



Original Research Article (Experimental)

# Ameliorative effect of *Annona reticulata* L. leaf extract on antihyperglycemic activity and its hepato-renal protective potential in streptozotocin induced diabetic rats

Vineela Pulivarthi <sup>a</sup>, Josthna P. <sup>b</sup>, C.V. Naidu <sup>a, \*</sup><sup>a</sup> Department of Biotechnology, Dravidian University, Kuppam, 517 426, A.P, India<sup>b</sup> Department of Biotechnology, Sri Padmavati Mahila Visvavidyalam, Tirupati, 517502, A.P, India

## ARTICLE INFO

## Article history:

Received 8 September 2020

Received in revised form

18 January 2021

Accepted 31 January 2021

Available online 16 June 2021

## Keywords:

*Annona reticulata* L.

Alpha amylase

Antihyperglycemic

Alpha glucosidase

GC–MS

Phytochemicals

## ABSTRACT

**Background:** *Annona reticulata* L. is a traditionally important plant due to its versatile source of medicine and industrial products. It is used to treat cardiac problems, wound healing, diabetes, ulcers and bacterial infections. As it is a commercial fruit bearing plant, wide range studies on this plant reaches the mankind efficiently.

**Objective(s):** The present study was focussed on antihyperglycemic potential of *A. reticulata* leaves under *in vitro* and *in vivo*.

**Material and methods:** The *in vitro* phytochemical analysis, total phenolic, flavonoid content, inhibition activity on alpha amylase and alpha glucosidase enzymes were determined for various solvent extracts, followed by *in vivo* oral toxicity, short term, dose dependant antihyperglycemic studies, oral glucose tolerance tests were performed. The activity of methanolic extract of *A. reticulata* (MeEAR)-500 mg/kg b.wt was studied for 28 days in diabetic rat model. Histopathological examinations and serum biochemical assays were performed. Gas chromatography–Mass spectrometry (GC–MS) analysis was performed to identify the compounds present in MeEAR.

**Results:** Among the various extracts, MeEAR possesses higher amount of phenols and flavonoids with effective inhibition on carbohydrate hydrolysing enzymes ( $P < 0.05$ ) and also exhibited higher glycemic control *in vivo*, with simultaneous improvement in the hepatic and renal activities in diabetic rats. GC–MS analysis revealed the presence of 63 bioactive compounds including carboxylic-acids, alcoholic groups, fattyacid esters, amino acid derivatives.

**Conclusion:** Altogether, our study demonstrated that leaves of *A. reticulata* possess better anti-hyperglycemic activity and could be developed in to a potential antidiabetic drug with further studies.

© 2021 The Authors. Published by Elsevier B.V. on behalf of Institute of Transdisciplinary Health Sciences and Technology and World Ayurveda Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Diabetes mellitus (DM) is characterized by persistent abnormal high blood sugar levels due to irregularity in pancreatic insulin secretion/action [1]. Insulin, a pivotal peptide hormone, coded on chromosome 11 and synthesized by the beta cells of pancreas and functions in carbohydrate, amino acid, lipid metabolism at cellular level. It regulates the proper glucose utilization of the cells, intracellular glucose transport in tissues and generates a homeostatic

environment in the body [2]. The decreased cellular sensitivity towards insulin metabolic actions, termed as insulin resistance, which plays a major pathophysiological role in Type 2 diabetes mellitus (T2DM) and is closely associated with complications such as hypertension, obesity, dyslipidemia, a cluster of cardiovascular and metabolic abnormalities and eventually defines DM as a metabolic syndrome [3]. The disease has developed as a precarious hitch in the contemporary world. According to International Diabetes Federation (IDF), in 2015, globally 415 million adults aged between 20 and 79 years were estimated with DM and the prevalence will progress to 642 million by 2040 with the rise from 8.8% to 10.4% [4]. Owing to its drastic prevalence, morbidity and mortality, in India diabetes has emerged as a seventh leading killer of

\* Corresponding author.

E-mail: [challagundlav@yahoo.co.in](mailto:challagundlav@yahoo.co.in)

Peer review under responsibility of Transdisciplinary University, Bangalore.

early death in 2016. Over 72 million cases of diabetes were reported in India 2017 [5]. The reports are alarming the world population to be cautious and aware of the disease progression.

Carbohydrates are the immediate sources of energy in the normal human diet. Alpha-amylase and alpha-glucosidase are the key enzymes in the digestive system that involves in catalysing of complex carbohydrates leading to the elevation of postprandial hyperglycaemia. Hence the hindrance of these enzymes slows down the absorption rate of glucose and in turn lowers the blood glucose levels in T2DM which created a better approach in the diabetes treatment [6]. Studies have conclusively reported that glucose control is the base to minimize the diabetic complications. In addition to the effective glycemic control, the drugs should balance their potential side effects that occur during the glucose lowering medication [7]. Acarbose, miglitol, voglibose are the present enzyme inhibitors that are commercially employed in the treatment of diabetes. The limitations of these synthetic drugs are bloating, flatulence, abdominal disturbances, diarrhoea etc. In addition, the other commercially available drugs in the market-Biguanides, Thiazolidinediones, Sulfonylureas etc., are also highly focused on the glucose control, failing to balance their associated side effects. Therefore management of diabetes with minimum or no concomitant side effects is an incredible challenge to the modern medicine. In this context, there is a relentless scientific investigation to find the alternate therapeutic strategies for the treatment and management of diabetes.

Plant derived medicines are gaining momentum as effective and preferable alternative treatment with the advantages of safety, affordability, adequacy and acceptability [8]. World Health Organization (WHO) acknowledged the significance of herbal medicines in chronic disease treatment, prevention, and health care management. Hence there is an increasing research studies to unveil the potential of traditional medicine in treatment or management of various disorders. The contribution of herbal medicine in pharmaceutical industry has been enormously enhanced all over the world. Keeping in view of the growing significance of herbal medicine, the present study was focussed on the antidiabetic potential of an important traditional medicinal plant *Annona reticulata* L., which is widely distributed and cultivated in India, belonging to the family Annonaceae.

*A. reticulata* L. is used in the treatment of various ailments and disorders such as dysentery, cardiac failure, ulcer, diabetes, cancer etc., [9]. The fruits of *A. reticulata* are commercially available in local markets with the name Ramaphal. As it is a commercially cultivated plant, an extensive research on pharmacological properties of the plant may definitely be beneficial and reach the mankind in an effortless manner. In West Godavari district of Andhra Pradesh, India, it was reported in the survey conducted by the researchers [10] that traditional healers recommend the intake of decoction of *A. reticulata* leaves along with cow's milk orally in order to treat diabetes. Studies of [11] determined the glucose lowering effect of *A. reticulata* leaves in diabetic rat model with single organic solvent. However, the available data is scarce in establishing the detailed antidiabetic profile of *A. reticulata* leaves using various solvent systems. Hence the present study was carried out with the objective of determining the ability of *A. reticulata* leaf extracts on anti-hyperglycemic activity under *in vitro* as well as in animal model along with phytochemical analyses of the extracts and biochemical assays. Besides, determining the antidiabetic potential of *A. reticulata* leaves, an attempt has been made to identify the bioactive compounds present in the crude leaf extract, such that the isolation of bioactive compounds with antidiabetic activity can be elucidated in further studies.

## 2. Materials and methods

### 2.1. Collection of plant material

Fresh leaves of *A. reticulata* were collected in the vicinity of Kuppam, Andhra Pradesh during the period May–June 2017. Herbarium specimen (voucher no. 0652, dated June-2017) was authenticated and deposited for reference in Department of Botany, Sri Venkateswara University, Tirupati.

### 2.2. Plant leaf extraction procedure

The leaves were rinsed thoroughly with water and shade dried at room temperature ( $25 \pm 5$  °C) for 10 days. Then the leaves were powdered and subjected for the extraction process with four different solvents based on their polarity i.e., methanol, aqueous, ethyl acetate and n-hexane by using soxhlet apparatus (Deschem 500 ml, 24/50, glass soxhlet extractor) at variable temperatures depending on their respective boiling points. The extracts (AqEAR – aqueous extract, MeEAR-methanol extract, EaEAR-ethyl acetate extract, nhEAR-(n-hexane extract) later were filtered and concentrated to dryness using rotary evaporator (Buchi R210 rotavapor, Switzerland) under reduced pressure to obtain semi dried solid masses. The extracts were tightly packed and stored at 4 °C temperature for further use.

### 2.3. Qualitative phytochemical screening

The plant extracts were screened for the presence of various phytochemicals like alkaloids, phenols, flavonoids, terpenoids, and glycosides by following the standard procedures [12].

### 2.4. Quantification of phytochemicals

#### 2.4.1. Estimation of total phenolic content (TPC)

The total phenolic content of various solvent leaf extracts of *A. reticulata* was quantitatively determined by using Folin–ciocalteu reagent method with gallic acid as a standard [13]. The TPC was expressed in milligrams of gallic acid equivalents (GAE) per gm of extract.

#### 2.4.2. Estimation of total flavonoid content (TFC)

Quantitative determination of flavonoids by Aluminium chloride colorimetric assay was performed according to Ref. [14]. The total flavonoid content was calculated by plotting a calibration curve with standard quercetin and expressed in terms of mg quercetin equivalent (QE) per gm dry weight.

### 2.5. *In vitro* antidiabetic study

#### 2.5.1. Inhibition of alpha amylase enzyme activity

The  $\alpha$ -amylase enzyme inhibition was performed using porcine pancreas isolated alpha amylase enzyme following the method [6]. A volume of 500  $\mu$ L plant samples of various concentrations (100, 200, 300, 400, 500  $\mu$ g/ml) were mixed with the 500  $\mu$ L of 0.02 M sodium phosphate buffer with 0.06 M NaCl, pH 6.9. The mixture was incubated for 10mins at after the addition of the 500  $\mu$ L enzyme solution (5 units/mL in the buffer). Then 500  $\mu$ L 1% soluble starch solution (in 0.02 M sodium phosphate buffer with 0.06 M NaCl, pH 6.9) was added as a substrate for the target enzyme which was followed by 10mins incubation at room temperature. 1 ml 3,5-dinitro salicylic acid (DNSA) reagent was added to halt the reaction. All the test tubes were placed in a water bath (at 80–90 °C)

for 5mins, a deep orange-red colour development was observed which were cooled down to room temperature. A volume of 10 ml distilled water was added to dilute the reaction mixture and the absorbance was measured at 540 nm. Acarbose, a commercial antidiabetic drug at the same concentrations (100, 200, 300, 400, 500 µg/ml) was used as a positive standard. The control sample was prepared by adding all the reagents except test samples and the blank solution was without enzyme and test samples. Their final volume was made up with buffer or solvent. The percentage of enzyme inhibition was calculated as follows:

$$\% \text{ of amylase enzyme inhibition} = (\text{Abc}-\text{Abs})/\text{Abc} * 100$$

Abc = Absorbance of control; Abs = Absorbance of sample.

### 2.5.2. Inhibition of Yeast's alpha glucosidase enzyme activity

The enzyme assay was performed according to the method [15]. Plant extracts, standard drug (Acarbose) of concentration 1 mg/ml was serially diluted and the final concentrations made were 100, 200, 300, 400, 500 µg/mL. A 96 micro well plate contains a mixture of 20 µL of test sample, mixed with 50 µL of phosphate buffer (0.1 M, pH 6.8), 10 µL of yeast isolated alpha-glucosidase enzyme (1U/mL in phosphate buffer) were pre-incubated for 10 min at room temperature. The pre-incubation was followed by the addition of the 20 µL substrate *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (5 mM in phosphate buffer) and incubated for 30 min. The enzyme acts on the substrate and releases *p*-nitrophenol in to the solution. After incubation, addition of 50 µL Na<sub>2</sub>CO<sub>3</sub> (0.1 M) cease the reaction and the absorbance of released *p*-nitrophenol in the solution was measured at 405 nm in microplate reader (Biorad iMark™-Microplate absorbance reader). The reaction mixture without test sample acts as a control and phosphate buffer was used as blank. Assay was done in triplicates. The extent of inhibition of alpha-glucosidase by plant extracts was calculated by the formula

$$\% \text{ of alpha glucosidase enzyme inhibition} = (\text{Abc}-\text{Abs})/\text{Abc} * 100$$

Abc = Absorbance of control; Abs = Absorbance of sample.

## 2.6. In vivo studies

### 2.6.1. Ethical approval

All the animal studies were performed at Sri Padmavathi Mahila University, Tirupati, India, with the approval of Institutional Animal Ethical Committee (with the No. 1677/po/a/CPCSEA/SPMVV-IEC/2014/14 dt.24-02-2014).

### 2.6.2. Test animals

Healthy adult wistar male albino rats of weight 180–220 g were procured for the present study. Before commencing the experimental study the rats were grouped and placed in the cages for acclimatization under standard laboratory conditions for 7 days with 12hr light/dark cycle, temperature at 25±2 °C with relative humidity at 45–55%. The rats were provided with standard pellet diet and water *ad libitum*.

### 2.6.3. Acute oral toxicity studies

The acute oral toxicity studies of various leaf extracts of *A.reticulata* was performed systematically according to Organization of Economic and Cooperation Development (OECD) – 423 guidelines (OECD/OCDE 423, 2001) [16]. After acclimatization period, doses of 250, 500 and 1000 mg/kg b.wt of each extract were tested on the normal rats. The extracts were dissolved in the distilled water and orally administered to the rats by gastric intubation using an oral gavage. After drug administration, the rats

were observed for every 2hrs periodically during the 24hrs of a day for any behavioural changes, toxic symptoms, mortality etc. Changes in body weights were measured. The amount of feed intake and water intake were observed regularly. The complete duration of the toxicity study was 14 days.

## 2.7. In vivo antidiabetic study

### 2.7.1. Induction of hyperglycaemia

A single low dose of streptozotocin (STZ) at a concentration of 45 mg/kg b.wt was dissolved in freshly prepared 0.1 M citrate buffer at pH 4.5, was administered intraperitoneal to 12hr overnight fasted rats. After 72hr of induction, a marked increase in blood glucose levels more than 250 mg/dL were considered as diabetic. The general symptoms of diabetes polyuria, polydipsia, and polyphagia were observed in the diabetic rats. After a week of stable hyperglycaemic conditions, the studies were initiated.

### 2.7.2. Evaluation of different solvent extracts of *A.reticulata* leaves for antihyperglycaemic activity (A short term in vivo study)

The rats were divided in to seven groups with six animals in each. Following the overnight fasting of normal and diabetic rats, Group 1 and Group 2 were served with only vehicle (water) whereas Group 3 was treated with glibenclamide (20 mg/kg b.wt) and from Group 4 to Group 7 the rats were served with respective leaf extracts with specified dose (250 mg/kg b.wt) concentration. Blood samples were collected from rat's tail vein and fasting blood glucose (FBG) levels were measured at intervals of 0, 1,3,5,7 h by using Accu chek active glucometer.

### 2.7.3. Evaluation of different doses of the methanolic extract of *A.reticulata* leaves (MeEAR) for anti-hyperglycaemic activity (A dose-dependent study)

The selection of the best extract by short term study was followed by dose dependant *in vivo* study. The rats were divided in to six groups with 6 rats in each. The normal and diabetic control group rats received vehicle whereas the other four diabetic groups each were served with oral dosage of glibenclamide (Glb-20 mg/kg b.wt), MeEAR (250, 400, 500 mg/kg b.wt.) respectively by using gavage needle. The FBG levels were measured at intervals of 0, 1st, 3rd, 5th, 7th hr by using Accu chek active glucometer.

### 2.7.4. Oral glucose tolerance test with effective dose 500 mg/kg b.wt in normal rats

The rats were divided in to three groups with 6 animals in each group named as Group I: Normal rats; Group II: Normal + Glb 20 mg/kg b.wt; Group III: Normal + MeEAR 500 mg/kg b.wt. All the rats were allowed for 12hrs overnight fasting. The group II and group III were fed with glibenclamide (20 mg/kg b.wt) and MeEAR (500 mg/kg b.wt) respectively. After 30mins of dosage, rats were fed orally with glucose solution with the concentration of 2 g/kg b.wt. FBG levels were determined prior to dosing and after 30, 60, 90, 120min of glucose administration by using Accu chek glucometer.

### 2.7.5. Treatment of *A.reticulata* leaf extract with effective dose (500 mg/kg b.wt) for 28 days

A total of 24 rats were used for the study. Out of which, 18 are diabetic rats. The rats were divided in to four groups- Normal and diabetic control groups, Diabetic + Glb and Diabetic + MeEAR 500 mg/kg b.wt. The best extract with effective dose and glibenclamide were given to the diabetic rats daily at the specified time for 28 days by gastric intubation using oral feeding cannula. Blood glucose levels and body weights of the rats were measured on weekly basis 0, 7th, 14th, 21st, 28th day. Rats were observed

regularly for their food intake and water intake. On the final day of study, blood samples were collected by retro orbital puncture from overnight fasted rats for biochemical assays.

## 2.8. Serum biochemical assays

Blood samples withdrawn from rats were collected in to sterile plastic tubes which were centrifuged at 3000 rpm for 15 min to separate serum and stored in  $-40\text{ }^{\circ}\text{C}$  for further assays. Various serum markers were evaluated using commercially available kits: Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase (ALP) - Aspen Laboratories Pvt. Ltd., Creatinine - AGD Biomedicals (P) Ltd., Urea - Reckon Diagnostics P. Ltd., Insulin - ELISA kit method, Bilirubin - Aspen Laboratories Pvt. Ltd., Albumin - Agappe Diagnostics Ltd., India.

### 2.8.1. Lipid profile

Total cholesterol (TC), High Density Lipid (HDL)-cholesterol, Low Density Lipid (LDL)-cholesterol, Very Low Density Lipid cholesterol (VLDL), triglycerides (TG) are the serum lipid markers which were estimated using commercially available kits: Total cholesterol, Triglycerides (AGD Biomedicals (P) Ltd., India), HDL-cholesterol (Reckon Diagnostics P. Ltd., India), LDL-cholesterol [17], VLDL cholesterol (calculated by formula  $\text{TG}/5$  i.e., 20% TG).

## 2.9. Histopathology study of kidney and liver

The dissected tissues kidney, liver were washed with distilled water and 10% saline and fixed in 10% formaline solution for 24hrs. Then the collected tissues were processed with paraffin and were sectioned at  $5\text{ }\mu\text{m}$  thickness approximately. The sections were stained with H&E (hematoxylin and eosin) stain to observe the histology of tissues under microscope.

## 2.10. Identification of bioactive compounds in MeEAR by GC–MS analysis

The identification of active constituents in MeEAR was performed by gas chromatography system (Agilent 7890 A, Agilent Technologies, USA) geared up with time-of-flight mass spectrometer (Pegasus HT TOF, LECO Corporation, USA). The chromatography system was equipped with a non-polar capillary column (Agilent J&W HP-5MS) with 5% phenyl-methylpolysiloxane and of dimensions  $30\text{ m} \times 0.25\text{ mm}$ , film thickness  $0.25\text{ }\mu\text{m}$ . Helium gas at its purity 99.999% acts as a carrier gas at the flow rate of  $1.2\text{ ml/min}$  in split-less mode of injection. The initial oven temperature was maintained at  $80\text{ }^{\circ}\text{C}$  for 5 min, later it was ramped (at the rate of  $15\text{ }^{\circ}\text{C/min}$ ) to  $280\text{ }^{\circ}\text{C}$ . A volume of  $1\text{ }\mu\text{L}$  sample was injected manually in to the inlet of the column which was maintained at  $250\text{ }^{\circ}\text{C}$  operating in a split-less mode. The ion source temperature was  $230\text{ }^{\circ}\text{C}$  whereas transfer line temperature was set at  $280\text{ }^{\circ}\text{C}$  and the acquisition rate was 10 spectra per second and a solvent delay of 2 min was used in mass spectrometer. The mass spectra within the range of  $50\text{--}1000\text{ m/z}$  were recorded by an electron ionization mass spectrometer with the ionization energy of  $70\text{ eV}$ . The total runtime was 34mins. LECO Chromatof software version 3.24 (LECO Corporation, USA) was employed for qualitative analysis. The obtained mass spectra were matched, compared and then identified by a library search, NIST/EPA/NIH Mass Spectral Library NIST.

## 2.11. Statistical analysis

All the *in vitro* experimental data were expressed as mean of triplicates  $\pm$  S.D whereas *in vivo* antidiabetic study's data and serum

biological assay's data was expressed as mean of six replicates  $\pm$  S.D. The comparison between the groups was assessed statistically by one way Analysis of Variance (ANOVA) followed by Post –Hoc Tukey Multiple comparison test using SPSS 16 software.

## 3. Results

### 3.1. Plant extract yield

The colour, texture and percentage (%) yield of the extracts were summarized in Table 1.

### 3.2. Phytochemical analyses

#### 3.2.1. Qualitative analysis

The preliminary phytochemical analysis of *A.reticulata* leaf extracts revealed the presence of phenols, alkaloids, flavonoids, glycosides, terpenoids and tannins and was mentioned in Table 2a.

#### 3.2.2. Quantitative analysis

**3.2.2.1. Total phenol and flavonoid content.** By following the Folin-ciocalteu reagent method, the phenol content was measured in all the solvent extracts against gallic acid standard curve with linear regression equation  $y = 0.004x + 0.725$ ,  $R^2 = 0.953$ . The MeEAR showed the highest amount of phenol content with  $177.3 \pm 12.3\text{ mg GAE/gm}$  of extract compared to other solvent extracts followed by EaEAR > AqEAR > nhEAR. By following the aluminium chloride method, total flavonoid content was estimated with quercetin standard curve ( $y = 0.0005x + 0.0359$ ,  $R^2 = 0.9895$ ). MeEAR showed the highest flavonoid content with  $69.6 \pm 2.7\text{ mg QE/gm}$  of extract followed by EaEAR > AqEAR > nhEAR. Table 2b represents the total phenol and flavonoid content.

### 3.3. In vitro antidiabetic activity

#### 3.3.1. Alpha amylase and alpha glucosidase enzyme inhibitory activities

The inhibitory effect of *A.reticulata* leaf extracts on the activities of the two carbohydrate hydrolysing enzymes porcine pancreas alpha amylase and yeast's alpha glucosidase were determined and the  $\text{IC}_{50}$  values were represented in Table 3. Among the four solvent extracts MeEAR has shown the prominent dose dependant inhibition for both the enzymes alpha amylase and alpha glucosidase with the  $\text{IC}_{50}$  values –  $713.09 \pm 2.37\text{ }\mu\text{g/ml}$  and  $1051.58 \pm 7.17\text{ }\mu\text{g/ml}$  respectively and with the % inhibition of  $16.45\% \pm 3.42$  on alpha amylase and  $11.71\% \pm 2.12$  on alpha glucosidase at  $200\text{ }\mu\text{g/ml}$  compared to the other extracts. The AqEAR showed positive for Alpha amylase activity with the  $2.29\% \pm 1.21$  inhibition and with no alpha glucosidase activity. EaEAR has shown mild inhibition on both the enzymes comparatively lower than Acarbose and MeEAR. The nhEAR had not shown any inhibition activity for both the enzymes.

### 3.4. In vivo studies

#### 3.4.1. Acute oral toxicity study

The symptoms of toxicity and mortality were not observed in any of the groups during the 14 days of observation study. The body weights of all the groups were increased slightly but not in a significant way. The intake of food and water was slightly increased in all the treated groups. In spite of absence of detrimental effects of the solvent extracts up to  $1000\text{ mg/kg}$  b.wt dosage in the normal rats, the minimum dosage of  $250\text{ mg/kg}$  b.wt was ascertained for antihyperglycemic studies.



**Table 1**

Represents the physical properties of the four plant extracts.

Types of Extracts	Colour	Texture	% yield (out of 250 gms)
AqEAR	Light green	Dried pellet form	21.40%
MeEAR	Black	Thick, semi-dried slurry paste	16.04%
EaEAR	Black	Semi dried slurry paste	7.13%
nhEAR	Black	Semi liquid form (Oils)	4.4%

AqEAR-aqueous extract, MeEAR-methanolic extract, EaEAR-ethylacetate extract, nhEAR-n-hexane extract.

**Table 2a**

shows the presence and absence of various phytochemicals in the plant extracts.

Phytochemicals	AqEAR	MeEAR	EaEAR	nhEAR
Phenols	+	++	+	+
Flavonoids	+	++	+	+
Tannins	+	++	+	+
Glycosides	+	++	-	-
Terpenoids	-	+	-	-
Alkaloids	+	+	+	-

AqEAR-aqueous extract, MeEAR-methanolic extract, EaEAR-ethylacetate extract, nhEAR-n-hexane extract. '+' indicates the presence of phytochemicals, '++' indicates the higher presence of phytochemicals, '-' indicates the absence of the phytochemicals.

**Table 2b**Total phenolic and flavonoid content of *A.reticulata* leaves extract.

Extracts	TPC (mg GAE/gm of extract)	TFC (mg QE/gm of extract)
AqEAR	26.16 ± 4.04 <sup>c</sup>	19.2 ± 1.63 <sup>c</sup>
MeEAR	177.3 ± 12.3 <sup>a</sup>	71.2 ± 2.7 <sup>a</sup>
EaEAR	29.16 ± 2.08 <sup>b</sup>	33.5 ± 3.0 <sup>b</sup>
nhEAR	4.33 ± 1.25 <sup>d</sup>	3.86 ± 1.41 <sup>d</sup>

The values in each column are the mean of three independent analyses with SD (n = 3), sharing different superscript letters (a,b,c,d) indicates the significant difference (P &lt; 0.05). AqEAR-aqueous extract, MeEAR-methanolic extract, EaEAR-ethylacetate extract, nhEAR-n-hexane extract.

**Table 3***In vitro* Alpha amylase and alpha glucosidase enzyme inhibition of different solvent extracts of *Annona reticulata*.L

Groups	$\alpha$ -amylase inhibition IC <sub>50</sub> (µg/ml)	$\alpha$ -glucosidase inhibition IC <sub>50</sub> (µg/ml)
Acr	58.03 ± 4.14 <sup>a</sup>	66.54 ± 10.24 <sup>a</sup>
AqEAR	4552.09 ± 6.82 <sup>b</sup>	—
MeEAR	713.09 ± 2.37 <sup>c</sup>	1051.58 ± 7.17 <sup>b</sup>
EaEAR	1326.86 ± 11.46 <sup>d</sup>	3818.92 ± 16.47 <sup>c</sup>
nhEAR	—	—

The represented data was the mean of triplicates with ±S.D (n = 3). Values sharing different superscripts (a,b,c,d) represents data significantly varies at P &lt; 0.05. Acr-Acarbose, AqEAR-aqueous extract, MeEAR-methanolic extract, EaEAR-ethylacetate extract, nhEAR-n-hexane extract.

### 3.4.2. A short term screening study on antihyperglycemic activity of various leaf extracts of *A.reticulata* in STZ induced rats

The acute antihyperglycemic effect of *A.reticulata* leaf extracts at a fixed dose (250 mg/kg b.wt) on fasting blood glucose levels at regular intervals of time was evaluated and shown in Table 4a. The FBG levels of diabetic untreated rats were significantly higher than those of the normal rats (P < 0.0001). When the four extracts each at the dose of 250 mg/kg b.wt were assessed for antihyperglycemic activity, the methanol extract (MeEAR) showed the statistical significant reduction of 30.76% in a progressive manner up to 7<sup>th</sup> hr of treatment whereas ethylacetate, aqueous and nhexane extract exhibited very poor glucose lowering effect. MeEAR showed significant reduction from 3rd hr to 6th hr. No extract has produced hypoglycaemia during the study.

### 3.4.3. Evaluation of different doses of methanol extract of *A.reticulata* leaves on antihyperglycemic activity in STZ induced rats (dose dependent study)

Fig. 1a showed the effect of different doses of MeEAR on anti-hyperglycemic activity. The MeEAR shows the proportional rise of antihyperglycemic activity with the increase of dosage without any hypoglycaemic effect. The MeEAR at 500 mg/kg b.wt exhibited a statistical significant reduction in FBG levels by 46.47% after 7hr treatment whereas MeEAR at the doses of 250 mg/kg b.wt and 400mg/kg b.wt reduced the FBG levels at the rate of 30.76% and 39.86% respectively and Glibenclamide at a dose of 20 mg/kg b.wt reduced 48.30% of FBG levels after 7hr treatment. All the doses exhibited significant glycemic control from 3rd hr to 7th hr (P < 0.01 to 0.001).

### 3.4.4. Effect of MeEAR 500 mg/kg b.wt of *A.reticulata* on oral glucose tolerance (OGT) test in normal rats

Due to the glucose load, the FBG levels were raised at first 30mins in all the groups. Fall in glucose levels was started from 1st hr in the rats that were treated with Glibenclamide and MeEAR of *A.reticulata* whereas in Normal control groups the FBG levels were reduced from 90mins. The FBG levels in Group II and Group III at 120mins were significantly reduced (P < 0.05) when compared to the 30mins FBG levels of respective groups. The MeEAR at 500 mg/kg b.wt effectively lessen the glucose levels. Fig. 1b showed the OGT activity.

### 3.4.5. Effect of *A.reticulata* leaf extract (MeEAR) with effective dose (500 mg/kg b.wt) for 28 days on fasting blood glucose levels

With the administration of MeEAR 500 mg/kg b.wt to the diabetes induced rats for 28 days, a clear significant decline in fasting blood glucose levels were observed with 57.37% whereas glibenclamide showed the decline of 64.28%. Fig. 1c depicts the pattern of FBG levels in normal, diabetic control and treated groups during the period of four weeks. The FBG levels were reduced significantly P < 0.001 compared to diabetic control by the end of the study. The reduced body weights with the induction of STZ, was increased gradually throughout the experimental period in both the treated groups. The observations in bodyweight change were mentioned in Table 4b.

### 3.5. Effect of MeEAR on liver and kidney markers

After the treatment of STZ induced diabetic rats with MeEAR for 28 days, a gross improvement in all the serum parameters were observed. There was a significant (P < 0.05) increase in the serum insulin and albumin levels in the MeEAR and Glibenclamide treated groups comparing to untreated diabetic group. The levels of creatinine, urea, and bilirubin were increased drastically in diabetic control group when compared to normal rats. The treated groups showed significant decrease in their levels at P < 0.05. Table 4 represented the changes in the serum markers. The change in the liver enzymes SGOT, SGPT, ALP levels in the serum compared to diabetic control group were showed in Fig. 2a.

**Table 4a**A short term screening study on antihyperglycemic activity of various leaf extracts of *A.reticulata* in STZ induced rats.

Groups	0 h	1st hr	3rd hr	5th hr	7th hr
Normal	85.67 ± 4.7	82.33 ± 3.2	79.33 ± 3.5	78.67 ± 6.5	82.33 ± 6.8
D.C	327.67 ± 18.5*	330.33 ± 24.0	343.00 ± 19.3	341.67 ± 27.5	332.67 ± 15.5
D + Glib 20mg/kgbw	292.67 ± 13.2*	261.67 ± 10.2	238.33 ± 9.6 <sup>#</sup>	198.67 ± 9.0	150.33 ± 6.6 (48.30%)
D + AqEAR 250mg/kgbw	288.67 ± 24.7*	275.33 ± 11.5	269.00 ± 12.6	251.33 ± 13.8	253.67 ± 15.3 (12.15%)
D + MeEAR 250mg/kgbw	273.67 ± 10.6*	257.00 ± 12.5	228.67 ± 14.5 <sup>#</sup>	192.33 ± 9.4	189.33 ± 7.0 (30.76%)
D + EaEAR 250mg/kgbw	316.67 ± 22.7*	310.33 ± 12.5	300.33 ± 21.22	282.33 ± 28.1	268.67 ± 13.6 (15.18%)
D + nhEAR 250mg/kgbw	319.33 ± 20.5*	321.67 ± 15.5	312.67 ± 21.5	309.33 ± 19.5	296.67 ± 13.1 (7.21%)

The depicted table was the mean values of three independent analyses with  $\pm$ SD (n = 6). Values in the paranthesis represents the % of glucose reduction from 0hr to 7th hr. Significance \*\*'- compared with 0hr values of Normal control (P < 0.0001). '#'- compared with 0hr values of corresponding group (P < 0.01). "'- compared with 0hr values of corresponding group (P < 0.001). D.C- Diabetic Control, D + Glib – Diabetic + treated with Glibenclamide, D + AqEAR-treated with aqueous extract, D + MeEAR-treated with methanolic extract, D + EaEAR-treated with ethylacetate extract, D + nhEAR-treated with n-hexane extract.

### 3.5.1. Effect of MeEAR on lipid profile

The levels of total cholesterol and triglycerides were increased drastically compared to normal control group (P < 0.001). When treated with glibenclamide and MeEAR, their levels were reduced at the significance level of P < 0.05 comparing to diabetic control group. The HDL-cholesterol levels were increased, LDL, VLDL-cholesterol levels were decreased comparing to diabetic control. Fig. 2b depicted the effect of MeEAR on the total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, triglycerides.

### 3.6. Effect of MeEAR on the histopathology of liver and kidney tissue

Fig. 3a and b represented the histopathology of liver and kidney tissues. The H&E stained slides were observed under microscope with the magnification 400X. The hepatic tissue in normal control (Fig. 3a-A) rat showed the presence of numerous hepatocytes, central vein, portal triad and sinusoidal lining signifies a normal cellular architecture. Fig. 3a-B was diabetic control liver tissue that showed necrotic changes, vascular congestion, with vacuolization around the central vein. The recovery of the hepatic tissue was observed in both the treated groups (D + Glib (Fig. 3a-C) and D + MeEAR (Fig. 3a-D) with the regeneration changes such as mild necrosis, less inflammatory cells around central vein, restored hepatocytes.

The histopathological observation of normal control kidney (Fig. 3b-a) showed the typical glomerulus with Bowman's capsule, proximal and distal convoluted tubules without any pathological alterations. Section of diabetic control kidney (Fig. 3b-b) displayed the shrinkage of cells and glomerular hypertrophy with discrete congestion. The treated diabetic groups (D + Glib (Fig. 3b-c) and D + MeEAR (Fig. 3b-d) demonstrated the recovery of mesangial cells, decrease in glomerular hypertrophy, less inflammatory cells. There is no significant variation in treated groups of kidney compared to normal control group.

### 3.7. GC–MS analysis of MeEAR

The chromatogram of MeEAR was shown in Fig. 4. By executing the NIST library search, the active compounds were identified based on the % peak area, molecular weight, and molecular formula, molecular structure etc. In the run time of 34mins, totally 63 major and minor compounds were identified with some repetitive ones and unknown compounds in MeEAR. All the identified compounds with retention time, %peak area, molecular mass and molecular formula were shown in Table 5 (Supplementary file).

## 4. Discussion

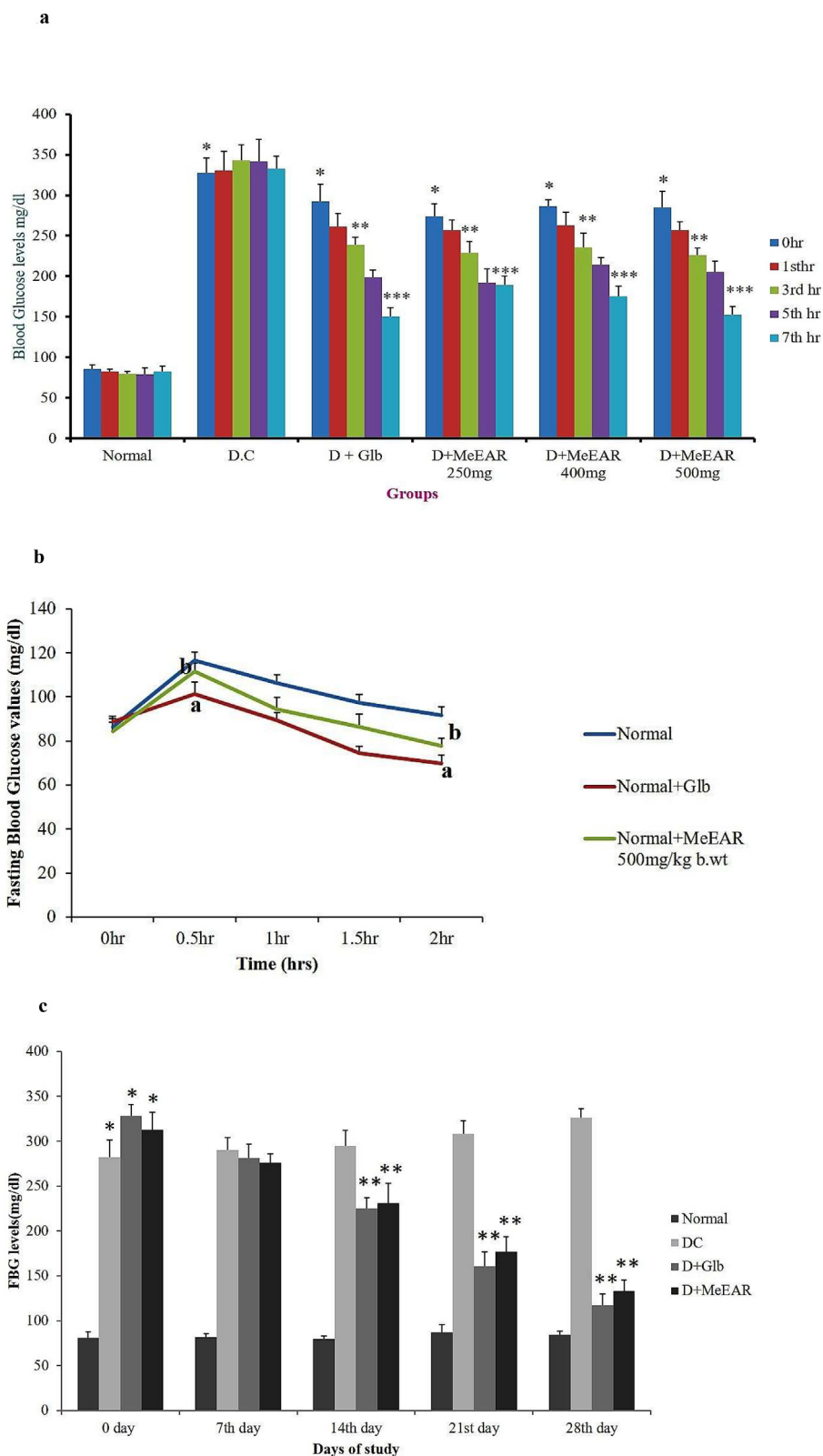
The undesirable effects of synthetic drugs endorse the modern medicine to search for the superior alternative for the treatment of

metabolic diseases. Herbal medicine turned out to be a hopeful therapy for the effective treatment of diabetes in foreseeable future [18]. In this context, the present study was essentially aimed at the evaluation of the *A.reticulata* leaf extracts on glucose lowering effects under *in vitro* and *in vivo* conditions.

Numerous studies have reported that phytochemicals are the bioactive elements of the plants that renders a new way-out to combat the onset and progression of oxidant stress, chronic diseases and aging etc. In order to process the phytochemicals, extraction of plant samples is crucial with various solvents. Extraction yield notifies the solvent efficiency to extract the active components from the sample. Percentage yields of solvent extract were mentioned (Table 1) [19]. reported that nutraceuticals of plant origin which were referred as phytochemicals could be the possible therapeutic tool to treat the metabolic syndrome. The report also listed out the phytochemicals and their impact on diabetes and its related complications. Hence keeping in view, the importance of phytochemicals in diminution of different ailments, the preliminary phytochemical analysis was carried out for alkaloids, flavonoids, phenols, terpenoids, and glycosides in all the four different solvent extracts (Table 2a). Among the phytochemicals, phenols and flavonoids are the most important phytochemicals which possess antibacterial, anti-inflammatory, antidiabetic, anti-cancer, antioxidant properties [20]. Hence the total phenols and total flavonoids were quantified in all the extracts under *in vitro* conditions. MeEAR showed the highest amount of total phenols and total flavonoids compared to the other extracts (Table 2b). The results were in accordance with the previous studies stating that phenolic compounds are more soluble in organic polar solvents than in water [21].

Inhibition of carbohydrate hydrolysing enzymes-alpha amylase and alpha glucosidase is one of the strategic approaches in retarding the digestion of carbohydrates and thus managing the T2DM. So evaluating the enzyme inhibition activities by drug compounds gained much more prominence for the treatment of diabetes. Table 3 illustrated the activity of individual solvent extracts of *A.reticulata* on inhibition of these enzymes. The variation and magnitude of inhibition activity of all the solvent extracts on both the enzymes depends on the nature of extract [22] and the activity is might be attributed to the presence of phenolic compounds, flavonoids due to their competitive binding ability to the active sites of the enzymes [23] and thus prevents the hydrolysis of the enzymes. In connection with this, the maximum % inhibition of MeEAR at 200  $\mu$ g/ml compared to other solvent extracts might be corroborated with the quantitative phenolic and flavonoid content of MeEAR. The pattern of our obtained results was in parallel with [22].

The *in vivo* studies were commenced with the acute oral toxicity studies that were carried out for all the extracts with the three selected dosages 250, 500, 1000 mg/kg b.wt. During the



**Fig. 1.** a. Evaluation of different doses of MeEAR on antihyperglycemic activity in STZ induced rats (Dose dependent Study): The figure represented the dose dependant study of MeEAR leaves. Values are mean of six independent variables with  $\pm$ S.D. D.C – Diabetic control, D + Glb – Diabetic treated with glibenclamide, D + MeEAR – Diabetic treated with methanol extract (doses- 250, 400, 500 mg/kg b.wt). Significance represented as \* - compared with the 0hr values ( $P < 0.0001$ ) of Normal control group, \*\* - compared with their respective group 0hr values ( $P < 0.01$ ), \*\*\* - compared with their respective group 0hr values ( $P < 0.001$ ). b. Effect of MeEAR 500 mg/kg b.wt of *A.reticulata* leaves on Oral Glucose Tolerance (OGT) Test in Normal rats: The figure represented the oral glucose tolerance of MeEAR. The curves that shares the same superscript letters (a), (b) are significantly different by ( $P < 0.05$ ). Values are mean of six independent variables with  $\pm$ S.D. c. Effect of *A.reticulata* leaf extract (MeEAR) with effective dose (500 mg/kg b.wt) for 28 days on fasting blood glucose levels in streptozotocin induced diabetic rats: The figure represented the effect of MeEAR on fasting blood glucose levels in streptozotocin induced diabetic rats for 28days. D.C – Diabetic control, D + Glb – Diabetic treated with glibenclamide, D + MeEAR – Diabetic treated with methanol extract. Data was mean of six replicates ( $n = 6$ ) with  $\pm$ S.D, and with the significant variance \*  $P < 0.001$ , compared to Normal control group, \*\*\*  $P < 0.001$ , compared to Diabetic control group.

study, a slight increase in the body weights of all the treated groups was observed which implies that all the bioactive compounds that were dissolved in the solvents aids the body growth of the rats without causing any pathophysiological changes. Significant body weight changes were not observed during toxicity study. As no toxic symptoms were observed up to dosage of 1000 mg/kg b.wt till the end of the study acute antihyperglycemic studies were initiated with the lower dosage of 250 mg/kg b.wt for all the solvent extracts.

Streptozotocin was used to induce diabetes mellitus in rats. The choice of STZ animal model was because of its resemblance to human hyperglycaemic non ketotic diabetes mellitus [23]. Induction of STZ caused an abnormal increase in blood glucose levels ( $P < 0.0001$ ) and also decline in the body weights when compared to normal control rats. The symptoms of polydipsia, polyuria was observed in the diabetic rats. After the stability of the disease in the rats, a short term study was conducted on fasting rats to evaluate the antihyperglycemic effect of four different solvent extracts at a single concentration (250 mg/kg b.wt). Among the four solvents, the administration of MeEAR at 250 mg/kg b.wt showed the significant fall of fasting blood glucose levels from 3rd hr ( $P < 0.01$ ) and continued up to 6th hr ( $P < 0.001$ ) compared to the respective initial 0hr FBG levels (Table 4a). The rest of the extracts also showed the antihyperglycemic activity but not in a significant way. No hypoglycaemic condition was observed in any of the extracts. The probable mechanism that lies in glucose lowering effect of the *A.reticulata* leaf extracts might be due to the action of phytochemicals that were suspended in each of the extract.

Since the maximum reduction of FBG levels (30.76%) was observed in MeEAR at 250 mg/kg b.wt dosage, it was considered as the best extract with active components compared to the other extracts. A short term dose dependent study was carried out with MeEAR (250, 400, 500 mg/kg b.wt) to check the dose dependant activity of MeEAR. All the doses reduced the fasting blood glucose values significantly (Fig. 1a). But MeEAR at dosage 500 mg/kg b.wt showed the enhanced antihyperglycemic activity (46.47%) which is comparable to the positive control Glibenclamide (48.63%). The dose dependant antihyperglycemic study results were similar to the previous reports of [25]. As the MeEAR 500 mg/kg b.wt showed higher antihyperglycemic activity, oral glucose tolerance test was conducted with the same in normal rats to evaluate the efficiency of the selected extract in glucose metabolism. After glucose load, the glucose values of normal rats were raised more than that of treated rats (Glibenclamide and MeEAR) (Fig. 1b). The MeEAR metabolised the glucose efficiently from the first hour after dosing and brought the increased glucose values to the normal glucose values ( $P < 0.05$ ).

Taking in to consideration of above results, the antihyperglycemic activity was carried out with the MeEAR at dosage 500 mg/kg b.wt for 28 days in diabetic rats. During the study period of 28days, the daily administration of the plant extract had shown a positive impact on all the pathophysiological alterations that were caused in the STZ induced diabetic rats. The treatment of STZ rats with MeEAR resulted in the 57.37% of significant fall in FBG levels, whereas glibenclamide 64.28% when compared to diabetic untreated rats (Fig. 1c). STZ induced diabetic control rats experienced more polyuria, polyphagia, polydipsia suggesting that the rats suffered from excess fatigue eventually resulted in the weight loss when compared to normal and treated rats. Reduction in body weight of diabetic rats might be the result of increased damage of structural proteins of muscles [26]. The increase in body weights of the animals was also observed in treated rats (Table 4b), which implied the blood glucose stabilization effect that prevents loss of body weights [27]. Along with the glycemic control, the biochemical alterations that were caused upon the induction of STZ in the rats

were tend to normalized in glibenclamide and MeEAR treated group of rats at the variance level of ( $P < 0.05$ ).

The probable mechanism of MeEAR of *A.reticulata* leaves involved in glycemic control and increase of insulin levels might be as follows. Glibenclamide is a Sulfonylurea drug that binds to the sulfonylurea binding site (SUR1) and stimulates the exocytosis of insulin to the extracellular matrix from  $\beta$  cells of pancreas, thus increases the insulin secretion and thus decreases the blood glucose values [28]. The same mechanism of Glb might be exerted by the *A.reticulata* leaf extracts on remnant  $\beta$  cells of pancreas and exhibits the potent antihyperglycemic activity. The researchers [28] listed several plants that increase the insulin secretion. The other probable mechanism according to Ref. [28] might be the increase of glucose uptake by the cells. The improper function of glucose transporters (GLUT) in the cells during DM leads to increase of blood glucose levels. Our plant extracts might enhance the translocation of GLUT to the plasma membrane of the cells and allows the blood glucose entry in to them efficiently and thus reduces the FBG levels. Another possible action of the plant extracts might be the extracts may possess the compounds which have insulinomimetic property and stimulates lipogenesis and glucose uptake in adipocyte cells of rats.

Upon the destruction of beta cells of pancreas due to STZ, insulin levels in the rats were decreased ( $P < 0.05$ ), which eventually increases the glucose levels. The hyperglycaemic condition and insulin deficiency or insulin resistance disturbs the carbohydrate, protein, fat metabolism and likely damages the liver and kidney tissues. Therefore the present study also aimed at the evaluation of protective role of MeEAR against renal and hepatic damages rendered by diabetic condition. Upon insulin deficiency, the raised levels of hepatic enzymes ALP, SGOT, SGPT that was discharged from cytosol of liver indicates the liver damage and causes the gluconeogenesis and ketogenesis [29]. Bilirubin, being an end product of heme catabolism needs to be processed in the liver and excreted from the body overtime. The impaired function of liver leads to the rise of bilirubin in the blood. The increased levels of these markers indicated the damage of the liver tissue. Insulin deficiency decreases albumin gene transcription, thereby reduces the serum albumin levels [30]. Serum creatinine and urea levels are considered to be the prognostic markers for renal function. Abnormal increase of these levels in the serum were observed in the diabetic control rats ( $P < 0.001$ ). Creatinine is the product of muscle metabolism which needs to be filtered and excreted out through kidneys. The impairment in kidney functions results in the increased levels of creatinine in blood. The augmentation of tissue proteolysis and decline in protein synthesis along with negative nitrogen balance results in the increased levels of serum urea and creatinine levels [24]. The researchers [31] evaluated an association between serum urea levels, creatinine levels with the blood glucose levels. With the treatment of diabetic rats with MeEAR 500 mg/kg b.wt, Glb 20 mg/kg b.wt causes a decline in their levels significantly  $P < 0.05$ . The present study provided a data on the aforementioned serum marker levels (Fig. 2a and Table 4b) which were quite regulated significantly ( $P < 0.05$ ) upon the treatment with MeEAR compared to diabetic control rats and indicates the ameliorating effect of MeEAR. In line with the study of [31], the glycemic control might be the cause of fall in serum urea levels in glibenclamide and MeEAR treated groups.

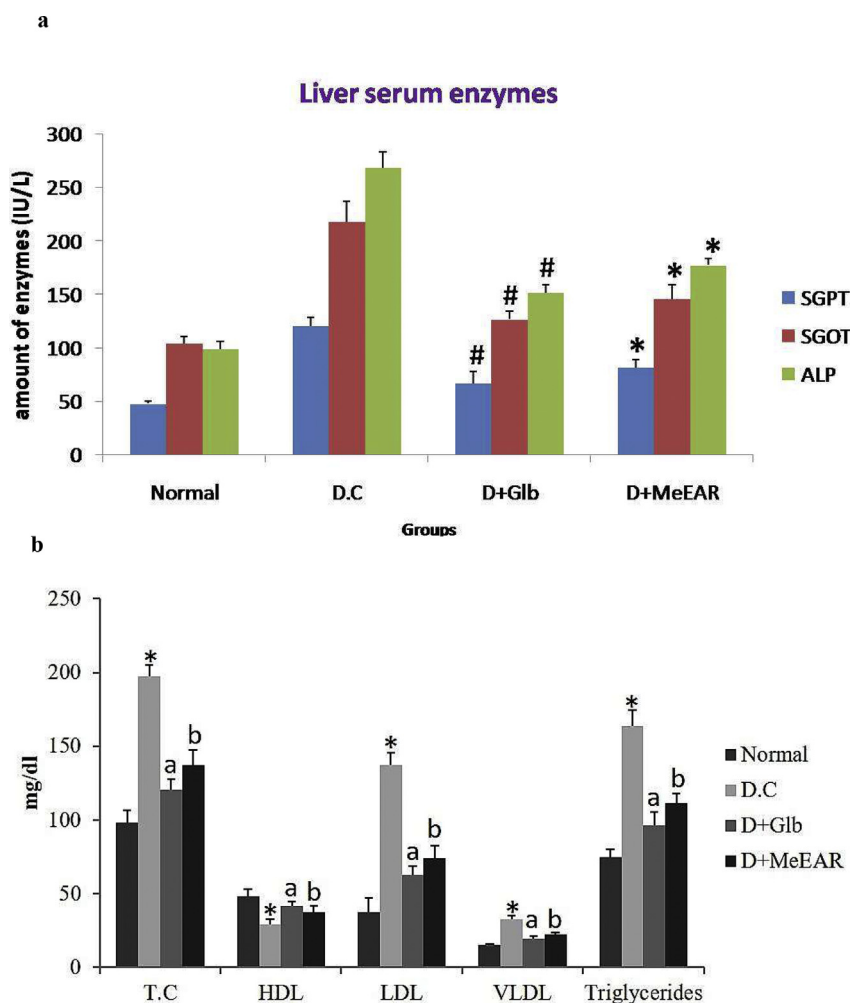
In association with hyperglycemia, lipolysis increases the free fatty acids mobilization from fat deposits that causes imbalance in lipoprotein metabolisms. The lipid metabolism was altered ( $P < 0.001$ ) drastically in diabetic rats. The low levels of insulin or insulin resistance affects the lipoprotein lipase activity and causes overproduction of TG from the liver, thereby increases VLDL levels in blood. Increased amount of TG promotes the



**Table 4b**  
Activity of MeEAR on change in body weights, Insulin, Creatinine, Urea, Albumin, Bilirubin, in STZ-induced diabetic rats.

Groups	Change in b.wt (in grams)	Insulin (mU/L)	Creatinine (mg/dl)	Urea (mg/dl)	Albumin (g/dl)	Bilirubin (mg/dl)
Normal	+10.84	15.26 ± 0.65	0.49 ± 0.07	37.5 ± 3.54	5.3 ± 0.63	0.31 ± 0.11
D.C	-20.74	7.27 ± 0.62	1.23 ± 0.06	73.16 ± 4.87	2.35 ± 0.47	1.21 ± 0.06
D + Glb	+18.55	13.28 ± 0.44 <sup>#</sup>	0.67 ± 0.04 <sup>#</sup>	47.33 ± 4.58 <sup>#</sup>	4.01 ± 0.79 <sup>#</sup>	0.64 ± 0.05 <sup>#</sup>
D + MeEAR	+10.74	11.9 ± 1.06 <sup>*</sup>	0.79 ± 0.04 <sup>*</sup>	53.33 ± 3.14 <sup>*</sup>	3.81 ± 0.3 <sup>*</sup>	0.86 ± 0.06 <sup>*</sup>

All the represented data was mean of six replicates (n = 6) ± S.D. The values with superscript symbols (\*, #) were significantly vary (P < 0.05) comparing with diabetic control group. D.C- Diabetic control, D + Glb – Diabetic treated with glibenclamide, D + MeEAR – Diabetic treated with methanol extract. Change in body weights (b.wt) is difference between initial day body weights and final day body weights (28th day). '+' value indicates increase in body weight in grams, '-' indicates reduction in body weight in grams.

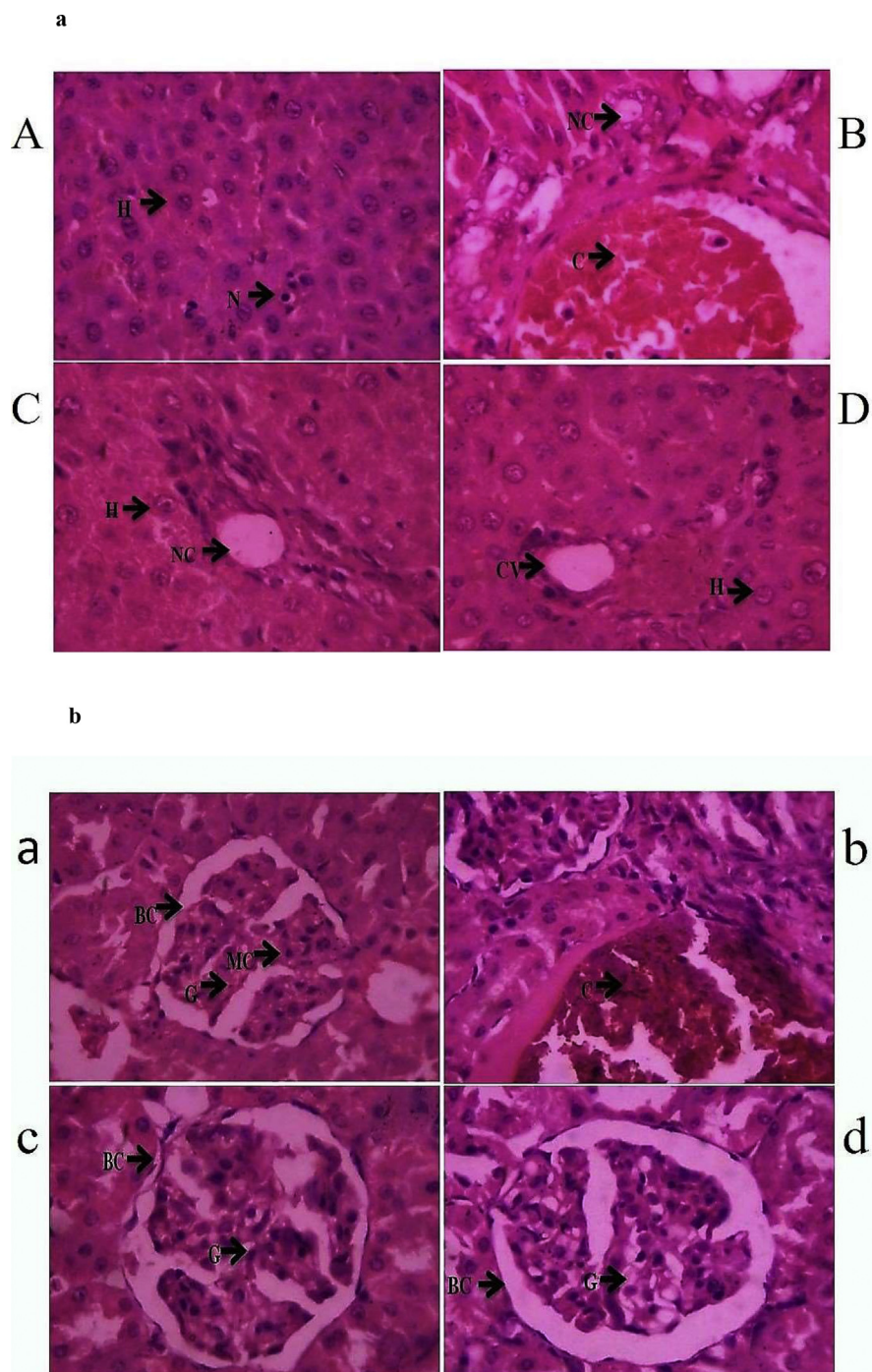


**Fig. 2.** a. Effect of MeEAR on liver enzyme markers in STZ induced diabetic rats: The figure represented the serum levels of SGOT - Serum glutamate oxaloacetate transaminase, SGPT - Serum glutamate pyruvate transaminase, ALP - Alkaline phosphatase in normal and streptozotocin induced diabetic and treated rats. D.C – Diabetic control, D + Glb – Diabetic treated with glibenclamide, D + MeEAR – Diabetic treated with methanol extract. Values with symbols (#,\*) were significantly vary at P < 0.05 when compared to diabetic control group. b. Effect of MeEAR on lipid profile of STZ induced diabetic rats: The figure represented the effect of MeEAR on the lipid markers. Values are mean of six independent variables with ±S.D. D.C – Diabetic control, D + Glb – Diabetic treated with glibenclamide, D + MeEAR – Diabetic treated with methanol extract. Significance was '\*' P < 0.001 compared to Normal control group. The letters 'a' and 'b' significantly different at P < 0.05 when compared to diabetic control group.

release of small dense LDL molecules accompanied with the decrease of HDL cholesterol concentrations [32]. Several studies demonstrated that glycemic control was the first line priority to overcome the changes in lipid metabolism by increasing the insulin levels. In the present study it was evidently observed that the MeEAR improved the lipid profile. With the increased level of serum insulin and down regulated glucose values, simultaneous improved levels of HDL, decline in LDL, VLDL, triglycerides, total cholesterol levels were observed significantly at P < 0.05 (Fig. 2b). The pathophysiological alterations that are

caused in diabetes directly or indirectly interlinks with the insulin deficiency or insulin resistance. So the up regulation of insulin levels by MeEAR might be the underlying mechanism for the regulation of the other serum biochemical parameters including FBG levels.

The histopathological alterations of liver and kidney tissues implicated that diabetic condition affects the crucial organs of the body in case of their structure and functions. The degenerative changes in liver such as necrosis, sinusoidal dilation with the symptoms of congestion and disarrangement of hepatocytes,

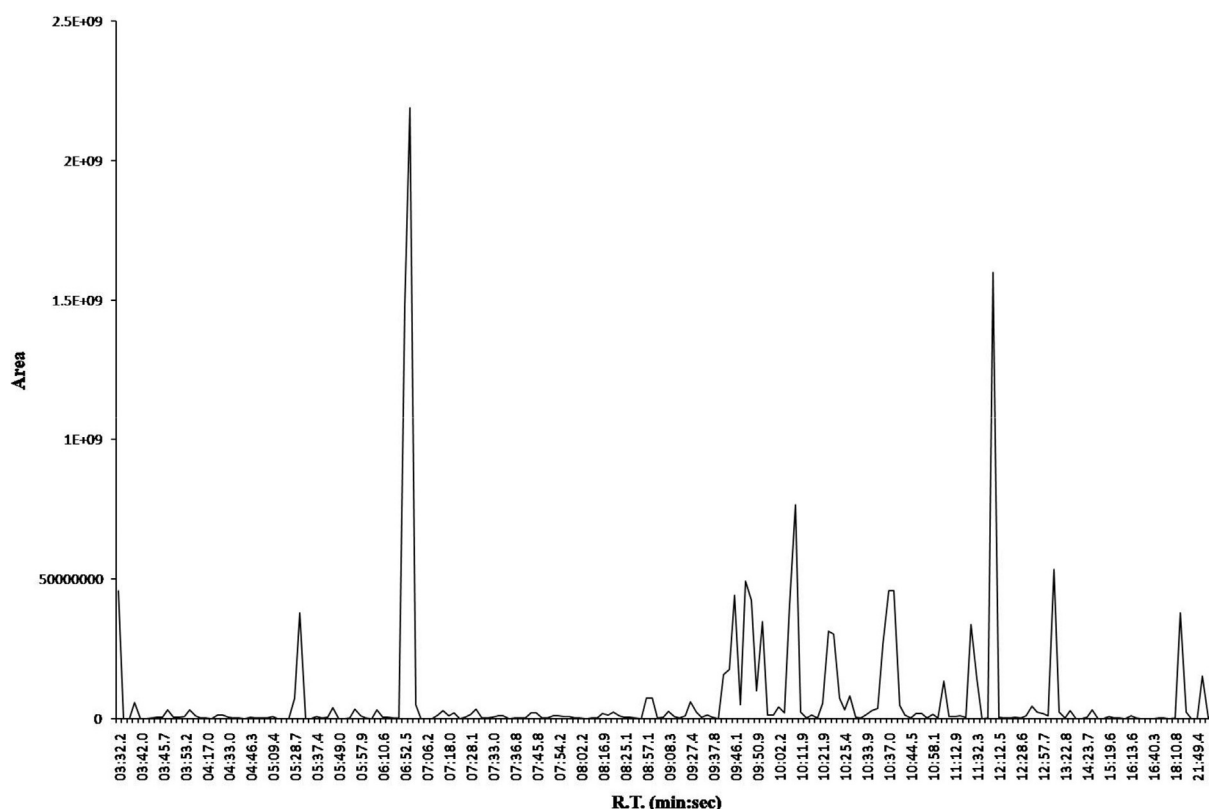


**Fig. 3.** a. Effect of MeEAR on the histopathology of liver tissue (400X) of normal and STZ induced diabetic control and treated rats: Histopathological observation of liver sections under microscopy (400X). **A** –Normal control, **B** – Diabetic control, **C** – Diabetic + treated with Glib (20 mg/kg b.wt), **D**– Diabetic + treated with MeEAR (500 mg/kg b.wt.). H- Hepatocytes, N- Nucleus, CV- Central vein, C- Congestion, NC- Necrotic change. b. Effect of MeEAR on the histopathology of kidney tissue (400X) of normal and STZ induced diabetic control and treated rats: Histopathological observation of kidney sections under microscopy (400X). **a** –Normal control, **b** – Diabetic control, **c** – Diabetic + treated with Glib (20 mg/kg b.wt), **d** – Diabetic + treated with MeEAR (500 mg/kg b.wt.). BC- Bowman's capsule, G- Glomeruli, MC- Mesangial cells, C- Congestion.

vacuolization were observed in diabetic control liver tissue. Glomerular hypertrophy, mesangial matrix accumulation, necrosis etc., demonstrated the diabetic control kidney histology. The elevated levels of liver serum (SGPT, SGOT, ALP, bilirubin), kidney markers (urea and creatinine) supported the hepatic and renal tissues damage. The ameliorating effect of MeEAR on the histology of tissues was quite distinct in Fig. 3a and b. Diminution in necrosis, shrinkage of cells, hypertrophy, congestion features, vacuolization,

inflammatory infiltration and other structural deformities in both kidney and liver implicated the apparent recovery of the tissues upon administration of MeEAR for 28 days compared to the diabetic control tissues and also near to the glibenclamide treated tissues.

Following the *in vivo* antidiabetic studies, GC–MS analysis was performed for MeEAR to identify the bioactive compounds in the crude extract. The observed active compounds in MeEAR include



**Fig. 4.** The GC–MS chromatogram of Methanolic leaf extract of *Annona reticulata* L. GC–MS total ion chromatogram of Methanolic extract of *Annona reticulata* L. with the peaks representing the presence of different compounds at different retention time.

carbohydrates, fatty acid esters, amino acid derivatives, carboxylic acid groups etc. Apart from identified compounds, there were some unknown compounds with high relative abundance. The independent or collective action of aforementioned compounds (Table 5 supplementary file) might aid the antihyperglycemic property of MeEAR. Some amino acids and its derivatives were also present in minute amounts. Studies reported that amino acids play a vital role in wound healing processes [33]. The identified compounds possess diversified pharmacological activities reported earlier. The effect of myo-inositol (10.64%) and chiro inositol (0.94%) on type 2 diabetes was evaluated by a pilot study of [34]. They put forwarded that myo-inositol improved the insulin sensitivity mainly in muscle and fat tissues along with a valid strategy in glycemic control in the diabetic patients. Tetracosane was reported to have antioxidant, cytotoxicity, cardiotoxic, antibacterial activity [35]. Propanoic acid (0.22%) has antioxidant and anti-proliferative activity [36], Cinnamic acid (0.02%) is an aldose reductase inhibitor, cancer preventive and possesses antibacterial properties (Dr. Duke's phytochemical and ethnobotanical databses). L-Alanine (0.06%) is a cancer protective, antiprostatic, antipancreatic. 5-Hydroxy-L-tryptophan (0.33%) treats insomnia, anxiety, obesity, parkinson's disease. L-Norvaline is known to promote tissue regeneration and muscle growth. L-Norleucine plays avital role in Alzheimer's disease. Dodecanoic acid (0.06%) also known as lauric acid, has antioxidant, antibacterial, hypercholesterolemic (HDL cholesterol) properties (Dr. Duke's phytochemical and ethnobotanical databses). Hexadecanoic acid (2.23%) possesses Antioxidant, 5-Alpha reductase inhibitor, hypocholesterolemic, haemolytic properties [37]. In addition to the above compounds, the other compounds in the extract also possess various pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant etc. WHO recognized the significance of herbal medicine to treat various ailments and

included metformin, a plant derived antidiabetic drug in essential medicine's list for treatment of T2DM. Similarly, studies on the identification of bioactive compounds in crude MeEAR would lead to the fractionation, isolation and purification of single active compound from leaf extract that may assist in the commercial drug development for the treatment or management of T2DM.

## 5. Conclusion

In conclusion of our study, the crude MeEAR of *A. reticulata* leaves is rich in phytochemicals, with potent antihyperglycemic activity along with the protective potential towards biochemical alterations associated with the diabetic condition. The present study laid a scientific evidence for the traditional use of the *A. reticulata* L. as antidiabetic plant. The identification of various bioactive compounds in the crude extract of *A. reticulata* by GC–MS analysis would pave a path towards the deep insights on isolation of active causative compounds which are liable for glycemic control, also evaluating the exact mechanism of the active constituent etc of *A. reticulata* leaves.

## Source(s) of funding

None.

## Conflict of interest

None.

## Acknowledgments

None.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaim.2021.01.010>.

## References

- [1] Wu Y, Ding Y, Tanaka Y, Zhang W. Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *Int J Med Sci* 2014;11(11):1185. <https://doi.org/10.7150/ijms.10001>.
- [2] Wilcox G. Insulin and insulin resistance. *Clin Biochem Rev* 2005;26(2):19.
- [3] Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab* 2008;294(1):8. <https://doi.org/10.1152/ajpendo.00645.2007>.
- [4] Fan W. Epidemiology in diabetes mellitus and cardiovascular disease. *Cardiovasc Endocrinol* 2017;6(1):8. <https://doi.org/10.1097/XCE.0000000000000116>.
- [5] Vijayalakshmi K, Selvaraj CI. Evaluation of antidiabetic potential of *Sarcostemma brevistigma* Wight & Arn. Using alloxan-induced diabetic murine model. *Appl Biochem Biotechnol* 2019;187(1):14–27.
- [6] Abirami A, Nagarani G, Siddhuraju P. In vitro antioxidant, anti-diabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from Citrus hystrix and C. maxima fruits. *Food Sci Hum Wellness* 2014;3(1):16–25. <https://doi.org/10.1016/j.fshw.2014.02.001>.
- [7] Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2015;38(1):140–9. <https://doi.org/10.2337/dc14-2441>.
- [8] Olubomehin OO, Abo KA, Ajaiyeoba EO. Alpha-amylase inhibitory activity of two Anthocleista species and in vivo rat model anti-diabetic activities of Anthocleista djalonensis extracts and fractions. *J Ethnopharmacol* 2013;146(3):811–4. <https://doi.org/10.1016/j.jep.2013.02.007>.
- [9] Jamkhande PG, Wattamwar AS. *Annona reticulata* Linn. (Bullock's heart): plant profile, phytochemistry and pharmacological properties. *J Tradit Complement Med* 2015;5(3):144–52. <https://doi.org/10.1016/j.jtcm.2015.04.001>.
- [10] Kadali VN, Sandeep BV. Anti-hyperglycemic plants used by the traditional healer of west Godavari District, Andhra Pradesh, India. *Int J Pharmacogn* 2015;2(9):473–7. [https://doi.org/10.13040/IJPSR.0975-8232.IJP.2\(9\).473-77](https://doi.org/10.13040/IJPSR.0975-8232.IJP.2(9).473-77).
- [11] Rout SP, Kar DM, Mohapatra SB, Swain SP. Anti-hyperglycemic effect *Annona reticulata* L. leaves on experimental diabetic rat model. *Asian J Pharmaceut Clin Res* 2013;6(1):56–60.
- [12] Herborne JB. Phytochemical methods. A guide to modern techniques of plant analysis. 1973. p. 5–11.
- [13] Alhakmani F, Kumar S, Khan SA. Estimation of total phenolic content, in-vitro antioxidant and anti-inflammatory activity of flowers of *Moringa oleifera*. *Asian Pac J Trop Biomed* 2013;3(8):623. [https://doi.org/10.1016/S2221-1691\(13\)60126-4](https://doi.org/10.1016/S2221-1691(13)60126-4).
- [14] Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *J Taibah Univ Sci* 2015;9(4):449–54. <https://doi.org/10.1016/j.jtusc.2014.11.001>.
- [15] Telagari M, Hullatti K. In-vitro  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity of *Adiantum caudatum* Linn. and *Celosia argentea* Linn. extracts and fractions. *Indian J Pharmacol* 2015;47(4):425. <https://doi.org/10.4103/0253-7613.161270>.
- [16] OECD O. Guidelines for the testing of chemicals, acute oral toxicity—acute toxic class method (423). Available online on. 2001. <http://www.oecdbookshop.org/oecd/index.asp>.
- [17] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *ClinChem* 1972;18(6):499–502. <https://doi.org/10.1093/clinchem/18.6.499>.
- [18] Okur ME, Karantas ID, Sifakia PI. Diabetes Mellitus: a review on pathophysiology, current status of oral pathophysiology, current status of oral medications and future perspectives. *Acta Pharm Sci* 2017;55(1). <https://doi.org/10.23893/1307-2080.APS.0555>.
- [19] Cicero AF, Colletti A. Role of phytochemicals in the management of metabolic syndrome. *Phytomedicine* 2016;23(11):1134–44. <https://doi.org/10.1016/j.phymed.2015.11.009>.
- [20] Patel DK, Kumar R, Prasad SK, Sairam K, Hemalatha S. Antidiabetic and in vitro antioxidant potential of *Hybanthus enneaspermus* (Linn) F. Muell in streptozotocin-induced diabetic rats. *Asian Pac J Trop Biomed* 2011;1(4):316–22. [https://doi.org/10.1016/S2221-1691\(11\)60051-8](https://doi.org/10.1016/S2221-1691(11)60051-8).
- [21] El-Chaghaby GA, Ahmad AF, Ramis ES. Evaluation of the antioxidant and antibacterial properties of various solvents extracts of *Annona squamosa* L. leaves. *Arab J Chem* 2014;7(2):227–33. <https://doi.org/10.1016/j.arabjc.2011.06.019>.
- [22] Kidane Y, Bokrezion T, Mebrahtu J, Mehari M, Gebreab YB, Fessehaye N, et al. In vitro inhibition of amylase and-glucosidase by extracts from *Psidium punctulata* and *Meriandra bengalensis* vol. 2018. *Evid Based Complement Alternat Med*; 2018. <https://doi.org/10.1155/2018/2164345>.
- [23] Laoufi H, Benariba N, Adjdir S, Djaziri R. In vitro  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity of *Ononis angustissima* extracts. *J Appl Pharmaceut Sci* 2017;7(2):191–8. <https://doi.org/10.7324/JAPS.2017.70227>.
- [24] Nabi SA, Kasetti RB, Sirasanagandla S, Tilak TK, Kumar MV, Rao CA, et al. Antidiabetic and antihyperlipidemic activity of Piper longum root aqueous extract in STZ induced diabetic rats. *BMC Compl Alternative Med* 2013;13(1):37. <https://doi.org/10.1186/1472-6882-13-37>.
- [25] Rao NK, Bethala K, Sisinthy SP, Rajeswari KS. Antidiabetic activity of *Orthosiphon stamineus* benth roots in streptozotocin induced type 2 diabetic rats. *Asian J Pharmaceut Clin Res* 2014;7(1):149–53.
- [26] Junejo JA, Rudrapal M, Nainwal LM, Zaman K. Antidiabetic activity of hydro-alcoholic stem bark extract of *Callicarpa arborea* Roxb. with antioxidant potential in diabetic rats. *Biomed Pharmacother* 2017;95:84–94. <https://doi.org/10.1016/j.biopha.2017.08.032>.
- [27] Soni LK, Dobhal MP, Arya D, Bhagour K, Parasher P, Gupta RS, et al. In vitro and in vivo antidiabetic activity of isolated fraction of *Prosopis cineraria* against streptozotocin-induced experimental diabetes: a mechanistic study. *Biomed Pharmacother* 2018;108:1015–21. <https://doi.org/10.1016/j.biopha.2018.09.099>.
- [28] Prabhakar PK, Doble M. A target based therapeutic approach towards diabetes mellitus using medicinal plants. *Curr Diabetes Rev* 2008;4(4):291–308.
- [29] Gayathri M, Kannabiran K. Antidiabetic activity of 2-hydroxy 4-methoxy benzoic acid isolated from the roots of *Hemidesmus indicus* on streptozotocin-induced diabetic rats. *Int J Diabetes Metabol* 2009;17:53–7.
- [30] Bae JC, Seo SH, Hur KY, Kim JH, Lee MS, Lee MK, et al. Association between serum albumin, insulin resistance, and incident diabetes in nondiabetic subjects. *Endocrinol Metab* 2013;28(1):26–32. <https://doi.org/10.3803/EnM.2013.28.1.26>.
- [31] Bamanikar SA, Bamanikar AA, Arora A. Study of Serum urea and Creatinine in Diabetic and nondiabetic patients in a tertiary teaching hospital. *J Media Res* 2016;2(1):12–5.
- [32] Asato Y, Katsuren K, Ohshiro T, Kikawa K, Shimabukuro T, Ohta T, et al. Relationship between lipid abnormalities and insulin resistance in Japanese school children. *Arterioscler Thromb Vasc Biol* 2006;26(12):2781–6. <https://doi.org/10.1161/01.ATV.0000245804.56871.31>.
- [33] Buszewska-Forajta M, Struck-Lewicka W, Bujak R, Siluk D, Kalisz R. Determination of water-soluble components of abdominal secretion of grasshopper (*Chorthippus* spp.) by GC/MS/MS in search for potential wound healing agents. *Chromatographia* 2014;77(15–16):1091–102. <https://doi.org/10.1007/s10337-014-2679-8>.
- [34] Pintauro B, Di Vieste G, Bonomo M. The effectiveness of myo-inositol and D-chiro inositol treatment in type 2 diabetes. *Int. J. Endocrinol.* 2016;2016. <https://doi.org/10.1155/2016/9132052>.
- [35] Banakar P, Jayaraj M. GC-MS analysis of bioactive compounds from ethanolic leaf extract of *Waltheria indica* linn. And their pharmacological activities. *Int J Pharma Sci Res* 2018;9(5):2005–10. [https://doi.org/10.13040/IJPSR.0975-8232.9\(5\).2005-10](https://doi.org/10.13040/IJPSR.0975-8232.9(5).2005-10).
- [36] Kifayatullah M, Sarker MM, Mustapha MS. Phytochemical investigation of ethanolic extract of *Pericampylus glaucus* leaves from Malaysia by GC-MS analytical technique. *Int J Pharmtech Res* 2017;10:234–40.
- [37] George LO, Radha HR, Somasekariah BV. In vitro anti-diabetic activity and GC-MS analysis of bioactive compounds present in the methanol extract of *Kalanchoe pinnata*. *Indian J Chem* 2018;57B:1213–21. <http://nopr.niscair.res.in/handle/123456789/45041>.