Evaluation of Antihypertensive activity of Punica Granatum Linn. in High Fat Diet and Streptozotocin Induced Diabetes in Rats

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Abstract: Diabetes Mellitus is one of the most prevalent metabolic disorders charactrised with increased blood sugar level and improper primary metabolism. It is charactrised by alteration in metabolism of carbohydrate, fat and protein, which are caused by inappropriate secretion of insulin or insulin resistance. The number of people with diabetes is increased due to population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity (Firdous et al., 2016).

Type 1 it is also called as Insulin Dependent Diabetes Mellitus (IDDM). It is due to failure of body for insulin production. It is often childhood disease so it is also called as Juvenile onset diabetes mellitus. In other words, it is a non-autoimmune, complex, heterogeneous and polygenic metabolic disease condition in which the body fails to produce enough insulin, characterized by abnormal glucose homeostasis. Its pathogenesis appears to involve complex interactions between genetic and environmental factors. It occurs when impaired insulin effectiveness is accompanied by the failure to produce sufficient ß-cell insulin (Shivasankar et al, 2011).

Type 2 it is also called as Non Insulin Dependent Diabetes Mellitus (NIDDM). In this type cells are unable for insulin usage. The other name of this type is adult onset diabetes mellitus (Soni, 2013). Type 2 diabetes is often, but not always, associated with metabolic abnormalities such as obesity, which itself can cause insulin resistance and lead to elevated blood glucose levels. Whereas type 2 diabetes is thought to be primarily heterogeneous and polygenic with low penetrance for the variants discovered, there exist monogenic types of non-autoimmune diabetes showing a Mendelian dominant pattern of inheritance, of which maturity-onset diabetes of the young (MODY) is the most common type 2 (Hertel, 2012).

1.INTRODUCTION

1.1 Diabetes mellitus

The terms "Diabetes" and “Mellitus” are derived from Greek. “Diabetes” denotes "a passer through a siphon" whereas the "Mellitus" denotes "sweet" (Piero et al., 2014). Diabetes represents a heterogeneous group of diseases characterized by changes in insulin secretion or action, resulting in chronic hyperglycemia and altered metabolism of carbohydrates, protein, and lipids (Vanessa E, et al, 2013). Chronicity of hyperglycemia is associated with long-term damage and failure of various organ systems mainly affecting the eyes, nerves, kidneys, and the heart (Chawla et al., 2016). A complex multifactorial disease increases the risk for macrovascular complications that are associated with cardiovascular diseases, mainly coronary artery disease, atherosclerosis, hypertension and stroke (Buraczynska et al., 2016).

1.2 Types diabetes mellitus

There are several forms of diabetes. Scientists are still defining and categorizing some of these variations and establishing their prevalence in the population. Types of diabetes include:

1.2.1 Type 1 diabetes (Insulin dependent diabetes mellitus):

It is much less common with only 5-10% of all diabetes cases being type 1. This type of diabetes usually present itself early in life though can occur at any age with some cases not being seen until the patient elderly (Simpson et al., 2014). Type 1 diabetes mellitus is a chronic autoimmune disease associated with selective destruction of insulin-producing pancreatic β-cells. The onset of clinical disease represents the end stage of β-cell destruction leading to type 1 diabetes mellitus (Ozougwu et al., 2013).

1.2.2 Type 2 diabetes (Non Insulin dependent diabetes mellitus):

Type 2 diabetes mellitus is chronic, progressive metabolic disease defined by the presence of hyperglycemia. It is characterized by hyperglycemia, decreased β cell numbers and maximal secretory

functions, rapid gastric emptying, increase apetite, obesity, systemic inflammation, elevated cytokines, hypercoagulation, and endothelial cell dysfunction (Faramarz, 2012). Type 2 diabetes mellitus is a multifaceted, widespread, chronic disease which clusters numerous comorbidities (e.g., insulin resistance, hypertension, and dyslipidemia), aggregates coagulability, impairs fibrinolysis, increases inflammatory burden, and promotes microalbuminuria (Kadoglou et al., 2009). People with type 2 diabetes are not dependent on exogenous insulin but may require it for the control of blood glucose levels if this is not achieved with diet alone or with oral hypoglycemic agents (Tripathi et al., 2006).

1.2.3 Gestational diabetes mellitus:

Gestational diabetes is a type of diabetes that develops only during pregnancy. The hormones produced during pregnancy increase the amount of insulin needed to control blood glucose levels. If the body can't meet this increased need for insulin, women can develop gestational diabetes during the late stages of pregnancy (NIH, 2014).

1.3. Complication of diabetes mellitus:

The vascular complications are sub-divided into two categories viz. Microvascular (nephropathy, neuropathy and retinopathy) and Macrovascular (Myocardial infarction, Hypertension, peripheral artery disease).

1.3.1. Diabetic nephropathy:

Diabetic nephropathy is the leading cause of renal failure. It is defined by proteinuria > 500 mg in 24 hours in the setting of diabetes, but this is preceded by lower degrees of proteinuria, or "microalbuminuria." Microalbuminuria is defined as albumin excretion of 30–299 mg/24 hours. Without intervention, diabetic patients with microalbuminuria typically progress to proteinuria and overt diabetic nephropathy. This progression occurs in both types 1 and type 2 diabetes (Fowler et al., 2008). The development and progression of nephropathy is highly complex given the diversity of cell populations present within the kidney and the various physiological roles of this organ. Indeed, aside from the filtration of toxins from the blood for excretion, it is difficult to pinpoint which other functional aspects of the kidney are most affected by diabetes. These include the release of hormones such as erythropoietin, activation of vitamin D, and acute control of hypoglycemia, in addition to maintenance of fluid balance and blood pressure via salt reabsorption. High glucose concentrations induce specific cellular effects, which affect various resident kidney cells including endothelial cells, smooth muscle cells, mesangial cells, podocytes, cells of the tubular and collecting duct system, and inflammatory cells and myofibroblasts (Forbes et al., 2013). The severity of glomerular damage is proportional to GFR value, DM duration, and blood glucose regulation. The main pathophysiological changes in diabetic nephropathy include the thickening of the glomerular basement membrane (GBM), mesangial expansion, nodular sclerosis – Kimmelstiel-Wilson change, diffuse glomerular sclerosis, tubular interstitial fibrosis, and arteriosclerosis and hyalinosis of kidney blood vessels Among other pathological lesions, we should mention hyalinosis, the so-called fibrin cap, which consists of accumulated hyaline material between endothelial cells and glomerular basement membrane(Vujicic et.al., 2012).

1.3.2. Diabetic neuropathy:

Diabetic neuropathy is the most common and troublesome complication of diabetes mellitus, leading to the greatest morbidity and mortality and resulting in a huge economic burden for diabetes care. Diabetic neuropathy is a set of clinical syndromes that affect distinct regions of the nervous system, singly or combined. It may be silent and go undetected while exercising its ravages; or it may present with clinical symptoms and signs that, although nonspecific and insidious with slow progression, also mimic those seen in many other diseases. Causative factors include persistent hyperglycemia, microvascular insufficiency, oxidative and nitrosative stress, defective neurotropism, and autoimmune-mediated nerve destruction (Aron et al., 2013).

Neuropathic pain is a persistent chronic pain resulting from damage to the central or peripheral pain signaling pathways. Neuropathy is the most common and debilitating complication of diabetes mellitus and results in pain and decreased motility. Diabetic Neuropathy is a comprehensible disorder, either subclinical or clinically evident that occurs in both autonomic and the peripheral nervous systems. Neuropathies are the most common complication of diabetes mellitus affecting up to 50% of patients with

Type 1 and Type 2 diabetes. In type 1 diabetes, distal polyneuropathy becomes sympto-matic after several years of diagnosis; in contrast, type 2 diabetes patients may have neuropathy at the time of diagnosis. Neuropathic pain develops as a consequence of lesions or disease affecting the somatic sensory nervous system moreover peripherally or centrally. Examples of neuropathic pain include painful polyneuropathy, post-therapeutic neuralgia, trigeminal neuralgia and post stroke pain. Neuropathic pain is characterized by spontaneous ongoing or shooting pain and evoked amplified pain response after noxious responses or non- noxious stimuli. Diabetic neuropathy is a heterogeneous disorder with high morbidity and can be classified as a number of different syndromes ranging from sub-clinical to clinical manifestations, depending on the classes of fibers involved (Khan et al, 2015).

The pathological mechanisms implicated in diabetic neuropathy, include microvascular damage, metabolic disorders, and changes in the interactions between neuronal and immunological systems in parallel with glial cell activation. Changes in the blood vessels supplying the peripheral nerves underlie the mechanisms involved in microvascular damage and hypoxia. These changes are based on increases in wall thickness with the hyalinization of the vessel walls and the basal lamina of arterioles and capillaries, leading to nerve ischemia. Through revised primary capillary membrane to the endoneurium penetrates the plasma protein, causing swelling and increased interstitial pressure in the nerves as well as capillary pressure, fibrin deposition and thrombus formation (Mika et al., 2013).

1.3.3. Diabetic retinopathy:

Diabetic retinopathy is the most microvascular complication of diabetes and remains one of the leading causes of blindness worldwide among adult's ages 20-74 yrs (Ahmed et al., 2009).

Diabetic retinopathy is classified into non proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR), characterised by the growth of new blood vessels (retinal neovascularization). NPDR is further divided into mild, moderate, and severe stages that may or may not involve the development of a macula diabetic macular oedema (DMO). The major causes of severe visual impairment are PDR and DMO. Nearly all patients with Type 1 diabetes and >60% of patients with Type 2 diabetes are expected to have some form of retinopathy by the first decade of incidence of diabetes (Chopra et al., 2013). Microaneurysms are the early signs of diabetic retinopathy that are ophthalmoscopically visible. These appear as red dots and are difficult to distinguish from small dot haemorrhages. The rupture of these aneurysms results in haemorrhages. The haemorrhages in the deeper layers of retina appear as blot haemorrhages. Superficial haemorrhages may appear as flame shaped haemorrhages as those seen in retinopathy. Even when there is no clinically detectable retinopathy, changes at hemodynamic and cellular levels take place. The endothelial [inner lining of the blood vessels] cell supporting cells called pericytes are affected early resulting in endothelial damage.

The retinal blood flow is decreased. The retinal autoregulation that maintains the level of blood supply to retina is impaired. Clinically evident retinopathy appears as the disease progresses. The various signs that appear are microaneurysms, dot and blot retinal haemorrhages, cotton wool spots, venous calibre changes and retinal capillary non-perfusion. Increasing retinal ischaemia [reduced blood flow] triggers the production of vasoproliferative factors that causes development of new vessels seen in proliferative diabetic retinopathy. Diabetes also causes retinal blood vessels to be more permeable resulting in transudation of serum components. This results in retinal thickening and causes macular oedema (Bailey et al., 2014).

1.4. Macrovascular complication:

Diabetes magnifies the risk of macrovascular diseases independently of conventional risk factors. The risk of developing coronary and peripheral artery disease are more than doubled in diabetes. In addition to increasing the risk of developing macrovascular complications, diabetic patients also experience adverse outcomes with high rates of recurrence and mortality (Beckman et al., 2002).

1.4.1. Cardiovascular complication:

Cardiovascular complications are a chief cause of mortality and morbidity in diabetic patient. Diabetic individuals are at significantly greater risk of developing heart failure independent from other risk factors such as coronary artery disease and

hypertension (Jiang et al., 2012). Hypertension is a major risk factor for cardiovascular disease and particularly, in diabetes mellitus (Michael et al., 2015). Hypertension is a common comorbid condition in patient with type 1 or type 2 diabetes (European Society of Hypertension, 2003). The development of vascular complications is determined by the presence of common risk factors in T2DM such as obesity, chronic hyperglycemia, insulin resistance, dyslipidemia and states of inflammation and oxidation (Fontana et al., 2016). Diabetes mellitus and Hypertension are several pathophysiological mechanisms including inappropriate activation of the renin-angiotensin-aldosterone system (RAAS), oxidative stress, secondary to excessive production of reactive oxygen species (ROS), inflammation, impaired insulin-mediated vasodilatation, increased sympathetic nervous system (SNS) activation, dysfunctional innate and adaptive immune responses, and abnormal renal processing of sodium. Obesity and increased visceral adiposity are key pathogenic factors of both Diabetes and Hypertension (Lastra et al., 2014).

The quality of diabetes care is not only able to control blood sugar levels but it is also involved in comprehension of risk factor management. Subsequently, it will help to prevent both macrovascular and micro-vascular complications, decrease mortality as well as improve quality of life in these patients (Sudchada et al, 2012).

Current strategies for controlling the cardiovascular complications of diabetes primarily target a cluster of well-defined risk factors, such as hyperglycemia, lipid disorders and hypertension (Lin et al., 2014).

Prevalence of the complications is greater among the lower socio-economic people due to lack of good control of glycaemia and hypertension and also due to behavioral factors. The direct and indirect costs involved in the treatment of the chronic disease especially when associated with the vascular complications are enormous (Brahmachari, 2011).

Numbers of researcher have worked on traditional Indian plant for treatment of diabetes (Chika et al., 2010). The ethnobotanical information reports about 800 plants that may possess anti-diabetic potential (Yasodamma et al., 2013). The oral hypoglycemic agents are usually produced serious side effect (Tahrani et al., 2010). Hence despite the presence of various antidiabetic medicines on the market, diabetes and related complications are continued to be a major problem (Ezzat SM, et.al, 2013). Traditionally used medicinal plants can provide effectiveness, less side effect and relatively low costs (Venkatesh S, et.al, 2003). This unique characteristic has contributed to the world wide interest in herbal medicine as an alternative therapy compared to currently available synthetic drugs which are associated with serious side effect and are usually not much effective in altering the course of diabetic complications (Patel et al., 2015).

Table1. List of plant used in Antihypertensive activity.

Sr.No	Plant Name	Plant Part	Used In	Reference
1	*Passiflora nepalensis*	Whole plant	Antihypertensive	Josh et al., 2012
2	*Olea europaea*	Leaves, oil, bark	Antihypertensive	Sedef et al., 2009
3	*Cynodon dactylon*	Leaf, root	Antihypertensive	Bharti et al., 2016
4	*Nauclea latifolia*	Stem	Antihypertensive	Odey et al., 2013
5	*Phyllanthus niruri*	Leaves, stem	Type 2 diabetic and hypertension	Bharti et al., 2016
6	*Indian snakeroot*	Root	Antihypertensive	Agarwal et al., 2010
7	*Cynodon dactylon*	Whole plant	Antihypertensive	Nagori et al., 2011
8	*Tribulus terrestris*	Fruits	Antihypertensive and vasodilator	Oludotun et al., 2006
9	*Bixa orellana*	Leaf, root	Antihypertensive, diabetes	Lans et al, 2006
10	*Origanum majorana*	Leaves	Diabetes, hypertension	Tahraoui et al., 2007
11	*Euphorbia hirta*	Whole plant	Diabetes, hypertension	Mensah et al., 2009
12	*Allium sativum*	Cloves	Antihypertensive	Kumar et al., 2011
13	*Annona muricata*	Fruits	Antihypertensive	Tabassum et al., 2011
14	*Erythrophleum suaveolen*	Bark	Antihypertensive	Lagnika et al., 2016
15	*Sonchus asper*	Aerial parts	Antihypertensive	Mushtaq et al., 2016

Punica granatum L. commonly known as pomegranate is a fruit bearing deciduous shrub or small tree, native to Asia and belongs to family Lythraceae (Altuner, 2011).The potential therapeutic

properties of pomegranate are wide-ranging and include treatment and prevention of diabetic complications like retinopathy, neuropathy, nephropathy, and cardiovascular diseases (Bhandary et al., 2012; Baliga et al., 2013). Pomegranate can be consumed as fresh, fruit juice, fermented fruit juice, dried aril, frozen aril, minimally-processed aril, canned aril, jam, jelly, wine, vinegar, paste, and fruit leather and in flavoring products (Bayizit et al., 2012). In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. In Ayurvedic medicine the pomegranate is considered "a pharmacy unto itself "and is used as a "blood tonic" (Mohammad et al., 2012).

Punica granatum flower extract have been reported to possess antihyperglycemic activity (Bhaskar et al, 2012). Therapeutically beneficial chemical constituent's simillar to that flower extract have been reported from leaves of *punica granatum* linn. Such as ellagitannins, gallic acid, anthrocyanins, piperidine alkaloids, flavonoids luteolin, apigenin, and quercetin (Garachh et al., 2012; Fozia et al., 2012). Further, our earlier work proved the preliminary antihyperglycemic and antioxidant activities of P. granatum leaves (Patil et al., 2013) and renal protective activity of flavonoid rich fraction of *punica granatum* (Patel et al., 2015). We, hypothesized that *punica granatum* may exert Cardioprotective activity through its antihyperglycemic and antioxidant properties. Thus, the aim of our present work was to investigate cardioprotective effect of flavonoid rich fraction of *Punica granatum* leaves in High fat diet (HFD) and Streptozotocin (STZ) induced diabetes in rats.

2. MATERIALS AND METHOD

2.1. Plant material

2.1.1. Collection and authentication of plant material

The leaves of *punica granatum* Linn. Of were collected from the pune in the month of June 2016. The plant was identified and authenticated by Department of Botany, Savitribai Phule Pune University, Pune and a Ref. No (A4/2016) was deposited in the herbarium for future references. The leaves were dried in shade and subjected to size reduction to a coarse powder by using dry grinder.

2.1.2. Preparation of fractions

The crude hydroalcoholic extract was prepared by cold maceration technique using 1000 ml of solvents ethanol: water (70:30) for 72 hours at room temperature. The liquid-liquid partitioning of the crude hydroalcoholic extract was carried out with ethyl acetate to obtain the ethyl acetate (EAPG) fractions. The fractions were heated at 40^{o} C to ensure complete removal of solvent from the fractions. The obtained EAPG fractions were stored at 10^{o} C in the refrigerator until the completion of pharmacological studies.

2.2 Experimental animals

Sprague Dawley (150-180 g) of either sex were procured from National Institute of Bioscience, Chaturshrungi, Pune. Animals were housed in standard polypropylene cages (32.5×21×14) cm lined with raw husk (renewed after 48h). The animal house was maintained on 12 h light/dark cycle at approximately 22 ± 2^{0}C, relative humidity 60–70% and the animals were provided with standard laboratory diet (Nutrivet Life sciences, Maharashtra, India) and water *ad libitum*. The animals were randomly assigned to different groups and a minimum period of 7 days was allowed for adaptation on each experiment. The animals described as fasting were deprived of food for 10 h but had free access to water throughout. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals i.e. CPCSEA (884/OP/05/ac/CPCSEA) and were approved by the Institutional Animal Ethics Committee (IAEC).

2.3 Drugs and chemicals

Streptozotocin (STZ) was purchased from SRL Pune. Glibenclamide was procured from oswal scientific Pune. All other chemicals were procured from local sources and were of analytical grade.

2.4 Preliminary phytochemical analysis

2.4.1 Qualitative phytochemical analysis

Preliminary phytochemical screenings of EAPG were carried out to detect the phytoconstituents using qualitative chemical tests (Khandelwal, 2006). Following tests were performed to determine the presence of various chemical constituents in the fractions.

A. Tests for Alkaloids

The dried fractions (20 mg) were added to dilute hydrochloric acid (1-2 ml), shaken well and filtered. With filtrate following tests were performed.

I. Mayer's test: To 3 ml of test solution add 3 drops of Mayer's reagent (potassium mercuric iodide). Appearance of reddish brown or cream precipitate indicated presence of alkaloids.

II. Dragendorff's test: Dragendorff's reagent (potassium bismuth iodide) was mixed with 3 ml of the test solution. Appearance of reddish brown precipitate indicated presence of alkaloids.

III. Wagner's test: Test solutions (3 ml) and Wagner's reagent (1 ml) were mixed in test tube. The appearance of reddish brown precipitate indicated presence of alkaloids.

A. Tests for Flavonoids

I. Shinoda test: The powdered fractions (10 mg) were added to 5 ml of ethanol (95%), 3 drops of hydrochloric acid and 0.5 gm magnesium turnings. Change of colour of solution to pink indicated presence of flavonoids.

II. Aqueous NaOH solution:

1 ml of aqueous NaOH solution was added to 1 ml of test solution. Formation of yellow colour indicated the presence of flavonoids.

III. Conc. H_2SO_4 test:

1 ml of conc.H_2SO_4 was added to 1 ml of test solution. Formation of red colour indicated the presence of flavonoids.

B. Tests for Tannins

I. Ferric-chloride test: 3 ml of test solution was treated with few drops of ferric chloride solution. Development of dark color indicated presence of tannins.

II. Lead acetate solution: The occurrence of white precipitate indicated presence of tannins or phenols.

III. Dilute iodine solution: Transient red colour indicated presence of tannins or phenols.

IV. Dilute Potassium permanganate solution: The discoloration of potassium permagnet solution indicated presence of tannins or phenols.

C. Tests for Saponins

Foam Test: Powdered extract (10-20 mg) was shaken vigorously with water (1 ml). Development of persistent foam which is stable at least for 15 minutes indicated presence of saponin.

D. Test for Steroids

I. Salkowaski test: Chloroform (2 ml) and 2ml of concentrated sulphuric acid were added to 2 ml of test solution, shaken and allowed to stand. Change in the colour of lower chloroform layer to red and acid layer to greenish yellow fluorescence indicated the presence of steroids.

II. Liebermann-Burchard reaction: T.S 2 ml was mixed with chloroform (2 ml). To the solution, 2 ml of acetic anhydride and 2 drops of conc. sulphuric acid from the side of test tube were added. Change in colour first red, then blue and finally green indicated presence of steroids.

E. Test for Triterpenoids

I. Salkowaski test: Concentrated sulphuric acid (2 ml) was added to 2 ml of test solution. The solution was shaken and allowed to stand. The colour of lower layer changed to yellow indicated presence of triterpenoids.

II. Liebermann-Burchardt Test: 3 ml of acetic anhydride was added to the 3 ml of test solution, mixed well and then 2 ml of concentrated sulfuric acid was added from the sides of the test-tube. Development of deep red colour indicated presence of triterpenoids.

F. Test for Glycosides

I. Keller-Killiani test: Glacial acetic acid (3-5 drops), one drop of 5% $FeCl_3$ and conc. Sulphuric acid were added to the test tube containing 2 ml of T.S. Appearance of reddish-brown color at the junction of two layers and bluish green in the upper layer indicated presence of glycosides.

II. Legals test: 2 ml of test solution was added to 1 ml of pyridine and 1 ml of sodium nitroprusside. Change in color to pink or red indicated presence of cardiac glycosides.

A. Test for Amino Acids

I. Ninhydrin Test (General test): Solution of extract (3 ml) and 3 drops of 5% lead acetate solution were boiled on water bath for 10 min. change in colour of solution to purple or blue indicated presence of amino acids.

II. Xanthoprotein test*:* Solution of extract (3 ml) and concentrated H_2SO_4 (1 ml) was mixed in test tube. The appearance of white precipitate which turn to yellow on boiling and orange on addition of NH_4OH (1 ml) indicated presence of proteins containing tyrosine and tryptophan.

2.5 Quantitative phytochemical analysis

2.5.1 Total phenolic content

Total phenolic content was determined using $KMnO_4$ colorimetric assay. An aliquot (1 ml) of EAPG (10-50 µg/ml) or standard quercetin (10-50 µg/ml) were added to 10 ml volumetric flask containing 9 ml of 60 µg/ml solution of $KMnO_4$. The solutions were well mixed and optical density was measured against prepared reagent blank at 520 nm. The total phenolic content was determined from the calibration curve and expressed as mg quercetin equivalents. All the determinations were carried out in triplicate and mean values were calculated (Madhu M et al., 2016).

2.5.2 Total flavonoid content

Total flavonoid content was measured by means of an aluminum chloride assay (Elberry et al., 2011) with slight modification. An aliquot (1 ml) of the extract (1 mg/ml) or standard solutions of quercetin (10-50 µg/ml) was added to a 10 ml volumetric flask containing 4 ml of 50% solution of methanol. To the flask, 0.3 ml 5 % $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ was added and at the sixth minute, 2 ml NaOH (1 M) solution was added, the total volume was made up to 10 ml with distilled water. The solution was well mixed and absorbance was measured against reagent blank at 510 nm. The total flavonoid content (mg/g) was determined from the calibration curve and expressed as mg quercetin equivalents. All the determinations were carried out in triplicate and the mean values were calculated.

2.5.3 Total tannin content

Total tannin content was determined by hide powder test according to the WHO procedure in which weight difference between tanned and untanned hide powder was used for quantitative determination of tannins (WHO, 1998). Briefly, accurately weigh a specific quantity of extracts to conical flask containing 150 ml of water and heat over boiling water bath for 30 min. cool, transfer the mixture to a 250 ml volumetric flask and dilute to volume with water. Allow the solid material to settle and filter the liquid through a filter-paper, diameter 12 cm, discarding the first 50 ml of the filtrate. To determine the total amount of material that is extractable into water, evaporate 50.0 ml of the plant material extract to dryness, dry the residue in an oven at 105 °C for 4 hours and weigh (*T*1). To determine the amount of herbal material not bound to hide powder that is extractable into water, take 80.0 ml of the herbal material extract, add 6.0 g of hide powder and shake well for 60 minutes. Filter and evaporate 50.0 ml of the clear filtrate to dryness. Dry the residue in an oven at 105 °C and weigh (*T*2). To determine the solubility of hide powder, take 6.0 g of hide powder, add 80.0 ml of water and shake well for 60 minutes. Filter and evaporate 50.0 ml of the clear filtrate to dryness. Dry the residue in an oven at 105 °C and weigh (*T*0). Calculate the quantity of tannins as a percentage using the following formula:

$[\{T1–(T2 – T0)\} \times 500]/w$

Where w = the weight of the extract in grams.

2.6 Thin layer chromatography and High-performance thin layer chromatography

2.6.1 Thin layer chromatography (TLC)

EAPG were subjected to thin layer chromatography using precoated silica plates of 250 µm thickness (TLC Silica gel 60 F_{254} Merck, Germany). The spots were developed in six different solvent systems as follows:

A. Ethyl acetate: formic acid: glacial acetic acid: water (100 : 11 : 11 : 2.6)
B. Toluene: dioxan: glacial acetic acid (90 : 25 : 4)
C. Chloroform: acetone: formic acid (75 : 16.5 : 8.5)
D. Toluene: acetone: formic acid (60 : 60 : 10)
E. Chloroform: ethyl acetate: formic acid (7.5 : 6.0 : 0.5)
F. Toluene: ethyl acetate: formic acid (5: 5 : 2.5)

The spots were visualized under long and short wavelength of UV chamber (Wagner and Bladt, 1996). R_f value was calculated using following equation

R_f = Distance travelled by the sample/Distance travelled by the solvent.

2.6.2 High performance thin layer chromatography

HPTLC chromatography of HACA was performed on 20×10 cm aluminum Lichrosphere HPTLC plates precoated with 200 µm layers of silica gel 60F254 (E. Merck, Germany). EAPG (10 µl) and standard biomarkers quercetin (600 ng/band) and gallic acid (800ng/band) were applied as bands 6 mm wide and 10 mm apart by means of Camag Linomat V sample applicator (Muttenz, Switzerland) equipped with a 100-µl syringe. The constant application rate was 160 nl s^{-1}. Linear ascending development with toluene: ethyl acetate: formic acid (5:4:1, v/v/v) as mobile phase was performed in a 20 cm×10 cm twin-trough

glass chamber (Camag) previously saturated with mobile phase for 15 min at room temperature (25±2° C) and relative humidity 60%±5%. The development was done using 20 ml of mobile phase. HPTLC analysis was performed at 270 nm in reflectance mode with a Camag TLC scanner III operated by Win CATS software (Version 1.2.0). The slit dimensions were 5 mm×0.45 mm and the scanning speed of 20 mm s^{-1} (Hussain et al., 2012).

2.7 High fat diet

All the experimental rats were fed on high fat diet except control rats for the duration of 4 weeks. The composition of high fat diet was considered as reported in literature with appropriate modification (Srinivasan et al. 2005).

Table 4.3: Formula of high fat diet

CONSTITUENTS	QUANTITY (%)
Lard	53.9
Casein	43.4
Cholesterol	1.73
DL- methionine	0.52
Yeast powder	0.17
Sodium chloride	0.17

2.8 Experimental design

2.8.1 Preliminary antihyperglycemic activity

2.8.2 Experimental induction of diabetes

The experimental rats were fed a high-fat diet for 4 weeks. 4 weeks later, streptozotocin (dissolved in 0.1 mol/L citrate buffer, pH 4.5) was intraperitoneally injected at 35 mg/kg. Post 48 hrs of STZ administration fasting blood glucose levels was measured using commercial glucometer (Accu-Chek). Experimental animals with fasting blood glucose levels ≥ 200 mg/dl were included in the experimental protocol. One week stabilisation period was considered prior to the treatment.

2.8.3 Experimental groups

The total of 36 rats will be used to accomplish the designated study.

They will be divided into 6 separate groups consisting of 6 animals in each group as follows:

1. Group I : Normal control rats receiving saline solution
2. Group II : Diabetic control rats receiving STZ and HFD
3. Group III: Diabetic control rats given 5 mg/kg standard drug (Glibenclamide)
4. Group IV : Diabetic control rats given 50 mg/kg of EAPG
5. Group IV : Diabetic control rats given 100 mg/kg of EAPG
6. Group V: Diabetic control rats given 200 mg/kg of EAPG

2.9 Plan of work

Work has been divided in two parts as follows:

A. Antihyperglycemic activity of EAPG

B. Antihypertensive study of EAPG

A. Evaluation parameters of antihyperglycemic activity

1. Oral glucose tolerance test

Oral glucose tolerance test was performed in overnight fasted (18 h) diabetic rats at the end of 28th day of study. Glucose (3g/kg) was fed 30 min after the administration of extracts or standard drug. Blood glucose was determined at 0, 30, 60, 90 and 120 min of glucose administration (Bandawane et al., 2013).

2. Fasting blood glucose

Fasting blood glucose was determined at 0, 7, 14, 21 and 28 day of study period using glucometer (Accu-Chek). Blood samples were collected by snipping the tail with the help of sharp razor.

3. Determination of body weight, food and fluid intake

During the study period of 28 days the rats were weighed daily using electronic balance and their body weights were recorded. Daily food and fluid intake were also recorded during 28 days study period.

4. Evaluation of liver function parameters

Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) were analysed by using commercial kits (Biolab diagnostic kit, India).

5. Evaluation parameters of antihyperlipidemic activity

Serum triglycerides (TG), total cholesterol (T-CH) and high density lipoprotein (HDL-CH) levels were estimated using standard commercial kits (Biolab diagnostic kit, India).Very low density lipoproteins (VLDL-CH) and low density lipoproteins (LDL-CH) levels were calculated using Friedewald formula (Friedewald et al., 1972).

VLDL-CH= TG/5

LDL-CH= T-CH – (HDL-CH+ VLDL-CH)

6. Serum Cardiac enzymes

At the end of experimental period, rats were anesthetized with diethyl ether. Blood was collected by retroorbital puncture and following serum was separated. Serum level of creatine kinase-MB isoenzymes (CK-MB), lactate dehydrogenase (LDH) was measured.

B. Antihypertensive study

1. Blood pressure measurement

1.1. NIBP (Non-inovasive) monitoring tail-cuff method

To measure changes in arterial blood pressure in conscious rat, NIBP technique was used (Biopac student lab instrument, Model NIBP-250). Conscious rats were acclimatized in the restrainer for 15 min, three times a day for a period of one-week before the starting of the testing schedule. The trained animals were placed in a restrainer and tails of rats were exposed to hot air blower. The rats were allowed to acclimatize inside the cage for 15 min before starting actual blood pressure measurement. Occlusion and sensor cuffs were placed around the base of tail and the cuffs were inflated and deflated several times to measure tail arterial blood pressure. A minimum of 8–10 cycles of inflation and deflation were made and the measurement of tail arterial blood pressure and heart rate was recorded and the data was averaged. The antihypertensive activity recorded on computer and analyzed with software (Sayed et al., 2016).

2. Cardiac biomarker:

2.1 MDA- malonaldehyde: (Badole S et al., 2013)

Principle : Thiobarbitoric acid (TBA) assay is the most commonly used method for determination of the MDA in biological fluids. The assay is based on a condensation reaction of two molecules of TBA with one molecule of MDA, in which the reaction rate depends on temperature, pH and concentration of TBA. The reaction is carried out in acidic solution and temperature of ~ 100°C within one hour time course and most of MDA is produced during reaction process from decomposition of products of lipid peroxidation.

Reagent preparation :

1. 50 % TCA (Trichloroacetic acid) : 50 gm of TCA + 100 ml distilled water
2. 0.1 M HCL: 0.36 gm of HCL + 100 ml distilled water
3. 26mM thiobarbituric acid: 0.37 gm of TBA +100 ml DW

Procedure :

200 µl of supernatant was added and briefly mixed with 1 ml of 50 % of TCA in 0.1M HCL and 1 ml of 26mM TBA. After mixing on a vortex, samples were maintained at 95 ºC for 20 min, after which sample were centrifuge at 960g for 10 min and superntant were read at 532 nm. The result were expressed as U/mg protein.

2.2. Catalase: (Moron et al., 1979)

Principle: One of the simplest qualitative procedures involves determination of the enzyme's presence in the test tissue isolate by using hydrogen peroxide, which is broken down to bubble-producing O2 by catalase-positive test. On the other hand, quantitative approaches focusing on careful measurements include colorimetric and spectrophotometric assay.

Reagent preparation:

65mM of phosphate buffer:

0.2M dibasic sodium phosphate (1L): 35.65 gm of diabasic sodium phosphate + 1 L distilled water

0.2M monobasic sodium phosphate (1L): 27.6 gm of monobasic sodium phosphate + 1L distilled water

29.73 ml of diabasic sodium phosphate + 2.74 ml of monobasic sodium phosphate

7.5 mM of hydrogen peroxide: 0.025 gm of H_2O_2 + 100 ml of distilled water

Procedure:

Catalase measurement was carried out by the ability of CAT to oxidize hydrogen peroxide (H_2O_2). 2.25 ml of potassium phosphate buffer (65 mM, pH7.8) and 100µl of the heart homogenate were incubated at 25 ◦C for 30 min. A 650µl H_2O_2 (7.5mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT mol/min mg of heart.

2.3 Glutathion (Ahmed et.al.2016).

Principle: The general thiol reagent, 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's Reagent) reacts with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TMB molecule and recycling the GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction

mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH.
Reagent preparation:
1. 10% TCA:10 GM OF TCA + 100 ml of water
2. 0.6 M of 5, 5'-dithiobis (2-Nitrobenzoic acid)
Procedure:
The equal amount of homogenate was mixed with an equal amount of TCA and centrifuge at 2000g for 10 min at 4 ºC. The supernatant was used for GSH estimation. To 100 µl processed tissue sample 2 ml of phosphate buffer 0.5 ml of DTNB and 0.4 ml doubled distilled water were added and the mixture was shaken vigorously on vortex mixer. The absorbance was read at 412 nm within 15 min. The results expressed as U/mg protein.

2.4 Superoxide dismutase: (Misera and fridovich, 1972)
Principle
(Cu-Zn) SOD activity was determined by use a simple and rapid method, based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA in the pH 8.2 is 50%. The principle of this method is based on the competition between the pyrogallol autoxidation by $O2^{\bullet -}$ and the dismutation of this radical by SOD
Reagent:
Tris –HCl buffer (50mM): 0.78 gm of tris-base + 0.26 gm of sodium chloride + 100 ml water.
Pyrogallol (24mM): 0.03 gm of pyrogallol + 100 ml of 10mM of HCl
Procedure:
Reaction mixture consist of 2.875 ml Tris-HCl buffer (50 mM P^H 8.5), pyrogallol and 100 µl of supernatant in total volume 3ml. enzyme activity measured at 420nm and was expressed as U/mg protein.

3. Nitric oxide: (Guevara et al., 1998)
Principle:
Nitric oxide (NO) is a molecular mediator of many physiological processes, including vasodilation, inflammation, thrombosis, immunity and neurotransmission. A number of methods exist for measuring NO in biological systems. One of these methods involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions. The detection limit for this method is 1.0 µM nitrite. The Griess reaction can also be used to analyze nitrate via its catalytic reduction to nitrite.
Procedure: Spectrophotometer Assay
1.1 Mix the following in a spectrophotometer cuvette (1 cm path length):
100 µL of Griess Reagent
300 µL of the nitrite-containing sample
2.6 mL of deionized water
Nitrite concentrations in the samples should fall within the linear range of the assay (approximately 1–100 µM).
1.2 Incubate the mixture for 30 minutes at room temperature.
1.3 Prepare a photometric reference sample by mixing 100 µL of Griess Reagent (above) and 2.9 mL of deionized water.
1.4 Measure the absorbance of the nitrite-containing sample at 540 nm relative to the reference sample.
1.5 Convert absorbance readings to nitrite concentrations as described in Calibration.

4. Histopathology
At the end of study period (last day of 4^{th} week), rats were sacrificed by cervical dislocation method and heart was isolated from all rats. The isolated heart tissue was trimmed into small pieces and preserved in 10% formalin for 24 hrs. Specimens were cut in section of 3-5 µm in thickness by microtome and stained by hematoxylin-eosin.

5. Statistical analysis:
All data are presented as mean ± SEM of measurement made on 6 animal in each group statistical analysis was performed with two way analysis of variance followed by bornferroni for multiple days study and one- way analysis followed by Dunnet's multiple comparison Test for one day study.

3. RESULT

3.1 Preliminary phytochemical analysis
3.1.1 Percentage yield of fraction:
The percentage yield of hydroalcholic extract of *Punica granatum* leaves was found to be 30.2% w/w while the percentages yield of ethyl acetate fraction of *Punica granatum* Linn. (EAPG) was found to be 10% respectively.
3.1.2 Qualitative phytochemical analysis:

The preliminary phytochemical screening of EAPG of *Punica granatum* Linn. leaves are enlisted in table 3.1. The qualitative phytochemical analysis of these extract showed the presence of active constituents such as glycosides, alkaloids, flavonoids compound and tannins.

Table 3.1: The preliminary phytochemical analysis of fraction of *Punica granatum* Linn. Leaves.

Sr. No.	Test/ Reagents	EAPG
1.	Test for alkaloids	
	a. Dragendorff test	+
	b. Mayer's test	-
	c. Wagner's test	-
2.	Test for tannins	
	a. Lead acetate test	+
	b. Ferric chloride test	+
	c. Iodine test	+
	d. Dil. Potassium permanganate test	+
3.	Test for flavonoids	
	a. Shinoda test	+
	b. Lead acetate test	+
	c. Sodium hydroxide test	+
4.	Test for saponins	
	a. Foam test	-
6.	Test for glycosides	
	a. Legal's test	+
	b. Keller-killani test	+
7.	Test for amino acid	
	a. Ninhydrin Test	+
	b. Tyrosine Test	+

(-): absent; (+): present; EAPG: Ethyl acetate fraction of *Punica granatum* Linn. leaves.

3.1.3 Quantitative phytochemical analysis:

Table 6.2. Depicts the total alkaloid content, total flavonoid content, and total phenolic content of EAPG.

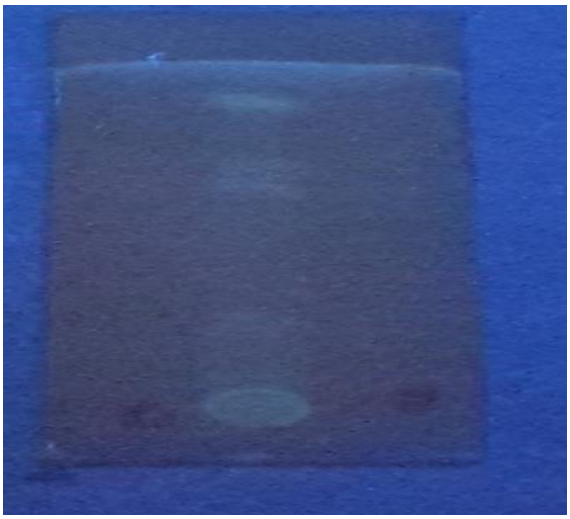
Table: 3.2 Quantitative phytochemical analysis of fraction of *Punica granatum* linn. Leaves

EAPG: Ethyl acetate fraction of *Punica granatum* Linn.

3.1.4 Thin layer chromatography (TLC):

The result of TLC analysis of EAPG is depicted in table. .while the TLC spot are show in figure.6 .1. The yellow spot show the presence of flavonoid present in EAPG.

Fig.3.1 Spot observed in thin layer chromatography of EAPG.



EAPG

Table:3.3. Rf values and colour of spot observed in thin layer chromatography of EAPG.

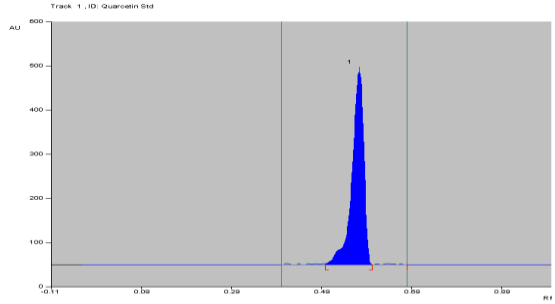
Sr. No.	Solvent system	Rf values (Retension factor)	Colour of spot
1	Toluene: Ethyl acetate: Ethanol (8:1:1)	0.61	Yellow

R_f: Retension factor, EAPG: Ethyl acetate fraction of *Punica granatum* Linn. Leaves.

3.1.5 High performance thin layer chromatography:

Fig.3.2. Overlay spectra observed in High performance thin layer chromatography of Quercetin standard.

QUANTITATIVE TEST	EAPG
Total alkaloid content % w/w	11.2
Total flavonoid content (mg quercetin equivalent/g of fraction)	95.4
Total phenolic content (mg quercetin equivalent/g of fraction)	38



HPTLC chromatographic overlay spectra of standard biomarker Quercetin.

Table: 3.4. Rf values and colour of spot observed in High performance thin layer chromatography of Quercetin standard.

Sr. No.	Peak No.	Concentration	Wavelength	Rf Value	Area
1.	1	5×10^5ng/spot	254nm	0.57	9509.2 AU

Mobile Phase – Toluene: Ethyl Acetate: Formic Acid (8: 1: 1)

Standard Preparation – 1 mg of Quercetin Standard dissolved in 1ml Methanol (1000ppm)

Fig.3.3: Overlay spectra observed in High performance thin layer chromatography of Sample_EAPG

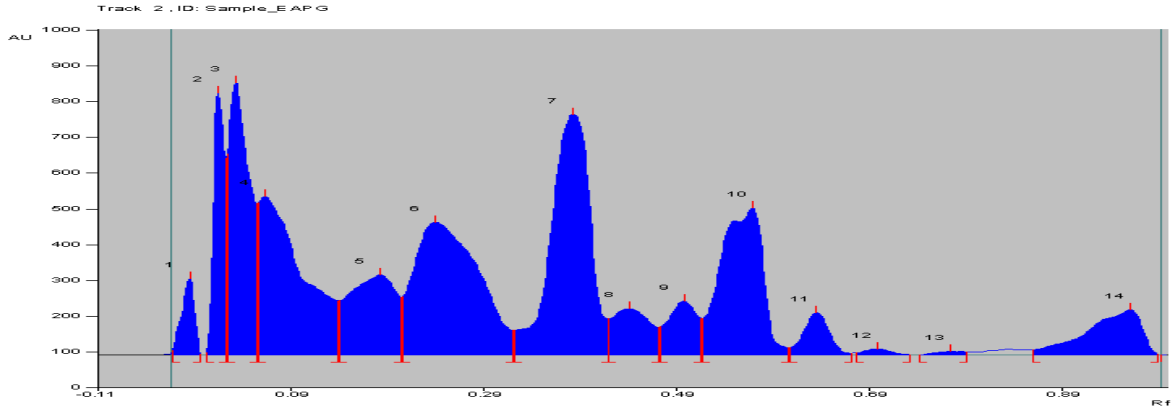


Table.3.5: Rf values and colour of spot observed in High performance thin layer chromatography of EAPG.

Sr. No.	Peak No.	Concentration	Wavelength	Rf Value	Area
1.	10	5×10^7ng/spot	254nm	0.57	14924.0 AU

Mobile Phase – Toluene: Ethyl Acetate: Formic Acid (8: 1: 1)

Sample Preparation – 5 mg of EAPG sample dissolved in 1ml Water (5000ppm)

The overlay spectra's of Quercetin standard and EAPG are shown in figures and respectively. Quercetin standard and quercetin resolved are R_f 0.57 and R_f 0.57 respectively. The spectrum reveals that EAPG contain same quercetin of standard quercetin.

3.2 Antihyperglycemic activity

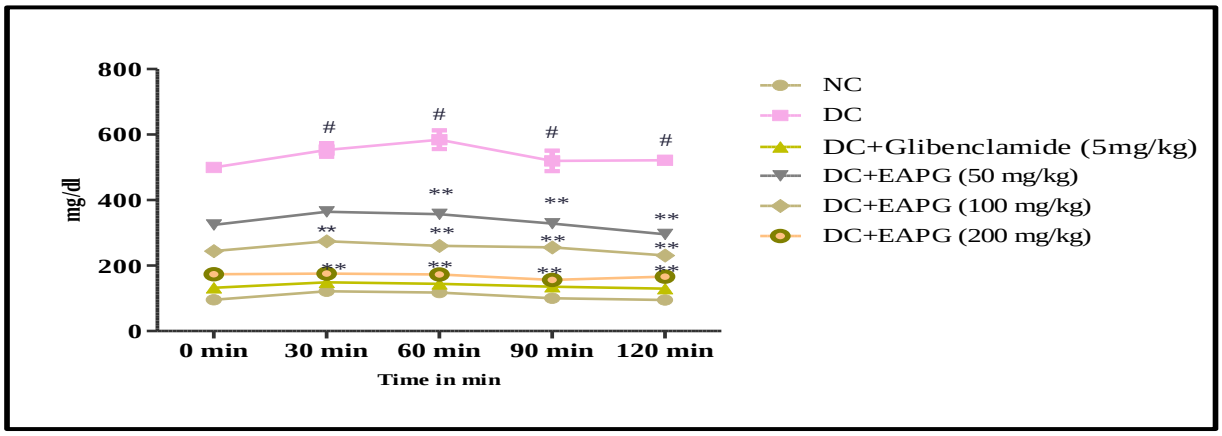
3.2.1 Oral glucose tolerance test (OGTT) in diabetic rats

Table.3.6: Effect of oral administration of EAPG on Oral glucose tolerance test in HFD-STZ induced diabetic rats.

Experimental groups	Fasting blood glucose level (mg/dl)				
	0 Min	30 min	60 min	90 min	120 min
Normal Control (NC)	85.671 ±1.22	111.75 ± 1.21	107.86 ±2.59	94.23 ±1.13	91.13 ± 1.57
Diabetic Control (DC)	489.58 ±6.05$^{\#}$	543.08 ±21.0$^{\#}$	575.29 ±11.6$^{\#}$	509.61 ±12.8$^{\#}$	511.26 ± 5.8$^{\#}$
DC+GL (5mg/kg)	63.95 ± 5.29	163.7 ±3.84*	162.8 ±3.26**	146.0 ±2.00*	157.4 ±2.47*
DC+EAPG (50mg/kg)	314.30 ± 9.62	354.66 ±2.61*	346.96 ± 4.30*	318 ± 0.93*	281.4 ±3.5**
DC+EAPG (100mg/kg)	234.13±2.50	264.06 ±1.20*	251.83 ±16.4*	245.53 ±1.49*	220.96 ±67.0*
DC+EAPG (200mg/kg)	134.05 ± 3.086	151.72 ±2.8**	223.5 ±13.0**	137.63 ±2.3**	133.84 ±1.4**

n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig 3.4: Effect of administration of EAPG on oral glucose tolerance test in diabetic rats.



n=6, Values are mean ± S.E.M. #p<0.05, ##p<0.01 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide, Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

The effect of EAPG on oral glucose tolerance test (OGTT) is shown in Fig.6.4. Blood glucose levels of normal and diabetic rats increased significantly (p< 0.01) at 30 min after glucose administration. EAPG at all dose levels significantly (p<0.01) reduced the increase in blood glucose at 90 min in glucose loaded rats compared with diabetic control rats which show elevation in blood glucose throughout the total measurement period (120 min).

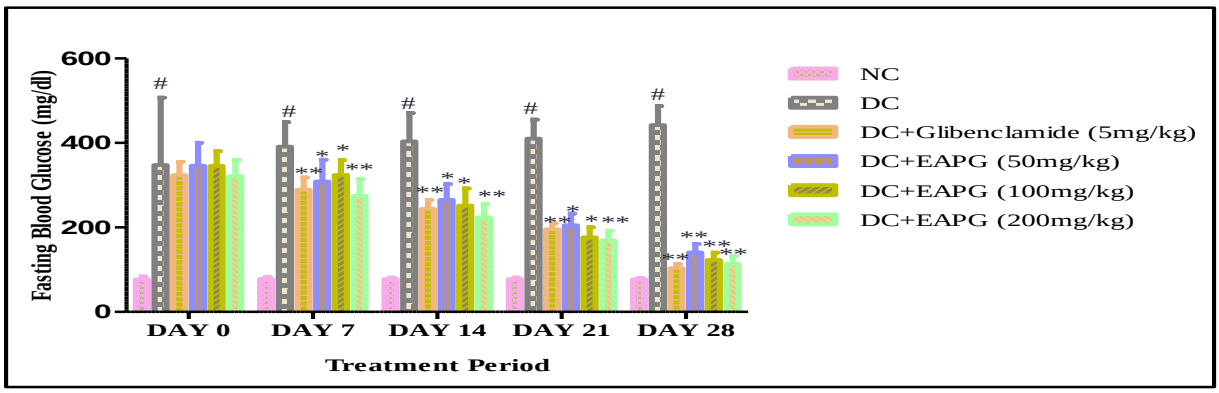
3.2.2 Fasting blood glucose level in diabetic rats

Table.3.7: Effect of oral administration of EAPG on Fating blood glucose in HFD-STZ induced diabetic rats.

Experimental groups	Fasting blood glucose level (mg/dl)				
	0 day	7thday	14thday	21thday	28thday
Normal Control (NC)	77.3 ±2.7	78.16 ±1.70	77.8 ± 1.44	77.66 ± 1.47	77 ± 1.31
Diabetic Control (DC)	347.5 ±65.2	391 ± 23.1#	403.83 ±27.2#	410.16 ±18.5#	442 ± 18.5#
DC+GL (5mg/kg)	323.5 ±13.9	289.1 ±11.9	244.18± 8.3**	195.5 ± 5.6**	103.6 ±3.8*
DC+EAPG (50mg/kg)	346.3 ±21.9	308 ±21.1	265± 15.4*	205 ± 11.3*	140.5 ±8.17
DC+EAPG (100mg/kg)	345 ±13.9	324 ±14.3	251.8 ±16.4*	176.6 ±9.72*	123. ±6.90
DC+EAPG (200mg/kg)	321.6 ±15.6	274.83 ±16.0**	223.5 ± 13.00**	169.3 ±9.15**	113.83 ±1.36**

n=6, Values are mean ± S.E.M. ,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.5. Effect of oral administration of EAPG on Fating blood glucose in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M. #p<0.05, ##p<0.01 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide, Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

As shown in Table 6 .7, treatment with EAPG for 4 weeks exhibited a significant (p<0.01) decrease in fasting blood glucose in HFD-STZ diabetic rats as compared to diabetic control (Fig.6.4). In diabetic rat's blood glucose level was reduced by 56.85%, 68.21% and 75.68% at 50, 100 and 200 mg/kg doses of the fraction respectively. The antihyperglycaemic activity of EAPG was found to be significant was comparable to diabetic control.

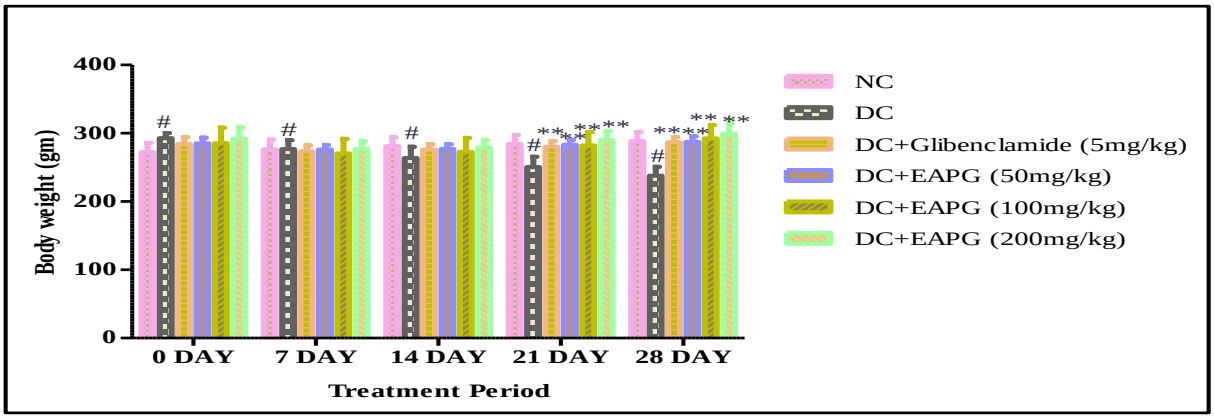
3.3 Body weight

Table 3.8: Effect of oral administration of EAPG on body weight in HFD-STZ induced diabetic rats.

Experimental Groups	Body weight (Gm)				
	0 Day	7 Day	14Day	21Day	28 Day
Normal Control (NC)	272.6 ±5.42	276.6 ±5.7	281 ± 5.29	284.1 ±5.39	288.66 ± 5.40
Diabetic control(DC)	292 ± 2.94#	276. ±5.3#	263 ± 6.61#	250.66 ±6.09#	237.8 ±5.30#
DC+GL (5mg/kg)	284.3 ±4.00	273.5 ± 3.49	276 ± 3.19	280 ± 3.39**	286.83 ±3.18**
DC+EAPG (50mg/kg)	285 ± 3.35	276. ±2.1	277 ± 2.65	283 ± 3.10**	287 ± 3.16**
DC+EAPG (100mg/kg)	285.6 ±8.98	270.5 ±8.4	272.8 ±8.31	282 ± 7.90**	292.5 ±7.94**
DC+EAPG (200mg/kg)	292.1 ±6.61	277.1 ±4.8	279 ± 4.47	290 ± 5.24**	299.6 ±6.41**

n=6, Values are mean ± S.E.M. #p<0.05, ##p<0.01 as compared to NC; *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.6.Effect of oral administration of EAPG on body weight in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

As shown in Table 6.8, HFD-STZ induced diabetic rats showed significant reduction in body weight as compared to normal rats. The diabetic rats treated with 50, 100 and 200 mg/kg of EAPG showed significant (p<0.01) increase in body weight by 8.42%, 8.71%, 12.7% respectively. After 4 weeks of treatment with EAPG body weight significantly (p<0.05) increased compared to diabetic control groups

3.4 Feed intake

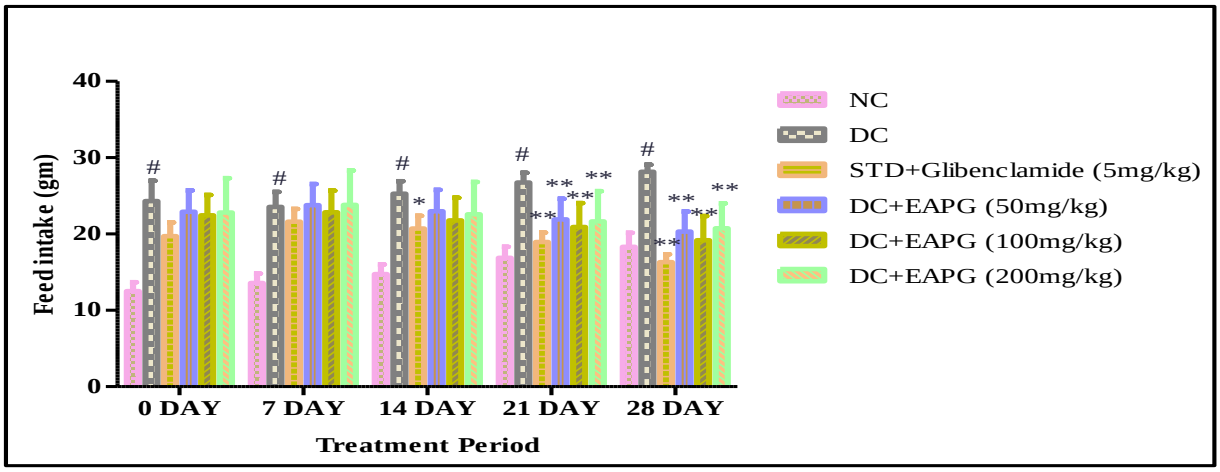
Table 3.9: Effect of oral administration of EAPG on Feed intake in HFD-STZ induced diabetic rats.

Experimental Groups	Feed intake (Gm)

	0 Day	7 Day	14 Day	21 Day	28 Day
Normal Control(NC)	12.5 ±0.48	13.58 ± 0.50	14.75 ± 0.51	16.88 ± 0.58	18.33 ± 0.73
Diabetic control (DC)	24.3± 1.07$^{\#}$	23.55 ±0.79$^{\#}$	25.3 ± 0.65$^{\#}$	26.76± 0.51$^{\#}$	28.16 ±0.38$^{\#}$
DC+GL (5mg/kg)	19.7 ±0.73	21.6 ± 0.69	20.71 ±0.68*	18.96 ±0.49*	16.33 ±0.42*
DC+EAPG (50mg/kg)	22.91 ±0.73	23.8 ± 1.12	22.98 ± 1.15	21.93 ±1.09*	20.31 ±1.08*
DC+EAPG(100mg/kg	22.45 ± 1.08	22.85 ± 1.16	21.8 ± 1.19	20.93 ± 1.27**	19.18 ± 1.29**
DC+EAPG (200mg/kg)	22.83 ±1.83	23.83 ± 1.83	22.58 ± 1.72	21.66 ±1.61*	20.75 ±1.34*

n=6, Values are mean ± S.E.M. #p<0.05, ##p<0.01 as compared to NC; *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.6.7.Effect of oral administration of EAPG on Feed intake in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control , EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

As shown in Fig.6.7.diabetic control rats showed significant (p<0.01) increase in feed intake as compared to normal rats. Diabetic rats treated with EAPG (50, 100 and 200 mg/kg) showed significant (p<0.01) reduction in feed intake from 14th day of treatment as compared to diabetic control rats. The effect of EAPG (50, 100 and 200 mg/kg) on feed intake of the animals was comparable to the diabetic control group .

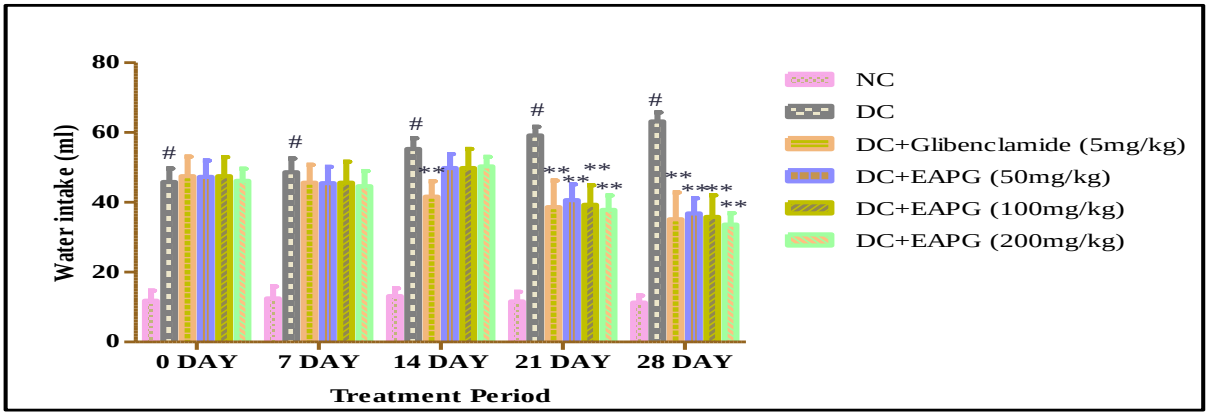
3.5 Water intake

Table 3.10: Effect of oral administration of EAPG on Water intake in HFD-STZ induced diabetic rats.

Experimental Groups	Water intake (ml)				
	0 Day	7 Day	14 Day	21 Day	28 Day
Normal Control (NC)	11.83 ±1.16	12.5 ±0.99	13.15 ±1.10	11.66 ±1.11	11.33 ±0.80
Diabetic control(DC)	45.83 ±1.5$^{\#}$	48.66 ±1.5$^{\#}$	55.33 ±1.2$^{\#}$	59.16 ±1.0$^{\#}$	63.16 ±1.0$^{\#}$
DC+GL (5mg/kg)	47.5 ±2.29	45.6 ± 2.07	41.6 ±1.7*	38.66 ±3.0**	35.16 ±3.1**
DC+EAPG (50mg/kg)	47.33 ±1.89	45.5 ± 1.91	49.83 ± 1.62	40.66 ±1.8**	36.83 ±1.7**
DC+EAPG (100mg/kg)	47.5 ±2.23	45.66 ±2.44	49.83 ± 2.22	39.3±2.27**	35.8 ± 2.52**
DC+EAPG (200mg/kg)	46.16 ±1.42	44.66 ±1.76	50.33 ± 1.11	37.83 ±1.6**	33.66 ±1.3**

n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control , EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.8. Effect of oral administration of EAPG on Water intake in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control , EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

As shown in Fig.6.8, diabetic control rats showed significant (p<0.01) increase in water intake as compared to normal rats. 28 days treatment with EAPG (50, 100 and 200 mg/kg) in diabetic rats showed significant (p<0.01) reduction in water intake from 21th day of treatment as compared to diabetic control rats. The effect of EAPG (50,100 and 200 mg/kg) on water intake of the animals was comparable to the diabetic control group.

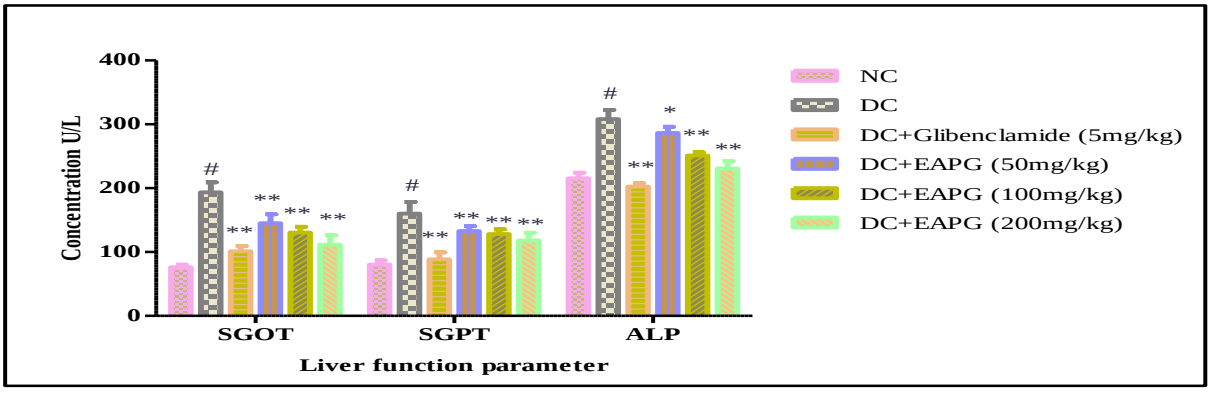
3.6 Serum function parameters

Table 3.11: Effect of oral administration of EAPG on serum SGOT, SGPT and ALP in HFD-STZ induced diabetic rats.

Experimental Groups	SGOT (IU/L)	SGPT(IU/L)	ALP (IU/L)
Normal Control (NC)	76 ± 1.63	80.5 ± 2.64	215.6 ± 3.53
Diabetic Control (DC)	193.5 ±6.35$^{\#}$	160 ± 7.55$^{\#}$	308 ± 5.81$^{\#}$
DC+GL (5mg/kg)	101 ± 3.51**	88.5 ± 4045**	202.41 ± 5.81**
DC+EAPG (50mg/kg)	145.16 ±5.67**	132.83 ± 3.09**	286 ± 3.93*
DC+EAPG (100mg/kg)	130.5 ± 3.7**	128 ± 3.03**	251.1 ± 2.09**
DC+EAPG (200mg/kg)	111.16 ±5.96**	118 ± 4.94**	230 ± 4.62**

n=6, Values are mean ± S.E.M, #p<0.05,as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.9: Effect of oral administration of EAPG on serum SGOT, SGPT and ALP in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M., #p<0.05 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide, SGOT: Serum glutamate oxaloacetate transaminse; SGPT: Serum glutamate pyruvate transaminase; ALP: Alkaline phoaphatase; Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

The effect of EAPG on the hepatic function markers such as SGOT, SGPT and ALP indifferent experimental groups of rats is shown in fig.6.9. In comparison with normal control group, the level of SGOT, SGPT and ALP were increased significantly in diabetic untreated groups and after treatment with EAPG for 28 days the level of SGOT, SGPT and ALP were significantly decreased to normal value as compared to diabetic rats.

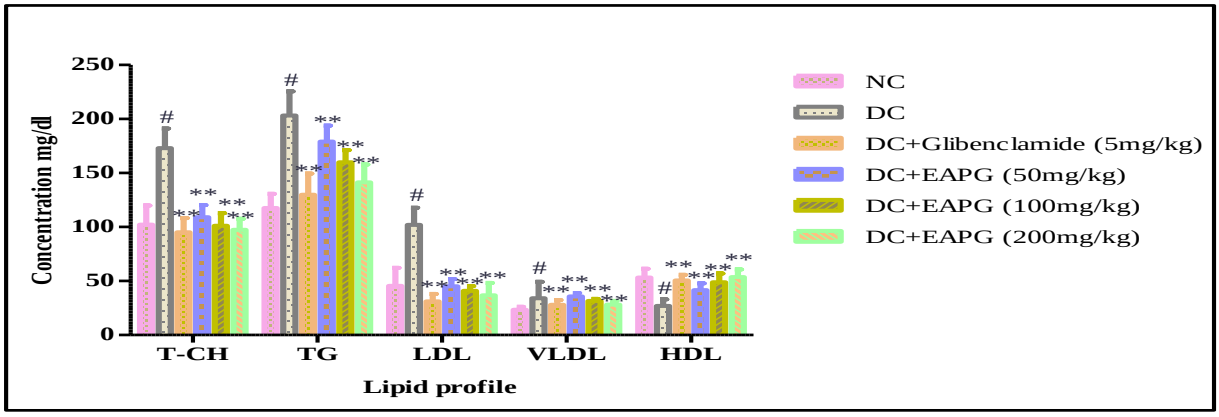
3.7 Evaluation parameter of antihyperlipidemic activity

Table 3.12: Effect of oral administration of EAPG on lipid profile in HFD-STZ induced diabetic rats.

Experimental Groups	T-CH (mg/dl)	TG (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)
Normal Control (NC)	102.27 ±7.20	117.5 ± 5.21	45.44 ± 6.84	23.5 ± 1.08	53.33 ± 2.42
Diabetic Control (DC)	173 ± 7.37$^{\#}$	203.16 ± 9.15$^{\#}$	102.03 ± 6.45$^{\#}$	33.96 ± 6.23$^{\#}$	27 ± 2.43$^{\#}$
DC+GL (5mg/kg)	95 ± 5.43**	129.83 ± 5.60**	31.24 ± 2.77**	27.95 ± 1.83**	50.49 ± 2.16**
DC+EAPG (50mg/kg)	109 ± 4.64**	179 ± 6.01**	45.08 ± 2.70**	35.83 ± 1.20**	41.66 ± 2.55**
DC+EAPG (100mg/kg)	101.16 ± 6**	159.83 ± 4.58**	41.04 ± 1.70**	31.6 ± 0.78**	48.86 ± 3.31**
DC+EAPG (200mg/kg)	97.33 ± 4.20**	141.33 ± 6.71**	36.73 ± 4.71**	28.26 ± 1.34**	53.63 ± 2.92**

n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison..

Fig.3.10: Effect of oral administration of EAPG on lipid profile in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M., #p<0.05 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide, T-CH: Total Cholesterol; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein. Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

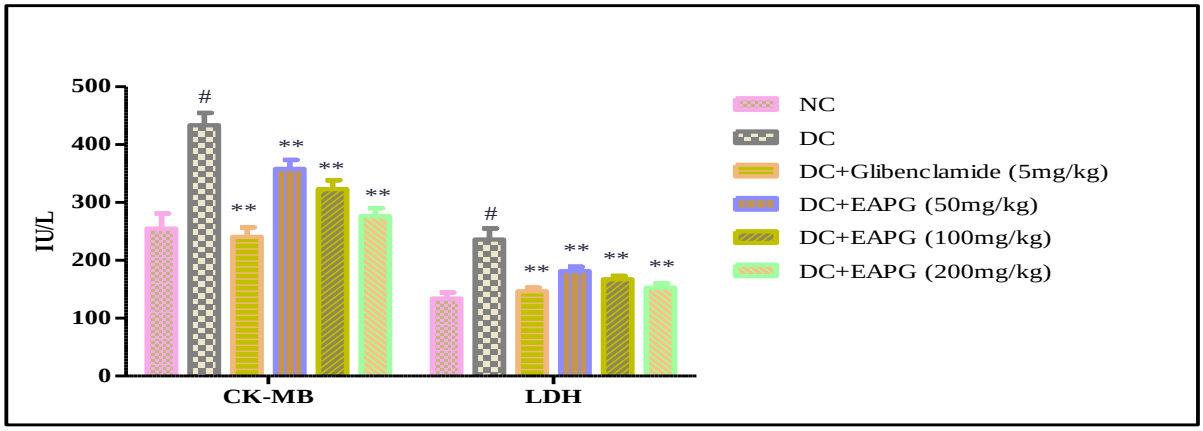
The protective effect of EAPG on lipid profile has been shown in Fig.6.10. There was a significant (p<0.01) decrease in T-CH, TG, LDL-CH, VLDL-CH and significant (p<0.01) elevation in serum HDL-CH in diabetic rats when compared to normal rats. EAPG (200 mg/kg) treated diabetic rats decreased T-CH by 26.02 %, TG by 61.52 %, LDL-CH by 90.61 % and VLDL-CH by 61.56 %. Whereas EAPG (200 mg/kg) treated group showed a significant (p<0.01) increase in HDL-CH as compared to diabetic control group. However, lipid profile remains unchanged in EAPG treated normoglycemic rats.

Table 3.13: Effect of oral administration of EAPG on Serum Cardiac enzymes in HFD-STZ induced diabetic rats.

Experimental Groups	CK-MB	LDH
Normal Control (NC)	254.66 ± 10.71	134.16 ± 4.14
Diabetic Control (DC)	433.5 ± 8.62$^{\#}$	236 ± 7.98$^{\#}$
DC+GL (5mg/kg)	240.5 ± 6.76**	146.33 ± 2.88**
DC+EAPG (50mg/kg)	358.33 ± 6.29**	181.5 ± 3.05**
DC+EAPG (100mg/kg)	323.16 ± 6.19**	167.16 ± 2.48**
DC+EAPG (200mg/kg	276.33 ± 5.73**	152.83 ± 2.93**

n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.11: Effect of oral administration of EAPG on Serum Cardiac enzymes in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M., $^{\#}$p<0.05, $^{\#\#}$p<0.01 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide; CK-MB: Creatinine kinase-myoglobin binding; LDH: Lactate dehydrogenase Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

Diabetic resulted in significant increase in the serum CK-MB activity level as compared to the diabetic control. Administration of EAPG decreased significantly the diabetes-induced increase in the serum CK-MB activity level in diabetic group. The serum LDH activity level of diabetic group was also increased significantly as compared to the diabetic control. EAPG administration to diabetic rats significantly attenuated the LDH serum activity level compared to diabetic control group.

3.8 Antihypertensive parameter

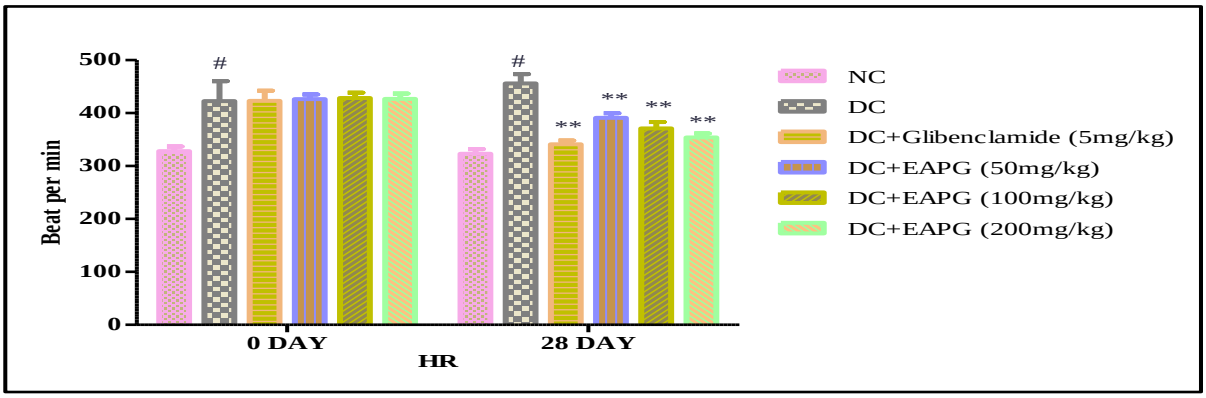
3.8.1 Blood pressure measurement

Table 3.14: Effect of oral administration of EAPG on Heart rate in HFD-STZ induced diabetic rats.

Experimental Groups	Heart rate	
	0 Day	28 Day
Normal Control (NC)	328 ± 3.60	323.33 ± 8.71
Diabetic control (DC)	422 ± 15.28	456.16 ± 7.09
DC+GL (5mg/kg)	423 ± 9.92	341 ± 7.04
DC+EAPG (50mg/kg)	426.33 ± 3.65	391 ± 8.94
DC+EAPG (100mg/kg)	428 ± 10.05	371 ± 12.12
DC+EAPG (200mg/kg)	426 ± 10.13	354.16 ± 2.93

n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.12. Effect of oral administration of EAPG on HR in HFD-STZ induced diabetic rats.



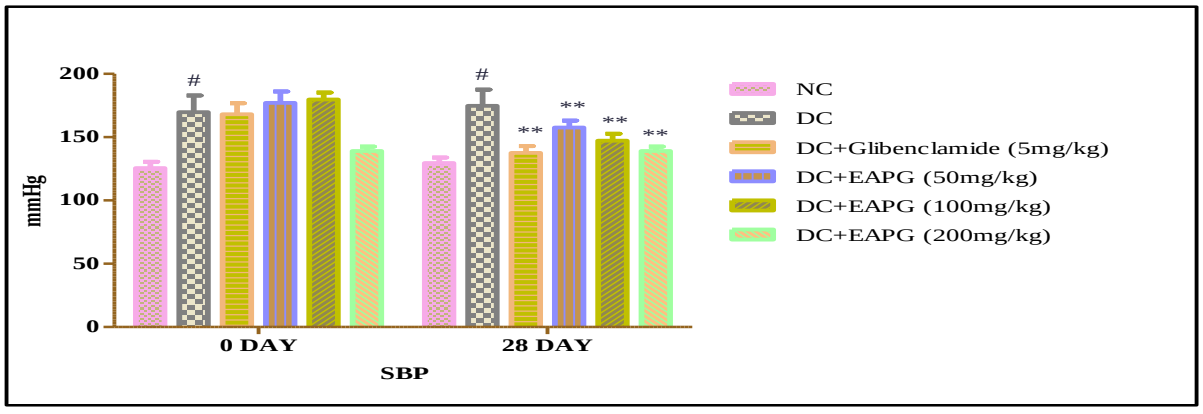
n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Table 3.15: Effect of oral administration of EAPG on SBP in HFD-STZ induced diabetic rats.

Experimental Groups	Systolic blood pressure	
	0 Day	28 Day
Normal Control (NC)	125.5 ± 5.01	129.66 ± 4.32
Diabetic control (DC)	169.8± 13.02	174.83 ± 12.76
DC+GL (5mg/kg)	168 ± 8.74	137.66 ± 5.27
DC+EAPG (50mg/kg)	177 ± 9.01	157.66 ± 5.42
DC+EAPG(100mg/kg)	179.8 ± 7.98	147.33 ± 5.39
DC+EAPG (200mg/kg)	173.83 ± 9.62	139 ± 1.48

n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.13. Effect of oral administration of EAPG on SBP in HFD-STZ induced diabetic rats



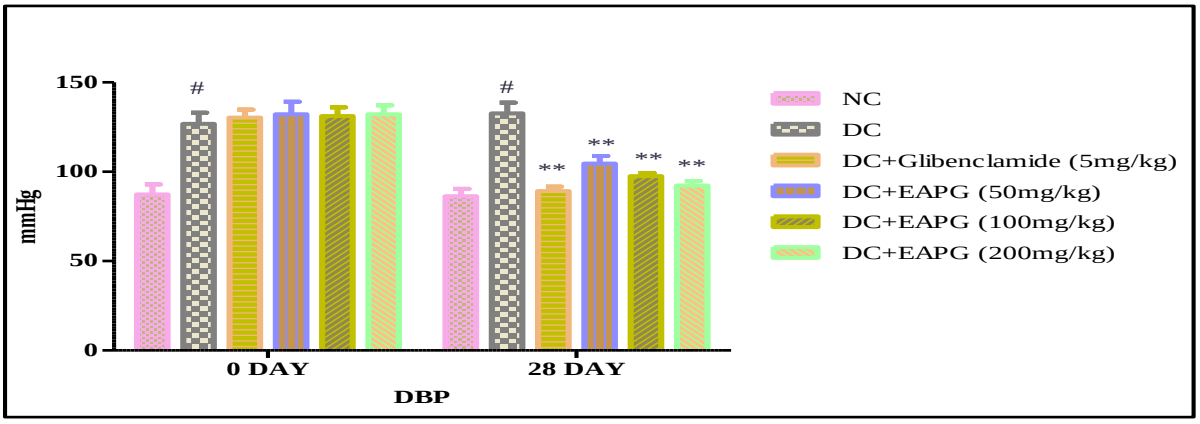
n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Table 3.16: Effect of oral administration of EAPG on DBP in HFD-STZ induced diabetic rats.

Experimental Groups	Diastolic blood pressure	
	0 Day	28 Day
Normal Control (NC)	87.33 ± 5.63	86.33 ± 4.08
Diabetic control (DC)	126.8± 6.24	132.66 ± 6.02
DC+GL (5mg/kg)	130.3± 4.54	89.16 ± 2.48
DC+EAPG (50mg/kg)	132.1 ±6.99	104.5 ± 4.37
DC+EAPG (100mg/kg)	131.1 ± 4.95	97.5 ± 1.87
DC+EAPG (200mg/kg)	132.1 ± 5.03	92.33 ± 2.50

n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control, EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.14. Effect of oral administration of EAPG on SBP in HFD-STZ induced diabetic rats



In fig (6.12) heart rate, systolic in fig (6.13) and diastolic blood pressure fig (6.14) of diabetic rats were significantly higher (p<0.01) than those of the normal control rats. After 28 days of the EAPG treatment doses of 50, 100 and 200mg/kg, heart rate, systolic and diastolic blood pressure were significantly decreased as compared to diabetic control rats.

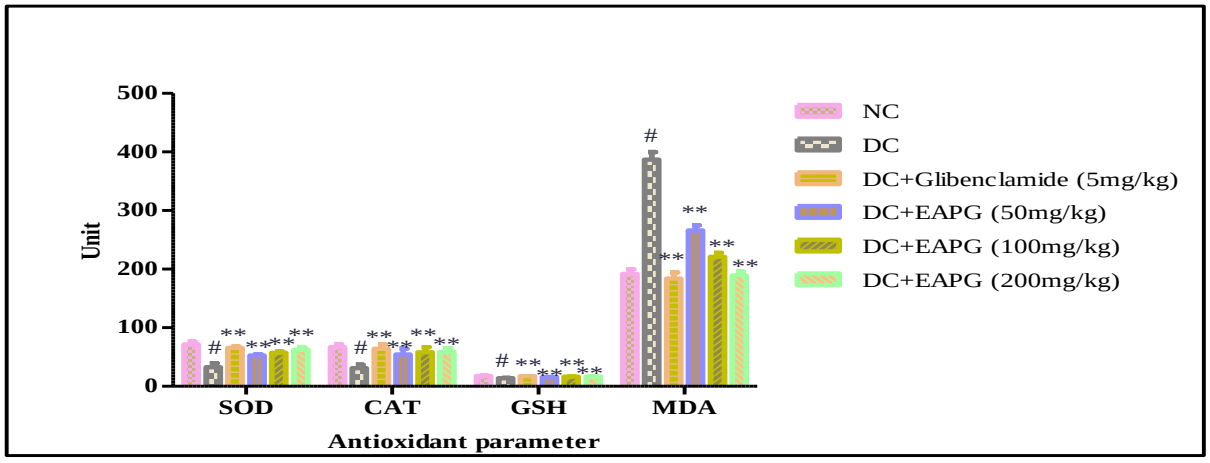
3.8.2 Antioxidant parameter in heart:

Table 6.17: Effect of oral administration of EAPG on Heart Oxidative stress markers in HFD-STZ induced diabetic rats.

Experimental Groups	SOD	Catalase	GSH	MDA
Normal Control (NC)	71.26 ± 2.19	65.83± 2.04	7.76 ± 0.10	191.82 ± 3.25
Diabetic Control (DC)	32.55± 2.78$^{\#}$	31.16± 2.38$^{\#}$	3.97 ± 0.12$^{\#}$	386.76 ± 5.26$^{\#}$
DC+GL (5mg/kg)	65.77 ± 0.96**	63.89 ± 3.15**	7.32 ± 0.07**	184.04 ± 4.25**
DC+EAPG (50mg/kg)	52.75 ± 0.76**	54.55± 4.07**	6.01 ± 0.05**	266.12 ± 3.60**
DC+EAPG (100mg/kg)	57.09 ± 0.92**	57.94± 3.70**	6.26 ± 0.04**	221.10 ± 2.69**
DC+EAPG (200mg/kg	62.47 ± 1.60**	58.80± 2.62**	7.65 ± 0.03**	189.44 ± 2.62**

n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.15. Effect of oral administration of EAPG on Heart Oxidative stress markers in HFD-STZ induced diabetic rats.



n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to normal control group; *p<0.05, **p<0.01 as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione MDA: Malondialdehyde; Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

Diabetic resulted in significant decreased SOD, Catalase, and GSH, enzyme levels in heart as compared to diabetic control group. Treatment with EAPG produced a significant increase in these enzyme levels at 50, 100 and 200 mg/kg doses. Diabetic resulted in significant increase in MDA levels in heart as compared to diabetic control group. Treatment with EAPG produced a significant decrease in the MDA level. There was a significant increase in antioxidant activity.

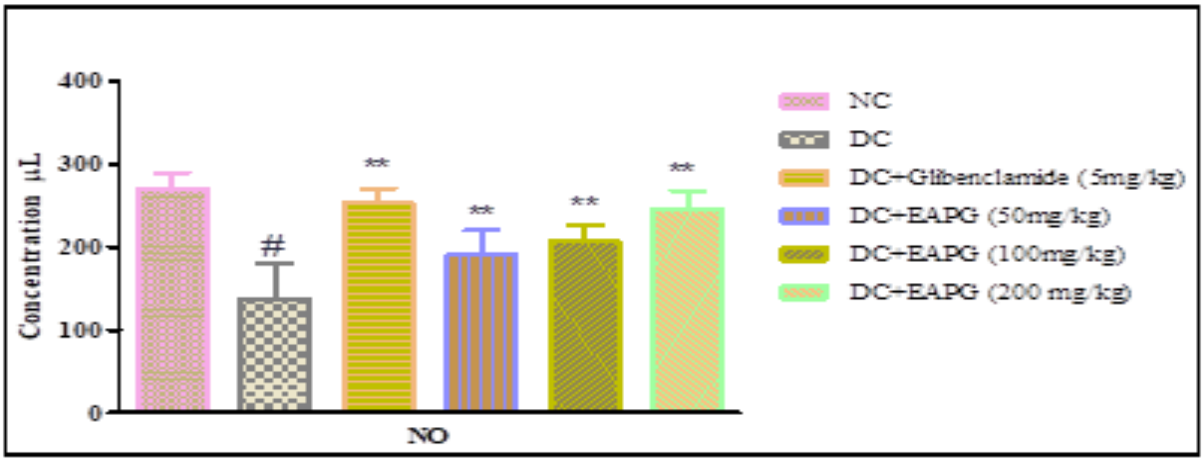
3.8.3 Nitric oxide (NO):

Table 3.18: Effect of oral administration of EAPG on Heart NO in HFD-STZ induced diabetic rats.

Experimental Groups	NO
Normal Control (NC)	271.16 ± 1.56
Diabetic Control (DC)	137.66 ± 17.46
DC+GL (5mg/kg)	253.16 ± 7.38
DC+EAPG (50mg/kg)	191.16 ± 12.23
DC+EAPG (100mg/kg)	207.16 ± 8.08
DC+EAPG (200mg/kg)	247.83 ± 8.53

n=6, Values are mean ± S.E.M.,#p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC, NC: Normal control, DC: Diabetic control ,EAPG: Ethyl acetate fraction of *Punica granatum*: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Fig.3.16: Effect of oral administration of EAPG on Heart NO in HFD-STZ induced diabetic rats.



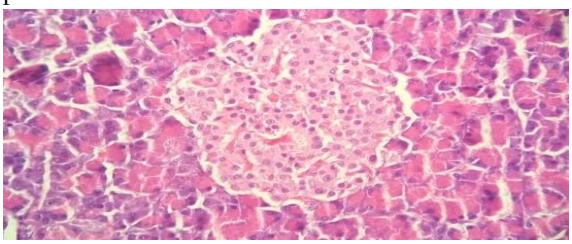
n=6, Values are mean ± S.E.M., $^{\#}p<0.05$, $^{\#\#}p<0.01$ as compared to normal control group; $^{*}p<0.05$, $^{**}p<0.01$ as compared to diabetic control group. EAPG: Ethyl acetate fraction of *Punica granatum*; GL: Glibenclamide; NO: Nitric oxide; Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

Diabetic resulted in significant decreased in NO level in heart as compared to diabetic control group. Treatment with EAPG produced a significant increase in these enzyme levels at 50, 100 and 200 mg/kg doses. There was a significant increase in NO activity.

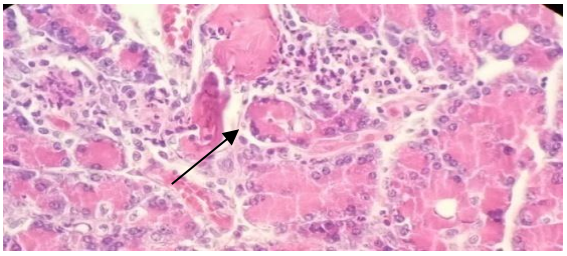
3.9 Histopathological study

3.9.1. Histopathology study of Pancreas

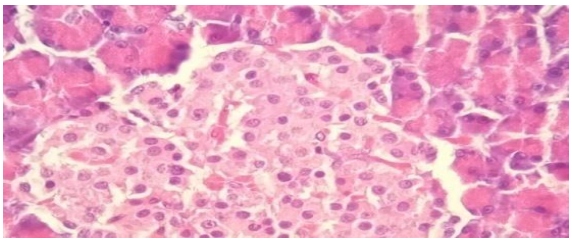
Fig.3.17: Effect of EAPG on histopathology of pancreas of HFD-STZ induced diabetic rats



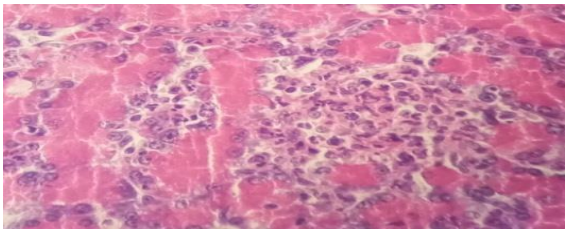
A. Normal



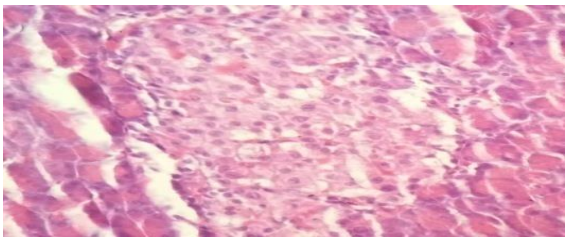
B. Diabetic control



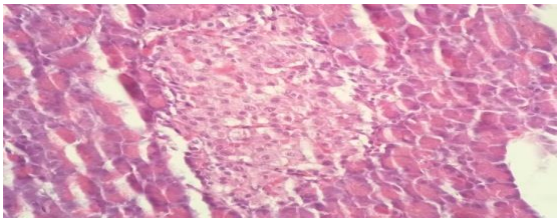
C. DC+Glibenclamide (5 mg/kg)



D. DC+EAPG (50 mg/kg)



E. DC+EAPG (100 mg/kg)



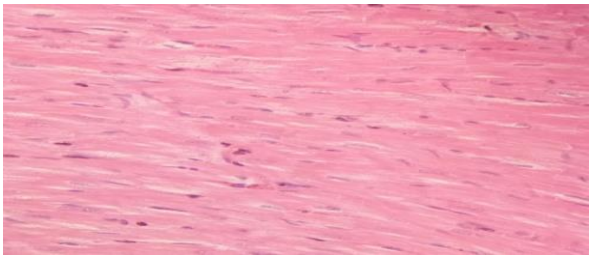
F. DC+EAPG (200mg/kg)

Pancreas section were stained with haematoxylin-eosin under 40X magnification of digital microscope. (A) Normal control with typical histological structure of pancreas. (B) Diabetic control group showing degenrative change in islets.
(C) DC+Glibenclamide (5mg/kg). (D) DC+EAPG (50 mg/kg). (E) DC+EAPG (100 mg/kg). (F) DC+EAPG (200 mg/kg).

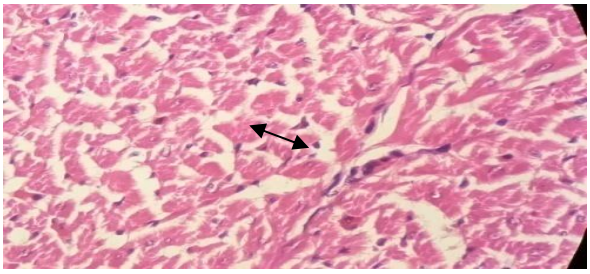
The histopathological changes of pancreas of diabetic rat showed necrosis of β- cells and degenrative change in islets. Treatment with EAPG showed improvement in β-cell restoration of architecture of pancreas that were earlier affected with HFD-STZ.

3.9.2. Histopathology study of Heart:

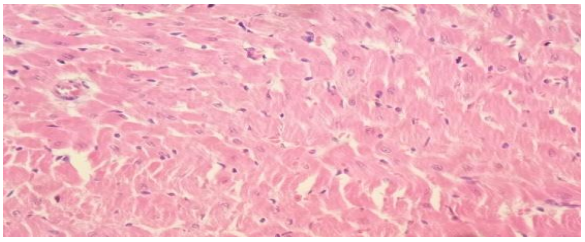
Fig.3.18: Effect of EAPG on histopathology of heart of HFD-STZ induced diabetic rats



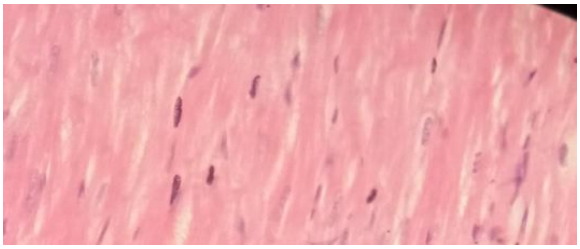
A. Normal



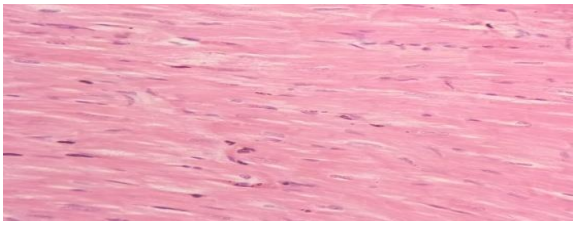
B. Diabetic control



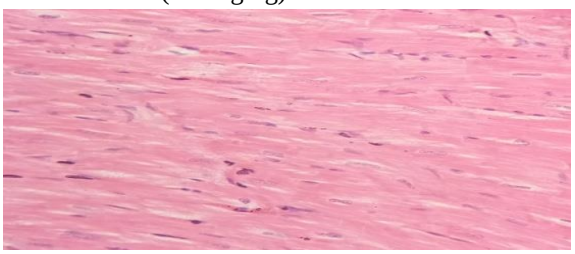
C.DC+Glibenclamide (5mg/kg)



D. DC+EAPG (50 mg/kg)



E. DC+EAPG (100mg/kg)



D. DC+EAPG (2000 mg/kg)

Heart section were stained with haematoxylin-eosin under 40X magnification of digital microscope. (A) Normal control with typical histological structure of heart. (B) Diabetic control group showing cardiac muscle fibre streched. (C) DC+Glibenclamide (5mg/kg). (D) DC+EAPG (50 mg/kg). (E) DC+EAPG (100 mg/kg). (F) DC+EAPG (200 mg/kg).

The histopathological changes of heart of diabetic rat showed edematous chang in the cardiac muscle fibre. Treatment with EAPG showed slightly improved and recovery of damage that were earlier affected with HFD-STZ.

4.DISCUSSION

Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically as antidiabetic and antihyperlipidemic remedies. Despite the presence of known antidiabetic medicine in the pharmaceutical market, diabetes and the related complications continued to be a major medical problem. Antihyperglycemic effects of these plants are attributed to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. More than 400 plant species having hypoglycemic activity have been available in literature, however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances which demonstrate alternative and safe effects on diabetes mellitus. Most of plants contain glycosides, alkaloids, triterpenoids, flavonoids, cartenoids, etc., that are frequently implicated as having Antidiabetic effect**.**

From the literature survey, *Punica granatum* flower extract have been reported to possess antihyperglycemic activity (Bhaskar et al, 2012). Therapeutically beneficial chemical constituent's simillar to that flower extract have been reported from leaves of *punica granatum* linn. Such as ellagitannins, gallic acid, anthrocyanins, piperidine alkaloids, flavonoids luteolin, apigenin, and quercetin (Garachh et al., 2012; Fozia et al., 2012). Further, our earlier work proved the preliminary antihyperglycemic and antioxidant activities of *P. granatum* leaves (Patil et al., 2013) and renal protective activity of flavonoid rich fraction of *punica granatum* Linn. (Patel et al., 2015). We hypothesized that *punica granatum* Linn. may exert Cardioprotective activity through its antihyperglycemic and antioxidant properties. Thus, the aim of our present work was to investigate cardioprotective effect of flavonoid rich fraction of *Punica granatum* Linn. leaves in High fat diet (HFD) and Streptozotocin (STZ) induced diabetes in rats.

5.CONCLUSION

The present invstigation, was designed to evaluate the effect of Punica Granatum linn. in high fat diet and Streptozotocin induced diabetes in rats. Punica granatum flower extract have been reported to possess antihyperglycemic activity (Bhaskar et al, 2012). Therapeutically beneficial chemical constituent's simillar to that flower extract have been reported from leaves of *punica granatum* linn. Such as ellagitannins, gallic acid, anthrocyanins, piperidine alkaloids,

flavonoids luteolin, apigenin, and quercetin (Garachh et al., 2012; Fozia et al., 2012). Further, our earlier work proved the preliminary antihyperglycemic and antioxidant activities of P. granatum leaves (Patil et al., 2013) and renal protective activity of flavonoid rich fraction of punica granatum (Patel et al., 2015). We, hypothesized that *punica granatum* may exert Cardioprotective activity through its antihyperglycemic and antioxidant properties. Thus, the aim of our present work was to investigate cardioprotective effect of flavonoid rich fraction of Punica granatum leaves in High fat diet (HFD) and Streptozotocin (STZ) induced diabetes in rats.

6.ACKNOWLEDGEMENT

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