative and quantitative availability of

phytoconstituents. The present study also shows the presence of trace amounts of carbohydrate, flavonoid, cardiac glycoside, coumarin glycoside, tannins, proteins, and phenolic compounds in the qualitative test of EEAA. *Adenocalymma alliaceum* Miers is responsible for widespread pharmacological actions such as antioxidant, anti-inflammatory, neoplastic, antimicrobial, etc.[25]

Our study has included both *in vitro* and *in vivo* models to screen *Adenocalymma alliaceum* Miers. In the cytotoxicity study, cell deaths were significantly lower in EEAA than in positive control. This implies the safety of EEAA in an animal study. Hence, the plant extracts are safe with no cytotoxicity, even at higher concentrations. Hence, they may be considered ideal for developing therapeutics for diabetes as far as *in vitro* toxicity is concerned. Furthermore, the cell death percentage exhibited no discernible pattern of increase in cytotoxicity with the increase in concentration.

The findings imply that the efficacy of EEAA on INS-I cells is similar to that of metformin in that it increases glucose absorption in the liver. Furthermore, since it has been observed that this concentration has no cytotoxic effect, it can be considered a therapeutic dose. However, it was still not comparable to metformin, which is widely used. Therefore, further investigation is needed to improve its efficacy to be widely accepted and translated from bench to bedside.

GLUT-4 redistribution assay indicates the pathway followed by any therapeutic molecule being investigated as an antidiabetic drug. Insulin-mediated glucose absorption requires the GLUT-4 glucose transporter. Therefore, the GLUT-4 glucose transporter vesicles can move towards the cell surface in the presence of insulin under normal conditions. However, this process is disrupted in disease conditions, and abnormal glucose uptake via this transporter is widely observed in patients suffering from obesity and T2DM. Hence, restoring GLUT-4 transporter activity indicates that the plant extracts influence this pathway.

Prior to the main study, an acute toxicity investigation on mice was conducted. There was no evidence of death at any of the doses tested. In our research, alloxan was chosen to induce diabetes. Alloxan is responsible for pancreatic β-cell damage, resulting in diminished insulin secretion.[26] Alloxan raises the fasting blood glucose levels significantly in laboratory animals. It is due to pancreatic β-cell toxicity and subsequent insulin insufficiency. The oxidative stress is due to reactive oxygen species (ROS) that cause pancreatic damage.[27] A two-step redox reaction is responsible for producing free radicals from alloxan. It starts by reducing alloxan into dialuric acid by the enzyme glutathione reductase (GSH). Here, GSH gets oxidized to glutathione disulfide (GSSG), and alloxan radical ($AH^{\bullet}$) is observed as an intermediate product. Reversibly, autoxidation of dialuric acid forms superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Further, hydrogen peroxide (H_2O_2) forms hydroxyl radical ($OH^{\bullet-}$). This is termed the "Fenton reaction," catalyzed by metal ions such as iron. Our study selected animals that reached >250 mg/dl of blood glucose for treatment. Furthermore, the fasting blood glucose of diabetic control animals was found to increase till the 21st day. Standard's effectiveness in diabetic rats lowers fasting blood glucose levels by boosting insulin secretion from an insulin-secreting cell. Glibenclamide works in pancreatic β-cells by binding to the sulfonylurea receptor 1 (SUR 1), a subunit of adenosine triphosphate-sensitive potassium channels (K_{ATP}). As a result, cells are inhibited, allowing sodium and calcium to enter the pancreatic cell. Subsequently, it raises intracellular calcium levels in β-cells, enhancing insulin secretion.[28] All the doses of EEAA lowered the fasting blood sugar levels. Furthermore, on the 1st, 7th, 14th, and 21st days, the extract revealed a substantially lower raised glucose level in the high dose group than in the diabetes control, low dose, and medium-dose group. EEAA was less significant in contrast to the Standard group. Polyphenol and flavonoids boost glucose uptake by increasing GLUT-2 expression in pancreatic β-cells, increasing insulin release, and encouraging GLUT-4 translocation, which can improve glucose uptake by muscle, liver, and adipose tissue, according to our findings.[29] Flavonoids also duplicate pancreatic β-cells, inhibit α glucosidase and β amylase, diminish aldose reductase, improve calcium ion absorption, slow stomach emptying, and inhibit aldose reductase.[30]

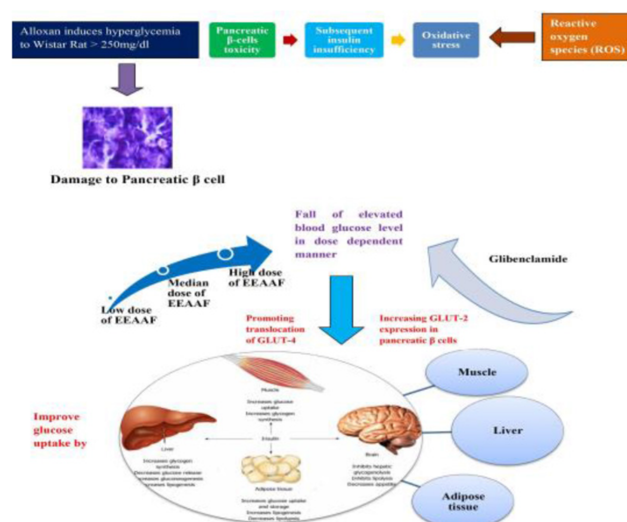

Figure 6: Mechanism of action of EEAA on Diabetes.

Numerous biological functions of EEAA were expressed in Figure 6.

Bodyweight is considered a standard parameter used in diabetic screening. However, animals were losing body weight during alloxan treatment. This loss in weight is due to the breakdown of available fat and its use as an energy source. Fat is used as a substitute energy source when the cell is not utilizing the glucose in the body. Our research discovered a decreased body weight in rats after they were given alloxan. In addition, the body weights of animals were restored for the standard group and EEAA in a dose-dependent manner for polyphenol, tannin, and saponins. However, from the clinical manifestation point of view, glycosuria has been observed. The possible mechanism for glycosuria is partial glucose reabsorption in proximal convoluted tubules.

Consequently, urine osmotic pressure increases with subsequent inhibition of water reabsorption. Finally, it results in polyuria, where the urge and tendency to urinate are exceedingly stimulated.[17] Both polyuria and glycosuria can cause dehydration. Dehydration is due to a redeeming mechanism to maintain normal urine osmolality. As a result, it causes a condition known as polydipsia, characterized by excessive thirst.[18]

Furthermore, the gluconeogenesis process is triggered by decreased glucose transport and use in body muscle cells, which leads to weight loss in diabetic patients. Another reason for fast weight loss in diabetics could be increased lipolysis due to lower insulin levels. Polyphagia is caused by both glycosuria and tissue catabolism. It is characterized by excessive appetite and food consumption.[18] The present investigations showed amelioration in weight loss, polyuria, and polydipsia after extract treatment. However, we noted a symbolic increase in polyphagia in diabetic groups. However, the high extract dose brought it almost to a normal state.

Many were reported to cause dyslipidemia in rats after alloxan treatment, leading to various cardiovascular complications.[4] It is because diabetes patients have higher serum total cholesterol, triglycerides, LDL levels, and lower HDL levels. In addition, people with diabetes use free fatty acid from peripheral loads at a higher rate than non-diabetics.[31] Furthermore, diabetes can cause a prominent decrease in total serum protein and increased serum creatinine and urea concentration, leading to kidney dysfunction.[32] Trio's doses of EEAA significantly restored biochemical parameters indicating its ability to protect against diabetic renal complications. These results imply the protective effect of carbohydrates on pancreatic β-cells.

SOD, GSH, and CAT are liver enzymes with antioxidant capacity against pancreatic β-cell injury. H_2O_2 is diverted from the oxidative pathway by GSH, which prevents the creation of further OH•. It can also directly neutralize OH• and limit the production of AH•. In contrast, EEAA regulates free radical production in alloxan-induced oxidative stress at low concentrations.[20]

Nevertheless, a high concentration inhibits oxygen utilization, dialuric acid oxidation, and $OH^{\bullet-}$ formation. The enzyme SOD inhibits the oxidation of dialuric acid. It inhibits oxidation where $O_2^{\bullet-}$ is involved. It inhibits the Fenton reaction by inactivating H_2O_2. Hence, the concentration of GSH and SOD can be one of the tissue markers for understanding the extent of alloxan-induced β-cell toxicity.[33] From our study, the presented data also supported the fact by reducing the concentration of GSH and SOD in diabetic groups than in the normal groups. However, the EEAA increased these biomarkers significantly in a dose-dependent manner. It can be assumed that polyphenol and polysaccharides' presence is the reason behind it. The high-dose extract was substantial and close to the standard group.[34]

The microscopical study of the pancreas reported the antihyperglycemic effect of EEAA. The normal group of animals exhibited no cell injury or structural changes in pancreatic islets of normal groups from the pancreas. In contrast, the DC group of animals expresses visible and injured cells resulting in hypertrophy, separation of interstitial cells, neutrophil infiltration, and reduction of islets cells. These signs distinctly mention the diabetic character or injury to pancreatic cells.[35]

EEAA showed a recovery rate of cell injury or visible changes in the pancreas's graded dose than diabetic histopathology. This information recommends that the EEAA at a high amount could be an excellent protective measure against ROS-initiated pancreatic islets toxicity. It is assumed that flavonoid attributes their properties to EEAA. It also came out that EEAA is safe, non-toxic, and has relative potency to Glibenclamide.

CONCLUSION

In our finding, *Adenocalymma alliaceum* Miers presents antioxidant and antidiabetic characteristics due to polyphenol and alkaloids, especially flavonoids. In addition, the cell line study reveals the cytotoxicity of EEAA in a graded manner, extensive Glucose utilization, and GLUT-4 expression using EEAA against hyperglycemic conditions. These active secondary metabolites regulate blood glucose levels, gaining bodyweight, scavenging free radicals' formation,

controlling lipid profile, and other diabetic-related complications. In addition, histological investigations significantly affected the β-cell injury at diverse doses of EEAA. However, more research is required to determine specific phytoconstituents involved in the protective impact. Furthermore, more outstanding research is needed to prevent *Adenocalymma alliaceum* Miers-related diabetic cardiovascular and renal issues.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DM: Diabetes Mellitus; **T1DM:** Type 1 Diabetes Mellitus; **T2DM:** Type 2 Diabetes Mellitus; **EEAA:** Ethanolic extract of *Adenocalymma alliaceum*; **NC:** Normal control; **DC:** Diabetic control; **STD:** Standard; **LD:** Low dose; **MD:** Median dose; **HD:** High dose; **TC:** Total cholesterol; **TG:** Triglyceride; **SOD:** Superoxide dismutase; **GSH:** Glutathione; **CAT:** Catalase; **ROS:** Reactive oxygen species; **IAEC:** Institutional animal ethic committee; **µg:** Microgram; **µm:** Micrometre; **ml:** Millilitre; **nm:** Nanometre; **hr:** Hour; **rpm:** Revolution per minute; **g:** Gram; **mg:** Milligram; **°C:** Degree centigrade; **cm:** Centimetre; **DMSO:** Dimethyl sulfoxide.

Author Contributions

Devid Chutia has prepared the manuscript and was involved in correcting the paper. C.K Tyagi has been involved in work being competition, scientific analysis, and manuscript writing, and N.R. Bhuyan was involved in scientific checking and manuscript writing. Dr. Sunil Kumar Shah was part of the editing.

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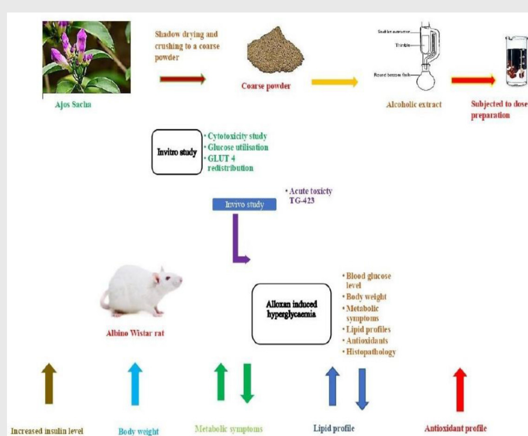
PICTORIAL ABSTRACT



SUMMARY

Adenocalymma alliaceum Miers is a shrubby herb habitant to rainy parts of India. The plant belongs to the Bignoniaceae family. Ajos sacha is documented for various potentials such as anti-inflammatory, antibacterial, antifungal, antitumor, and antioxidant. On the phytochemical screening of ethanolic extract of *Adenocalymma alliaceum,* Miers flowers reported polyphenol, tannin, and flavonoid. During *in vitro* study, EEAA shows minimal toxicity in cytotoxicity study, elevated glucose utilization, glucose uptake rate on INS-I cells, and effective *in vivo* antidiabetic property.

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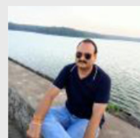

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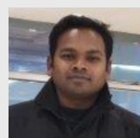

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