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Centratherum Anthelminticum (L.) Kuntze Seed Oil Restoring Hepatic Balance in Type 2 Diabetic Rats through Nrf2/Keap1/HO-1 and NF-κB Pathway Modulation

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Abstract

Introduction

Diabetes Mellitus (DM) contributes to global morbidity and mortality. The liver also takes the brunt in the form of diabetic hepatopathy (DH). The spectrum of liver involvement varies from glycogen-hepatopathy, non-alcoholic fatty liver disease (NAFLD), fibrosis and cirrhosis to hepatocellular carcinoma. The insufficient choices for managing DH necessitates the exploration of other novel treatment options. Therefore, fixed oil (FO) extracted from the seeds of *Centratherum anthelminticum* (CA) and its hexane (HF), chloroform (CF) and ethanol (EF) fractions were explored for their anti-inflammatory, antioxidant, antidiabetic and anti-apoptotic properties in mitigating liver damage in diabetic rats.

Methods

The STZ-induced diabetic model was developed with challenging the rats first with high-fat and fructose diet followed by STZ (60mg/Kg). Following the development of DM rats were subjected to four-weeks of treatment with CA seed's FO and their HF, CF and EF soluble fractions alongside with gliclazide (Glic) as a treatment-control. The efficacy of oil's anti-inflammatory, antioxidant and anti-apoptotic pathways were evaluated through a comprehensive array of biochemical, histological and molecular parameters.

Results

Mitigation of inflammation (TNF-α, IL-1β, COX-1, and NF-κB) was observed. However, the antioxidant effects (expression of HO-1 and Keap1/Nrf2) were opposite to what has been observed in the kidney (previous-work). The seed's oil and its all soluble-fraction dampen and reverse the deleterious perturbation of hyperglycemia (FBS, Insulin, and HbA1c) induced inflammation and oxidation (SOD, CAT, GPx, LPO, GSH and HMG CoA/Mevalonate ratio) in the liver to near-normal constitutive-levels. The liver-function-tests, lipid-profile also improved along with mitigation of apoptosis (Bcl2 and Bax)

Conclusions

Our study holds the promise of identifying a putative natural adjuvant-remedy with a potential to slow or reverse the progression of liver insult in diabetes. It also identifies the gap and gives confident for further exploration of DM models developed chronically; as a means for appropriate representation of the pathophysiology of diabetes and DH.

1. INTRODUCTION

Diabetes Mellitus (DM); a global multifactorial pandemic is a public health challenge. It is primarily of two types: insulin-dependent (Type 1) and non-insulin dependent (Type 2). The condition is characterized

by abnormal levels of circulating blood glucose which can be either due to impaired functioning and/or deficiency of insulin. The said abnormality in the metabolic hormone is further compounded by the physical inactivity, life style choices, body mass index (BMI) and genetics [1–3]. According to the figures released by the International Diabetes Federation (IDF) in 2021 approximately 536.6 million (10.5%) people between the ages of 20 to 79 were suffering from DM. The projected prevalence is much grim as the number is expected to increase to 783.2 million (12.2%) by the year 2045 [4].The disease has a fair share not only in global morbidity rather it was reported in 2019 that 11.3% (an estimated 4.2 million) of mortality is also attributed to it. The cause of concern is that out of 4.2 million people approximately 46.2% (1.9 million) were younger than 60 years of age [5–6]. The aforementioned observed mortality of DM is preluded either by microvascular (e.g., nephropathy, retinopathy and neuropathy) and/or macrovascular complications (cerebrovascular and coronary artery diseases) [7]. Furthermore, liver is not spared either as it takes the brunt of DM in the form of diabetic hepatopathy (DH).

Being one of the largest organ; the liver accounts for 2.5% of the overall body weight in an adult human [8]. Its size delineates the significant role it has in immune, excretory, metabolic, coagulation and in detoxification systems [9–10]. Involvement in protein, carbohydrate and lipid metabolism makes it a “nutritional hub.” Besides pancreas, brain and skeletal muscle glucose homeostasis is also regulated by the hepatic tissue. In terms of glucose hemostasis it not only stores extra glucose in the form of glycogen rather also de novo synthesizes glucose. It also burns fatty acids into ketones, synthesizes both lipids and proteins; and also detoxify ammonia by deamination [11–12]. The severity of liver involvement in DM varies from simple abnormal enzyme levels to glycogen hepatopathy, non-alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis, hepatocellular carcinoma, acute liver failure and/or chronic liver disease [13–17]. In 2020 Banday et.al, cited a stronger correlation between liver disease and DM; the two most commonly reported diseases [18]. Whereas; in 2023 Devarbhavi et.al, categorized it as liver disease associated with diabetes, hepatogenous diabetes (HD) and liver disease that develops concurrently with diabetes [19]. It is a serious risk both for the progression and evolution of chronic liver disease [20]; and has also been linked to morbidity and mortality [21]. Therefore, addressing liver health while actively treating DM attenuates the disease progression and its associated complications [22–24].

Globally NAFLD has been identified as the fastest growing cause of liver-related mortality and is also reported to affect approximately 47.3%-67.3% diabetics [25–26]. Both H.M. El-Karaksy et.al. (2010) and R. Farhan et.al. (2018) in their respective cohorts of type 1 diabetic children and adolescents reported that 4.5% had hepatomegaly and 10% had NAFLD; respectively [27–28]. While the prevalence of hepatic steatosis was reported to be 24–53% in type 1 adults [29]. The scenario is no better for type 2 DM (T2DM); as compared to general population NAFLD is 2-fold higher i.e. 37–55% more common in them [30]. Moreover, individuals with obesity and NAFLD have twice the risk of developing hepatic fibrosis if they happen to suffer from T2DM [31]. In addition, increased levels of γ-glutamyltransferase (GGT), alanine transaminase (ALT) and aspartate transaminase (AST) are reported in 23.1%, 16% and 8.8% of type-2 diabetics; respectively [32]. In 1999 R. De Marco group explored a cause specific mortality in T2DM through a study called “The Verona Diabetes Study”; it’s reported that cirrhosis increases the mortality risk more than twofold in diabetics as compare to the general population [33–34]. On its own

T2DM is also one of the strong risk factor for atherosclerotic cardiovascular disease. However, evidences cited in the literature identifies that severity is compounded with existing NAFLD [35–38]. There seems to be not only a bidirectional relation between the two rather, they have synergistic association; as well [35, 39–40]. Not much is clearly delineated in the literature in the context of the mechanisms linking NAFLD with cardiovascular disease in diabetics. However, both of them being proathergenic are potentially conducive in not only creating and maintaining low grade inflammatory states rather, also increasing levels of cholesterol (CHOL), very low density lipoprotein (VLDL) and small dense particles of low density lipoprotein (LDL) [41–42]. Furthermore, NAFLD alone also has detrimental effect on glycemic control in patients with T2DM [43]. Thus, this synergism and bidirectional relationship between the two increases the risk of cardiovascular disease and all-cause mortality in diabetics which is supporting the findings of the “The Verona Diabetes Study” [44].

Persistent hyperglycemia precipitates ketoacidosis, glucose auto-oxidation, depletion of reduced glutathione (GSH); suppression of cellular antioxidant defense mechanisms and; impair mitochondrial membrane permeability and ATP production. All these perturbations have domino effect in activating number of reactive oxygen species (ROS)-generation pathways at cellular level like polyol and hexosamine phosphate; advanced glycation end products (AGEs) and PKC/NAD(P)H oxidase etc. [45]. These perturbations and triggering mechanisms presents itself as hepatic insult in the form of inflammation, steatosis, fibrosis, DNA fragmentation and apoptosis [46–48]. Suppression of cellular antioxidant defense players and depletion of GSH further precipitates master inflammatory transcription factor nuclear factor kappa B (NF-κB) and enhances its downstream pro-inflammatory cytokines, collagen deposition through transforming growth factor-1 (TGF-1) and mitochondrial-mediated (intrinsic) cell apoptosis through p53/Bax axis [49].

In the light of accumulating evidence nuclear factor erythroid 2-related factor 2 (Nrf-2), a transcription factor has been identified to play a key role in cellular antioxidant mechanisms and its down-regulation has been implicated in DH [50–53]. Nrf2 recognized as a pivotal antioxidant transcription factor in hepatic cells is involved in enhancing antioxidant potential by activating expression of phase II antioxidant enzymes like catalase (CAT), heme oxygenase-1 (HO-1), superoxide dismutase (SOD) and glutathione peroxidase (GPx); and reduced glutathione (GSH) [54–55]. It also suppresses NF-κB and the NRLP3 inflammasome associated inflammation thus; also acting as a potent anti-inflammatory factor [54, 56]. In addition to antioxidant and anti-inflammatory potential it can up-regulate anti-apoptotic protein Bcl2 thus suppressing cellular apoptosis [56]. Interestingly, Nrf2 being a transcription factor is located in the cytoplasm where its function is under tight regulation by its substrate adaptor Kelch-like ECH-associated protein 1 (keap-1). It ensures that under basal conditions Nrf2 is not only restricted within cytoplasm and protected from degradation by E3 ubiquitin ligase rather; it also stimulates Nrf2 proteasome-mediated degradation in the cytoplasm. However, under electrophile, ROS and xenobiotic stresses Keap-1 gets phosphorylated and release Nrf2. Nrf2 than translocate into the nucleus and binds to the enhancer sequence present in the regulatory region of the antioxidant genes. This sequence is called as antioxidant response element (ARE) [57–60, 56]. Of note, damages/insults witnessed in number of organs (including liver) in DM is due to decrease in the levels and transcriptional activation of

Nrf2, as well as decrease in the expressing of its downstream antioxidant enzymes [61–66, 54]. Opposing this, Nrf2 activation in the experimental DM model either through pharmacological or transgenic manipulation has mitigated liver damage, nephropathy, retinopathy, atherosclerosis and neuropathy by modulating inflammation, apoptosis and oxidative stress independent of correcting hyperglycemia. These accumulating data highlights the significance of Nrf2 as a putative drug target which if stimulated holds the potential of alleviating DM-induced complications [54, 60, 64, 67–68]. In a nutshell, both the inducible transcription factor Nrf2 and NF-κB and their downstream proteins are important elements of antioxidant response pathways.

The limited and insufficient availability of choices for managing liver health during diabetes in the long haul necessitates the exploration of other novel and non-conventional treatment options. Therefore, the terpene- and flavonoid-rich fixed oil (FO) extracted from the seeds of *Centratherum anthelminticum* (CA) and its hexane (HF), chloroform (CF) and ethanol (EF) fractions [unpublished work, 69] were explored for their potential therapeutic benefits in mitigating liver damage in high-fat-high-fructose-streptozotocin-(HF-HFr-STZ) induced diabetic rats. The focus of the present study is to explore the potential hepato-protective effect of CA seeds by evaluating its anti-inflammatory, antioxidant, antidiabetic and anti-apoptotic properties in the liver. Previously, these properties have garnered substantial attention and are supported by ample of evidences cited in the literature [70–80]. Both terpenes (natural unsaturated hydrocarbons) and flavonoids (polyphenolics) found in FO extracted from the seeds of CA are known for modulating the inflammatory, apoptotic and oxidant signaling pathways along with scavenging the free radicals. The plant has a rich and long history for tending to myriad of aliments [81–87]. The central hypothesis of the study has been formulated to explore the role of inflammatory: NF-κB, innately existing anti-oxidant: Nrf2 and HO-1 signaling pathways in diabetic liver and what are the potential ameliorative effects of CA on these aforementioned signaling mechanisms in diabetic liver.

To evaluate our hypothesis we developed the time-tested STZ-induced diabetic rat model. However, the model was developed with little modification of challenging the rats first with HF-HFr diet which was followed by STZ in order to optimally represent the metabolic milieu of T2DM [70]. Following the development of DM in rats they were subjected to four weeks of treatment with CA seed's FO and their HF, CF and EF soluble fractions alongside with gliclazide (Glic) as a treatment control group. The efficacy of oil and its fractions in mitigating the liver insult induced through STZ and their effect on inflammatory (NF-κB) and innately existing antioxidant (Nrf2/Keap1/HO-1) pathways were evaluated through a comprehensive array of biochemical, histological and molecular parameters (Figure.1). In the wake of exploring alternative therapeutic options; our study holds the promise of not only identifying a putative natural adjuvant remedy with a potential to slow or reverse the progression of liver insult in diabetes rather, also help in understanding the underlying molecular mechanisms of terpene and flavonoid-rich seeds of CA in protecting the liver. It will also help to substantiate the cited evidences on the therapeutic potential of modulating the Nrf2/Keap1/HO-1 pathway. It can also enhance our understanding of the pathogenesis of DH and what are the other management avenues. We anticipate that our study has the possibilities of exploration and the significance of being extended beyond animal experimentation.

2. MATERIALS AND METHODS

2.1. Materials and Reagent

SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), cDNA synthesis kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit; Catalog no. K1691 USA), RNA extraction kit (Catalog no. K0731 GeneJET ThermoFisher Scientific, USA) were purchased from Thermofisher Scientific. Whereas, kit USCN Catalog no. SEB824Ra from USCN and rat enzyme-linked immunoassay (ELISA) test kit (Bioassay Technology Laboratory Insulin ELISA kit Catalog no e0707RA) were procured from Bioassay Technology. Ellman's reagent (DTNB, 5, 5'-dithiobis nitrobenzoic acid, Cat. no: D8130), TRIzol® Reagent (Cat.no: T9424) were procured from Sigma Aldrich Corp (St. Louis, MO, USA), epinephrine (Cat. no: E4250), glutathione (Cat. no: G4251), sodium azide (Cat. no: 13412), sodium arsenate (Cat. no: A6756), perchloric acid (Cat. no: V001526), hydroxylamine reagent (Cat. no: 159417), dichromate-acetic acid reagent (Cat. no: 223964) were purchased from Sigma Aldrich Corp (St. Louis, MO, USA). Chloroform (Cat. no: 102447), glucose (Cat. no: D9434, dextrose), and trichloroacetic acid (TCA, Cat. no: T6399) were obtained from Merck & Co (New Jersey, US). Hexane (Cat. no: 296090, ethanol (Cat. no: V001229), DMSO (Cat. no: 276855), STZ (Cat. no: S0130), ketamine (Cat. no: K1884), xylazine (Cat. no: X1126), gliclazide (Cat. no: G2167), hydrochloric acid (HCl, Cat. no: 320331), hydrogen peroxide (H2O2, Cat. no: H1009) were also purchased from Sigma Aldrich Corp (St. Louis, MO, USA).

2.2. Procurement, Preparation and Oil Extraction of Centratherum anthelminticum (L.) Kuntze seeds by Cold Press Method

Seeds of CA (bitter cumin) were procured, prepared and fixed oil of the seed was extracted by cold press method; and subsequently fractionated with three different solvents i.e. from the most non-polar solvent hexane to the least non-polar ethanol for the detail refer to our earlier work [70].

2.3. Animal Procurement, Acute Toxicity Study and Oral Glucose Tolerance Test (OGTT)

The Wistar Albino rats used in the acute toxicity, OGTT and in *in-vivo* T2DM studies were procured from Dow Institute of Advance Biological and Animal Research (DIBAR), DUHS and kept in a well-ventilated Animal House of DIABAR (DUHS), Karachi. According to the protocol two animals were housed per cage which was made of polycarbonate. The rats were acclimated for a week to twelve hour cycle of light and dark; humidity was maintained around 50 ± 10% and ambient temperature was kept at 22 ± 3°C. The animals according to the study protocol during the period of acclimatization and before interventions had access to standard rat diet and clean sterilized drinking water. All the procedures and interventions performed during the study were approved by the Ethical Review Board for Animal Research and Ethics, Dow University of Health Sciences (AR.IRB-21/DUHS/Approval/2021/037). Acute toxicity and (OGTT) were carried out in both male and female Wistar Albino rats of about 6–10 weeks old and weighing 160g

to 280g each. A single increasing dose (i.e. 500, 1000, 1500 and 2000 mg/kg*)* of the oil extracted from the seeds and fractionated in hexane, chloroform and ethanol was used in acute toxicity study whereas; for OGTT recruited animals were given five different doses of the experimental drug ranging from: 50, 100, 200, 400 to 600mg/kg (Figure.1) [70]

2.4. Induction of Diabetes Mellitus and Experimental Design

Sixty five aged matched male Wistar Albino rats (8–12 weeks old and weighing 180–280 ± 20g) were recruited in the study, following the period of acclimatization they were divided into seven groups. Six groups out of seven were assigned 10 rats each whereas; only one group comprised five rats. The group with five animals was labeled as a control group. Besides control rats all the others were fed high-fat (45%) and high fructose (30%) diet i.e. "*HF-HFr diet*" for the entire period of the study (i.e. 63 days). The rest of the six groups were: Diabetes mellitus Control **(DM Control)**, Diabetes mellitus Reference Control **(DM Glic)**, Treatment-Fixed oil **(FO)**, Treatment-Hexane Fraction **(HF)**, Treatment-Chloroform Fraction **(CF) and** Treatment-Ethanol Fraction **(EF)**. The experimental groups were fed for 28 days i.e. 4 weeks; on day 29, prior to STZ injection all rats were fasted for 8 to 12 hours water was provided as normal. A 1-ml 23-G syringe was used to inject the prepared STZ intraperitoneally into the experimental groups at 60 mg/kg (1.0 ml/kg). Rat with blood glucose of 230–270 mg/dl was considered to have developed DM (Under this treatment regimen, 85% of the animals developed diabetes with a mean fasting blood glucose of 252 mg/dl). Control animals were injected an equal volume of citrate buffer (pH 4.5) (Figure.1) [70].

2.5. Dose Selection and Administration

Following development of diabetes in experimental groups; the dose of 200mg/kg was selected based on the results of acute toxicity study and OGTT. The animals in the treatment groups were initiated with the test oil i.e. *C. anthelminticum* seeds FO, and it's HF, CF and EF at dose of 200mg/kg and continued for 4 weeks. Whereas, the animals in the reference control were give the reference drug i.e. Gliclazide at dose of 200mg/kg. This protocol was followed in order to evaluate the hypoglycemic potential of the test oil and compare it to diabetic rats given either 0.05% DMSO 1mL/kg and reference drug; as well compare it to the rats belonging to control group [70].

2.6. Animal Sacrifice, Blood and Hepatic Tissue Collection

At the end of the study period i.e. at 63rd day animals from all the groups were euthanized by intraperitoneal injection of xylazine (*7mg/kg*) and ketamine (*60 mg/kg*). From the dissected animals blood samples were collected from the heart through cardiac puncture and serum separated [70]. Liver tissue after excision was washed with ice-cold saline. The liver tissue was preserved in 10% formalin for histology and in phosphate buffered saline (PBS) for various assays and PCR (Figure.1).

2.7. Hepatic Tissue Weight and Homogenate Preparation

The dried weight of all the resected liver was recorded. Following this they were washed with and preserved in ice PBS at -80°C until further processing. Approximately 4g of tissue was weighed and homogenized in 10-fold volume of ice cold PBS (pH = 7.4) at 1000 RPM. Then the homogenate was

centrifuged at 10.000 × *g* for 15 min at 4°C, to remove the debris and the collected supernatant was stored at − 80°C until further experimentation [88].

2.8. Assay for Fasting Blood Glucose, Hepatic Function and Serum Lipid Profile

The separated serum was used for the measurement of HbA1c and for the assessment of hepatic function: indirect, direct and total bilirubin, AST, ALT, GGT and alkaline phosphatase (ALP) were quantified using commercially available spectrophotometric assay kits *("Atellica Solutions, Siemens Healthcare")*. Additionally lipid metabolism parameters e.g. LDL, high density lipoprotein (HDL), total cholesterol (TC), triglycerides (TG), VLDL were also measured using the commercially available spectrophotometric assay kits (*"Atellica Solutions, Siemens Healthcare"*). Blood glucose levels were measured using a glucometer ("*ACCU-CHEK Roche, Switzerland"*) in each group.

2.9. Measurement of Serum Insulin, HOMA-IR and HOMA-

Insulin concentration in the separated serum were measured using the protocol specified by the manufacture ("*rat insulin enzyme-linked immunoassay (ELISA) test kit (Bioassay Technology Insulin ELISA Kit Catalog # e707RA").* The mathematical assessment of a balance between hepatic glucose output and insulin secretion was done by computing the fasting glucose and insulin concentration into a model for assessment of homeostasis; abbreviated as HOMA. It is a cost- effective mathematical tool which can be used in clinical and epidemiological studies since it requires basal insulin and glucose measurement only once [89]. Hence it is used as an alternative to the costly 'gold standard' methods that requires not only the dynamic data rather, is also invasive. Refer previous work for the formulae used in calculating Insulin sensitivity (IS) and HOMA models for beta cell function (HOMA-) and insulin resistance (HOMA-IR) [70, 90–91].

2.10. Determination of Hepatic Antioxidant and Inflammatory Stress Parameters

2.10.1. Lipid Peroxidation (LPO)

In our study we used the modified method of Niehius and Samuelsson [92]. For lipid peroxidation 0.37% thiobarbituric acid (TBA), 15% trichloric acetic acid (TCA) and 0.25N hydrochloric acid (HCl) were prepared. Once all the chemical were prepared they were mixed in the ratio of 1:1:1. Following the preparation and mixing of all the reagents and chemicals 0.1 mL of liver homogenate (i.e. 1%) were mixed with 2 mL of "*TBA-TCA-HCl reagent"*; in the blank only distilled water was mixed with the above mentioned reagent. After mixing the tubes were kept for thirty minutes in boiling water bath at a temperature of 100℃ and then allowed to cool. The amount of MDA formed in each of the tubes was measured in the clear supernatant by assessing the absorbance using a spectrophotometer at 535 nm against the reference blank. The method is rapid and easy; however, there can be interferences in the

method due to presence of aldehydes other than MDA, which may produce derivatives that absorb light in the same wavelength range [93].

2.10.2. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) Reductase

In our study we used an indirect method of assessing HMG-CoA reductase activity. HMG-CoA and mevalonate concentrations in the liver tissue samples (homogenates) were estimated in terms of absorbance and calculated per gram of wet tissue, as measured by Klett-Summerson. The activity of HMG CoA reductase converts HMG CoA to mevalonate in the presence of NADPH and was measured by calculating the ratio of absorbance of HMG-CoA to absorbance of mevalonate. 0.1% saline arsenate, physiological saline, 0.05% Perchloric acid (PCA), 4.5M NaOH, 0.65 M HCl solution, ferric chloride reagent and 2M hydroxylamine hydrochloride reagent (HHR) were prepared. After preparation of all the above reagents 1 mL of 1% liver homogenate was mixed with 1 mL of PCA and incubated for 5 minutes at room temperature. Following incubation samples were centrifuged for 10mins (3000 rpm) and supernatant was collected. Supernatants from liver were mixed and shaken well with HHR reagent (pH of 5.5) and allowed to stand for 5 minutes. This was followed by measuring the absorbance of HMG-CoA at 540 nm. The procedure was repeated with supernatants of liver homogenate but with the HHR reagent at pH 2.1 in order to measure the absorbance of mevalonate at 540 nm. Once the absorbance were measured following calculations were performed by adapting the Ramakrishnan and Rao method [94].

Mevalonate and HMG-CoA = 20 x Absorbance (Klett reading) = Absorbance per gram of wet tissue

Ratio = Absorbance per gram of wet tissue of HMG-CoA/Absorbance per gram of wet tissue of Mevalonate

2.10.3. Reduced Glutathione (GSH) level

The most popular assays to determine glutathione concentration is based on enzymatic reactions discovered by Ellman, Owens and Belcher and Tietze [95–96]. In Ellman's method glutathione reductase (GR) present in the tissue homogenate is used to reduce GSSG to GSH, and the Ellman's reagent, 5, 5-dithiobis-(2-nitrobenzoic)-acid (DTNB) to measure the absorbance at 412 nm. TNB (5-thio-2-nitrobenzoic acid) is a chromogenic compound with yellow color; its presence in the reaction mixture results in a color yield. The rate of excess color development depends on the concentration of GSH. 0.2M phosphate buffer with a pH of 8.0 was prepared by using monobasic sodium phosphate and dibasic sodium phosphate; and labeling them as solution A and B, respectively. 5% tricarboxylic acid, 1% sodium citrate solution and Ellman's reagent were freshly prepared. Once the reagents were prepared freshly, then 0.5 mL of 1% liver tissue homogenates were mixed with 2 mL of 5% TCA whereas, in case of blank no homogenate was used and TCA was replaced with water. Following this both the test and blank samples were centrifuged (3000 rpm) for 15 minutes and supernatant was collected. 1 mL of supernatant from both the test and blank samples was mixed with 3 mL and 0.5 mL of phosphate buffer and Ellman's reagent, respectively. Upon mixing the supernatants with the above mentioned reagent the absorbance

were measured in spectrophotometer at 412 nm for 3 consecutive minutes with one minute interval. The measured absorbance was used to estimate the level of GSH.

2.10.4. Superoxide Dismutase (SOD)

In our study SOD was measured by the method cited by Fridovich and Misra (1972) [97]. According to this assay it has been observed that SOD acts as a potent inhibitor of the spontaneous auto oxidation of epinephrine at elevated pH in the presence of oxygen. 0.1M carbonate-bicarbonate buffer having a pH 10.2 was prepared by weighing and dissolving 10.6g and 8.4g of sodium carbonate in 1000 mL of distilled water and was labeled as **solution A** and **B**; respectively. Furthermore, 0.6mM EDTA and 1.8mM epinephrine was prepared. Following the preparation of all the reagents 0.1mL of 1% liver homogenate were mixed with 0.75 and 0.15mL of ethanol and ice chilled chloroform and the mixture was centrifuged at 3000 rpm at 4°C for 10 minutes The supernatant was collected. Collected 0.5 mL of supernatant was mixed with 0.5 mL, 1 mL and 0.5 mL of EDTA, buffer and epinephrine. The absorbance of the supernatant mixed with above mentioned reagents was measured spectrophotometrically at 480 nm for every 30s for 150s. The blank without the homogenate was prepared and absorbance measured in a similar way. The 50% inhibition of the rate of autoxidation of epinephrine measured as a change in absorbance /min was used to measure one unit of enzyme activity

2.10.5. Catalase (CAT)

CAT was estimated in liver tissue homogenate by method reported by Sinha [98]. The method is based on the principle of reduction of dichromate to chromate acetate and an unstable intermediate perchromic acid; when it is heated with acetic acid in the presence of H_2O_2. Phosphate buffer (0.01M; pH 7.0) was prepared by dissolving sodium dihydrogen phosphate and disodium hydrogen phosphate (1.735g) in distilled water (1000 mL). 6.8 mL of H_2O_2 was dissolved in distilled water (93.2 mL) to prepare hydrogen peroxide (2M). Fresh dichromate-acetic acid reagent was prepared by mixing 5% potassium dichromate and glacial acetic acid in 1:3 ratio. Following preparation of all the reagent; each liver homogenate (1mL) were mixed with phosphate buffer (1mL) and H_2O_2 (0.4 mL). After mixing of the aforementioned reagents the reaction of the mixture was stopped by the addition of dichromate-acetic acid reagent (2 mL). Once the reaction was halted the absorbance was spectrophotometrically measured at a wavelength of 620 with an interval of one minute for 3 minutes consecutively and percent inhibition was calculated. Since the reagent dichromate has no absorbance in the region of 570–620 nm therefore, its presence in the assay is least likely to interfere with the photometric determination of chromic acetate.

2.10.6. Glutathione Peroxidase (GPx)

The GPx activity in the study was measured by method described by Rotruck *et al*, 1973 [99]. 0.1% of sodium nitrate, 10% tricarboxylic acid, 0.1% sodium nitrate, Ellman's reagent, 0.4M Tris-HCl buffer (pH 7), 10mM sodium azide and 0.2mM hydrogen peroxide were all freshly prepared. Once all the reagent were prepared, then GPx activity was measured by mixing the liver homogenate with all the reagents and incubating the contents for 10 minutes at a temperature of 37°C. Following 10 minutes of incubation the

reaction is arrested by 0.4mL of 10% TCA and centrifuged. In the retrieved supernatant Ellman's reagent is added in order to assay for glutathione at 412 nm.

2.10.7. NF-κB p65 using ELISA

In the study ELISA was used to measure the levels NF-κB p65 in the hepatic tissue homogenates using the kit *"USCN Catalog no. SEB824Ra"*. Following the instructions specified in the kit provided by the manufacture the optical-density was read at 450nm by spectrophotometer for the protein-bound NF-κB.

2.11. Analysis of Antioxidant, Anti-inflammatory and Anti-apoptotic genes: Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

RNA Extraction and Purification

Total cellular RNA was extracted from 30-50mg of the liver tissue following the protocol of the RNA extraction kit (*Catalog no. K0731 GeneJET Thermo Fisher Scientific, USA*). Trace contaminations were removed according to the protocol by ethanol precipitation of the RNA followed by two washes of the pellet with iced cold 75% ethanol and in the end nuclease free water was added.

Removal of Genomic DNA from RNA Preparation

For cDNA synthesis and qRT-PCR the extracted RNA used as a template must be free of DNA contamination. Therefore genomic DNA was removed using RNase free DNase I following the protocol in which the RNase free tube was prepared by adding the following reagents/chemicals

Nucleic Acid (RNA)	***1µg***
10X reaction buffer with $MgCl_2$	***1µl***
DNase I, RNase I	***1µl***
DEPC-treated water	*Upto 10µl*
Total Volume	*10µl*

Following the preparation the tube was incubated at 37℃ for 30 minutes. Then 1µl of 50mM EDTA was added and incubated for another 10 minutes at 65℃. EDTA was added to ensure that RNA does not hydrolyzes when heated. The RNA was free from genomic DNA and can be used as a template for reverse transcriptase. While removing the genomic in the reaction mixture to inactivate type A RNase which is potentially present in the initial RNA solution RiboLockTM RNase Inhibitor (1µ/µl) can be added. RiboLock was used initially in extraction of RNA therefore, not required here. Integrity and purification of extracted RNA was confirmed by running 1% agarose gel electrophoresis and quantified by Nano drop.

cDNA synthesis

In the first step of real time-RT-PCR protocol; the initial i.e. first strand of complementary DNA was made by using total RNA (approx. 0.1ng -5µg) or poly (A) mRNA (1ng-500 ng). The kit and *("Thermo Scientific RevertAid First Strand cDNA Synthesis Kit; Catalog no. K1691 Thermofisher Scientific, USA")* its protocol was used to make complementary DNA strand. Following reverse transcription reaction, the reaction product i.e. first strand of cDNA was directly used in PCR or it can be stored at -20℃ (for ⍰ 1week). However, storage for longer periods should be done at -80℃. The cDNA synthesized in the study was used in qPCR with eight different sets of primers [70].

PCR Amplification: Inclusive in the 50µl of total volume of PCR is 2µl of the first strand cDNA mixture, "*SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA)"* and eight primer sets. Expression of the mRNA in a sample was measured and related to the expression of a house-keeping or reference genes; HPRT-1 "*(internal standard)"*. The qRT-PCR was performed using the following thermal cycling i.e. amplification program using an "*Applied Biosystems, Foster City, CA, USA"*: 1 cycle of 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C for 40 sec, and 72°C for 30 sec, and a final elongation cycle at 72°C for 5 min. Reproducibility and robustness of the protocol was ensured by running the duplicates. The data obtained were plotted as fluorescence vs. cycle number using the software provided with the Applied Biosystems instrument, Foster City, CA, USA. The software reported the level of amplification as the mean of the replicate PCR cycles at which the fluorescence is first detected, which is known as Cycle threshold (Ct). Ct is inversely proportional to the initial DNA template concentration. It calculated the relative gene expression level according to the comparative cycle of threshold (Ct) method using HPRT1, as a housekeeping. A blank control after normalizing it with the housekeeping gene using a formula 2-ΔΔCt was used to determine the fold increase or decrease relative to the blank control [100]. The data were shown as the relative expression of TNF-α, Nrf2, COX-1, IL-1β, Keap1, Bcl2, HO-1, Bcl2 and Bax.

2.12. Histological Evaluation of Hepatic Tissue

For histopathological staining the basic protocol was followed. Following dissection of the liver it was fixed in 10% neutral buffer formalin for 48 hours. Following fixation, hepatic tissue sections were made and washed thoroughly in 30% alcohol. This was followed by gradual dehydration of the sample by placing them in ascending series of alcohols. The tissue was then placed in wax, made into blocks and finally cut into sections of 5 micron thickness. Sections were adhered on the glass slide by treating with Mayer's solution, stretched at approximately 40℃ of temperature and preserved at room temperature. The sections prepared and adhered on glass slides were then stained with hematoxylin and eosin for general morphological observations. This was followed by mounting of tissue with Canada balsam and preservation with cover slip. The compound microscopy was used for analysis of the changes in the liver architect.

2.13. Statistical Analysis

The results from this study are represented as the "*mean ± standard error of mean (S.E.M)*". "*One-way ANOVA"* followed by "*least significant difference (LSD) multiple comparisons post-hoc test"* was carried out for statistical analyzes of the data using IBM SPSS v. 26 software ("*Chicago, IL, USA"*). The analytical results were considered statistically significant with a p value of 0.05. The data obtained from OGTT were expressed as "*mean ± standard error of mean (SEM)"*. Statistical comparisons were performed by "*one-way ANOVA"* followed by "*Dunnett's Multiple Comparison Test"* and the values were considered statistically significant when $p < 0.05$. The graphs and statistical calculations were prepared using "*GraphPad Prism version 4.00"* for Windows ("*GraphPad Software, San Diego, CA, USA*, www.graphpad.com").

3. RESULTS

3.1. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on Hepatic Weight

Upon induction of diabetes the weight of the liver decreased; however, upon treatment it was gained back to near initial weight. The liver mass decrease in DM control which did not improved much after treatment with FO. However, upon treatment with Glic, HF, CF and EF improvement in liver weight was seen as compare to DM control (Figure.2).

3.2. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on the Concentration of Fasting Blood Glucose (FBG), Serum Insulin (SI), HOMA, HbA1c

A significant increase in fasting blood sugar levels in diabetes control (DM Control) as compare to control groups (C) shown in the (Figure.2) depicts that it is an initial indicator of HF-HFr-STZ induced DM. Reduction in fasting sugar levels were observed when the DM Glic group and each of the treatment groups were administered 200mg/kg of Glic, FO and its HF, EF and CF soluble fractions, respectively. Similar to glucose levels seen at fasting there was an increase in glycated hemoglobin level ($p < 0.05$) in DM Control group when compared to control whereas; upon treatment for 4 weeks with Glic i.e. reference control (5.3%), FO, HF, CF and EF HbA1c levels were decreased. However, the group treated with the chloroform and ethanol soluble fraction did not show substantive decrease in the HbA1c as compare to FO and HF groups (Figure.2). Figure.3 shows levels of serum insulin in C, DM Control, Reference control (DM Glic) and Treatment (FO, HF, CF and EF) groups. Both glucose levels and insulin resistance are indicators of T2DM induced by STZ. Insulin was decreased in diabetic control rats; whereas control rats showed increased secretion of insulin as measured in serum. Improvement ($p < 0.05$) in serum insulin levels of rats following treatment with Glic, FO, HF, CF and EF were observed (Figure.3) [previous work; 70].

Insulin resistance index as measured by HOMA-IR was elevated in DM Control group with a considerable difference observed ($p < 0.05$) between control and DM Glic. However, it also did not improve in HF, CF and EF groups. Furthermore, HOMA-β and IS shows normal functioning of *beta-cells* and *insulin*

sensitivity in control group with deterioration seen in DM control group. Significant improvement was observed in HOMA-β and IS in DM Glic, FO, HF,CF and EF treatment groups as compared to the significant decreased observed in DM control group (Figure.3) [Previous work, 70].

3.3. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on the Acute Toxicity and Oral Glucose Tolerance Test

The study was carried out in both male and female Wistar Albino rats as explained previously [70]; showed no sign and symptoms of toxicity, and there was no mortality reported within the first 24 to 48 hours. Neither was it observed for the 15 days thereafter. Results of 15 days observations and biochemical parameters i.e. complete blood count, urea, creatinine, AST, ALT, ALP, GGT, direct, indirect and total Bilirubin and complete blood counts were within the reference ranges and showed no significant deviation from the norm. Therefore, based on the observations and biochemical parameters it can be deduced that the LD50 of the fixed oil and its soluble fractions could be greater than 2000 mg/kg body weight.

The oral glucose tolerance was carried out as mentioned previously [70]. The hypoglycemic effect was observed for all the doses ($p \leq 0.05$). The maximum decrease however, was recorded after 30 to 60 minutes in comparison to 60–120 minutes for all the doses i.e. from 50 to 600 mg/kg. The glucose levels were maintained in euglycemic concentrations between 60 to 120 minutes when compared to the reference antidiabetic drug; Gliclazide. Cumulatively from the results of the present studies and those cited previously it was deduced to select a dose of 200 mg. This dose was then used to study the antioxidant, anti-apoptotic, anti-inflammatory and antidiabetic activities in T2DM rat model [70, 72, and 74].

3.4. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on Serum Lipid Profile and Liver Function Test

Serum total and indirect bilirubin showed slight decrease in HF, FO and EF as compared to control and DM control group whereas; no change was observed in direct bilirubin levels (Figure.4). Liver enzyme ALT decreased in FO, HF and EF group as compared to DM Control; and AST increased in CF and FO groups as compared to Control DM Control study groups (Figure.4). On the other hand ALP and GGT increased in all the four treatment group of CA when compared with control, DM Control (Figure.4). In lipid profile significant decrease was observed in serum cholesterol in HF, EF and FO groups as compared to Control, DM Control and DM Glic whereas; levels of serum TG and VLDL decreased in HF, CF and EF. However levels of LDL increased significantly ($p \leq 0.05$) in CF and HDL levels showed no change in any of the treatment groups. On the other non-HDL levels were significantly decreased ($p \leq 0.05$) in HF and EF treatment groups as compared to Control, DM Control and DM Glic (Figure.5).

3.5. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on Hepatic Antioxidant and Inflammatory Stress Parameters

3.5.1. Lipid Peroxidation (LPO)

The liver tissue homogenate from the DM Control group showed significant increase in LPO (the MDA level) (Figure.6). After 4 weeks of treatment LPO decreased in contrast to no treatment intervention in diabetic rats. Levels of MDA decreased in DM-Glic, EF, CF, FO and HF groups.

3.5.2. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) Reductase

The ratio was raised in diabetes i.e.DM Control. The rat groups following treatment showed decreased in the ratio as compare to DM-C. However, among the treatment groups significant improvement (*p≤0.05*) was observed in the groups given standard anti-diabetic drug (i.e. Glic), FO, and HF. Similar improvement was observed in ethanol and chloroform soluble fraction groups (Figure.6).

3.5.3. Reduced Glutathione (GSH) level

The percent inhibition of reduced GSH was significantly increased (i.e. decreased levels) in control rats whereas it decreased in the DM C (i.e. increased level). Treatment with 200mg/kg of Glic was did not show any improvement However treatment with FO and its solvent-soluble portions HF, CF, and EF for 4 weeks once daily increased the levels (i.e. reduced the percent inhibition) of reduced GSH (Figure.6).

3.5.4. Superoxide Dismutase (SOD)

The increased percent inhibition in HF-HFr-STZ induced diabetic control group (DM Control) reflected as a decrease in the activity of the enzyme as compare to control group. There was a significant decrease observed in percent inhibition (i.e. increase in enzyme activity) in DM Glic as compare to DM Control. Whereas, FO, HF, CF and EF groups showed increase activity (decreased in percent inhibition) in comparison to DM-C (Figure.7).

3.5.5. Catalase (CAT)

The hydrogen peroxide scavenging activity of CAT was increased (i.e. decreased percent inhibition) HF, FO, CF and EF groups in contrast to rats with no intervention and on regular diet. In DM Control treatment group increased CAT reflected as increased in percent inhibition (i.e. decreased activity) of the enzyme (*p≤0.05*) in comparison to control and DM-C (Figure.7). However, CAT activity in DM-control managed to decrease oxidative stress as reflected by decrease in percent inhibition.

3.5.6. Glutathione Peroxidase (GPx)

The antioxidant activity of GPx in hepatic tissue homogenates from DM control increased (i.e. decrease in percent inhibition) as compare to DM Glic and Control. The enzyme activity increased (*p≤0.05*) in all of the treatment groups when compared with Control, DM Control and DM Glic groups respectively (Figure.7).

3.5.7. NF-κB p65 using ELISA

In the DM control rats the levels of this pro-inflammatory transcriptional factor increased significantly ($p < 0.05$) in contrast to control. Treatment with standard antidiabetic drug Glic failed to subside the inflammatory response rather the levels in liver tissue increased significantly ($p < 0.05$). There was comparatively much decrease in NF-κB p65 in rats treated with FO. However, the NF-κB p65 reached to control levels in the groups treated with HF and CF as compared to EF (Figure.8).

3.6. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on Hepatic mRNA Expression of Antioxidant, Anti-inflammatory and Anti-apoptotic genes

3.6.1. Expression Profiles IL-1β, TNF-α genes and COX-1

Expression levels of genes (IL-1β, TNF-α) were increased in rats from DM Control as opposed to the control rats. On the other hand COX-1 decreased as compared to control. Increased expressions were observed upon subsequent treatment with Gliclazide in the DM Glic rats when comparison was done to DM-Control and Control group; COX-1 expression increased upon treatment with EF, FO HF and CF. TNF-α and IL-1β level decreased (p 0.05) in groups treated with Glic, FO, HF and CF. In the EF treatment groups the expression pattern of IL-1β bizarrely increased as compared to both DM Control and Control however, the mRNA levels of TNF- α were found to be decreased significantly (p 0.05) as compare to Control and DM Control groups. COX-1 levels showed neither marked decrease nor increase in HF, FO and CF treatment groups rather the expressions were almost same to the one observed in Control group (Figure. 9). The accompanying Heatmap of these three inflammatory marked depicts the similar pattern of expression (red being the highest and light pink being the lowest)

3.6.2. Expression levels of Anti-apoptotic marker: Bcl-2 and Pro-apoptotic marker: Bax

The Figure illustrates the effect of diabetes and its subsequent treatment. Bax in DM Control rats increased (compared to Control group). After four weeks of treatment with Glic HF, FO and CF Bax decreased (p 0.05) in contrast to DM Control group. Bizarrely, the levels increased significantly in the EF group. On the other hand the mRNA levels of anti-apoptotic marker Bcl-2 decreased in DM Control group significantly ($p < 0.05$); the decreased expression of Bcl-2 improved significantly ($p < 0.05$) in the CF treatment group only. The other treatment groups inclusive of DM Glic, FO, HF and EF showed no improvement rather the expressions were lower (p 0.05) in contrast to control rats. The accompanying Heatmap of these apoptotic markers is in agreement with the bar graph (Figure.10).

3.6.3. Expression levels of Antioxidant genes: Nrf2, Keap1 and HO-1

There was decline in the expression of both Nrf-2 and HO-1 in DM Control rats shown in the Figure.11; whereas the Keap1 levels increased significantly in DM-C (p 0.05). The mRNA levels of Nrf2 measured in EF, DM Glic, FO, CF and HF treatment groups increased in descending order following 4 weeks of treatment increased as compared to the rats with DM rats with no intervention (i.e. DM Control). However, the most significant increase ($p < 0.05$) was seen in EF, DM Glic and CF groups followed by FO,

HF groups; respectively. Very interestingly, the expression pattern of HO-1 parallels the Nrf2 levels in all the treatment group showing increase in the antioxidant capacity. The most significant increase was observed in the EF group followed by DM Glic, FO and HF. The expression pattern of Keap1 in all the treatment groups increased significantly ($p < 0.05$) as compared to both Control and DM-C (Figure.11). Similar to the expression pattern HO-1 & Nrf2, Keap1 also showed elevated levels in EF group as opposed to remaining treatment groups. The Heatmap of Nrf2, Keap1 and HO-1 depicts the expression profile of innate antioxidant pathway and the figures are in agreement (Figure.11)

3.7. The Effect of C. anthelminticum Seed's Fixed oil and its Fractions on Hepatic Tissue Histology

Figure.12 shows the liver sections from the Control, DM-C, and DM Glic, FO, HF, CF and EF rats. Hepatic parenchyma (HP) of the Control rats (A and B) shows hexagonal lobules with portal spaces and centrally located portal vein (PV) can be seen with no obvious signs of liver injury. In the T2DM model group (Figure.12: C and D) pathological findings of hyperemia (HE) with the rupture of vessel in central view can be observed. Loss of cell boundaries along with distorted sinusoidal spaces can be seen, as well along with nucleus (N) towards the periphery. An increased degeneration from portal vein to portal triad (PT) and various white lipid droplets are also seen showing the evidence of microvesicular steatosis (MS). However, there is no to very little evidence of inflammation (IC: inflammatory cells). These findings confirm the establishment of T2DM diabetes in the rats after 8 weeks of HF-HFr diet followed by STZ injection. Compared with DM-Control the morphological finding in the treatment control i.e. DM-Glic (E) and CA treatment groups (F-J) improved. However, microvesicular steatosis did not improve much especially in HF and EF groups (Figure.12: G and J). In the figure HA stands for hepatic artery seen within the portal triad in Figure: 12: H.

4. DISCUSSION

Both the cellular uptake of micro-molecules (glucose, amino acids, and fatty acids); and the synthesis of their respective macromolecules (carbohydrates i.e. glycogen, proteins and lipids) are regulated by insulin; the most significant metabolic hormone [101]. Its absence, insufficient secretion and impaired functionality is the aetiological crux of DM. The STZ-induced T2DM is the commonly employed animal model to study the pathogenesis and discover novel therapeutic options. Not only STZ damages 60–80% of pancreatic beta (□)-cells by generating carbonium and reactive oxygen radicals rather; clinical and pathological manifestations like polyphagia, polyuria, polydipsia, weight loss, muscle wasting, lipolysis of adipose tissue, hypoinsulinemia and fasting hyperglycemia are very akin to what are observed in humans [102]. Yet, to quantitatively substantiate □-cell mass and function; HOMA-□ index is mentioned as the most justifiable theoretical index [103]. In our study besides the observations of overt clinical signs; reduction in the weight of liver (Figure.2), as well as in serum insulin, beta cell functioning index (HOMA-β), insulin sensitivity (IS) (Figure.3) with concomitant increase in FBS, HbA1c (Figure.2) and insulin resistance index (HOMA-IR) (Figure.3) validated the successful development of HF-HFr-STZ induced T2DM model. Furthermore, the pronounced decrease in FBS along with the improvements in circulatory

levels of insulin and HOMA-□ in diabetic rats treated with CA seed's FO and it soluble fractions—HF, CF and EF were our clearest indication of not only the hypoglycemic effect of CA rather; also the plausible improvement and/or regeneration of pancreatic □-cell mass.

The HbA1c; a widely used index of glycemic control is both a good biomarker to assess nonenzymatic glycosylation of proteins and also evaluate the causal relationship between hyperglycemia and vascular complications associated with diabetes [104]. Increased HbA1c due to persistent hyperglycemia was observed in the rats in our model which subsequently improved upon treatment with CA seed oil and it fractions. These findings could have been further validated by observing the improved morphology of the pancreatic □-cell mass under the microscope from the treated rats. However, cumulative effects of the FO and its HF were more pronounced as compared to the rats administered CF and EF. These observed improvements could be attributed to attenuation of inflammation and oxidative stress which will be discussed later. Unfortunately, in our study we did not probed the other probable explanations like reduction in hepatic glucose output, increase peripheral glucose uptake, decreased glucose digestion and absorption for the improvement seen in FBS, HbA1c, HOMA-□ and liver weight etc. [105]. These require further research for the better understanding of the putative benefits of the seeds of CA.

Inhibition of mitochondrial Complex I and II causes electron leakage in STZ-induced diabetic rats which causes the incomplete reduction of oxygen to superoxide. Thus persistent hyperglycemia leads to overproduction of ROS which impairs both mitochondrial membrane potential and ATP production, thereby favoring cellular damage and death [106]. Our histological (Figure.12) and biochemical observations of liver are in line with these findings. Both the transaminases i.e. ALT and AST responsible for normal functioning of the liver cells were increased in untreated (i.e. DM control) rats indicating hepatic insult (Figure.4). ALT was more significantly increased since it is more specific and precise marker of liver inflammation as compare to AST which is also secreted from tissues other than liver [107–108]. However, there was reduction in the ALP levels in diabetic control rats and these finding are in agreement with the findings of Mandal. A et.al. (Figure.4) [109–110]. Not tissue specific; ALP is also secreted from bone, intestinal lining and bile duct etc.; however, it serves as a marker of liver function. Khajuria. P et.al identified its distinctive role in diabetes and hepatopathy therefore, monitoring ALP levels can provide better insight into diabetes-associated complications (e.g. DH) [111–115].

Both the alkaline (ALP) and acid phosphatases (ACP) have been implicated to be involved in response to STZ-induced toxicity at cellular level by participating in molecular signaling pathways [116]. They tend to regulate glucose homeostasis and insulin signaling by dephosphorylation of key enzymes involved in the glycogen and glucose metabolism, and affect phosphorylation of insulin receptors; respectively [117–118]. Furthermore, they are also involved in oxidative stress response by modulating the phosphorylation status of phase II detoxification and antioxidant proteins (i.e. enzymes) which are under the control of important transcription factor Nrf2—a key player in the innately existing Nrf2/Keap1/HO-1 antioxidant signaling pathway [119–120]. The CA seed oil and it fractions' attempted to restore ($p \leq 0.05$) both the energy balance and hormonal regulation in diabetic rats by improving the activity of ALP enzyme. The ALP activity showed marked improvement in HF, CF and EF treated groups as compared to FO and

gliclazide treated rats (Figure.4). Marked reduction in the activity of ALT was observed upon treatment with CA seed oil's EF and CF whereas; it was restored to control levels in rats subject to gliclazide (DM Glic), FO and HF (Figure.4). However, no significant improvement resulted in AST activity in the HF and EF treated rats, the reason could be AST being non-specific biomarker of liver may be raised as a result of DM affecting organs such as skeletal or cardiac muscles (Figure.4) [121]. Whereas; GGT reflection of biliary tract function and being involved in number of pathologies inclusive of DM [122–124] improved across all the treatment groups (Figure.4). Previously, Qureshi, S. A. et.al, reported the potential hepato-protective effect of CA seed in carbon tetrachloride-induced hepatotoxicity in rats [73]. Our study had more or less similar findings however, the findings could have been substantiated with more confidence by not only measuring the activities of the aforementioned enzymes in the serum rather; also measuring both the cellular activity, quantity and expression levels. This could also have helped in better evaluation of STZ-induced toxicity and therapeutic potential of CA seed oil in diabetic rat models.

The tetrapyrrole ring of heme cleaved by heme oxygenase produces biliverdin which is eventually reduce to bilirubin by biliverdin reductase. Bilirubin—previously regarded as the toxic end product of heme metabolism is now being recognized and cited by Stocker as a potent antioxidant who's scavenging capacity is more stronger than α-tocopherol [125–128]. It prevents the generation of oxidant molecules by inhibiting protein kinase C and NAD(P)H pathways besides being considered as an effective antioxidant of lipid peroxidation. At mildly increased levels; it has been recently cited as cytoprotectant in diseases of cardiovascular system inclusive of kidneys, diabetes and its associated complication. This cytoprotection is attributed to its antioxidant and anti-inflammatory properties as evident by comparison of hyerbilirubinemic Gunn and genetically controlled STZ-induced diabetic rats [129–131]. Its beneficial effects have also been reported in human disease like metabolic syndrome [132–134], DM [135–136] and cardiovascular diseases etc. [137].

Jenko-Pražnikar, Z et.al, Hwang, H. J et.al, and Kwon, K. M et.al, have reported inverse relation between insulin resistance and bilirubin which was evaluated by assessing serum triglyceride concentration and HOMA-IR [132–136, 138]. Varied relationships have been reported between bilirubin and total cholesterol, LDL and HDL-c depending on the experimental conditions [132–136]. These evidences point to the fact that bilirubin is closely related to both insulin resistance and lipid metabolism. Very long ago Shepherd et.al in 1979 subjected the fat cells of rat to high concentration of bilirubin and reported not only inhibition of lipolysis rather; also stimulation of glucose oxidation [139]. Unfortunately, very few mechanistic studies have been carried out to probe the role of bilirubin in regulating dyslipidemia and explain its association with insulin resistance. In our study no significant differences have been observed in the serum total, direct and indirect bilirubin levels between all the study groups (i.e. Control, DM C, DM Glic and; the FO, HF, CF and EF treatment groups) (Figure.4).This is encouraging since it can be the plausible explanation of liver attempting to protect its cell against oxidant and inflammatory insults incurred in HF-HFr-STZ-induced diabetic rats.

Lack of insulin or resistance to it in T2DM leads to decrease activity of lipoprotein lipases which facilitates increased mobilization of FFA from adipose tissue thus, precipitating hyperlipidemia and

hepatic steatosis [140–142]. The viscous cycle of generation of oxidant and inflammatory cytokines in the face of persistent hyperglycemia further precipitates hepatic steatosis by stimulating the TG synthetic genes and the lipogeneic transcription factors SREBP1/2 along with concomitant suppression of mitochondrial beta oxidation of fatty acid through PPAR-α [143]. Treatment of diabetic rats in our study with CA seed oil and its soluble fractions positively affected the lipid profile by decreasing the levels of serum cholesterol, TG, VLDL, non-HDL-c and increasing HDL-c (Figure.5). The aforementioned changes in lipids were either similar to or less than control group. These effects can be attributed to either decrease intestinal absorption of cholesterol or it could be a secondary effect due to antioxidant, anti-inflammatory or hypoglycemic activities of CA oil and its fractions. Or the more plausible explanation could be that the not perturbed effects of bilirubin observed in our study seems to be in agreement to what has been reported about the varied relationship of bilirubin with lipid metabolism along with its putative antioxidant and anti-inflammatory activities. Moreover, suppression of the TG synthetic genes and the lipogeneic transcription factors SREBP1/2 along with concomitant stimulation of mitochondrial beta oxidation of fatty acid through PPAR-α could be a reasonable explanation. However, these are all observational findings which require well designed mechanistic studies to confirm and validate these assumptions.

In order to probe the antihyperlipidemic activity of CA seed oil and its fractions, HMG-CoA reductase activity was assayed and determined by measuring the HMG-CoA/mevalonate ratio. Cholesterol homeostasis in the body is maintained by two processes. First is the synthesis, two-third of which occurs in the liver under the regulation of HMG-CoA reductase; second is the exogenous dietary absorption and endogenous liver clearance through biliary secretion [144–145]. The activity of the enzyme in hepatic cells have inverse relationship with HMG-CoA/mevalonate ratio [146]. Cholesterol synthesis decreased in diabetic rat as reflected by increased ratio. The activity of enzyme did not show significant difference in EF, CF and HF treated rats when compared with diabetic rats; on the contrary there in improvement when compared with control rats (Figure.6). Comparatively, enzyme activity improved in rats treated with FO and gliclazide. These are inconclusive finding which needs to be dissected further.

Redox-sensitive proteins—Nrf2, NF-κB and phosphatases (e.g. ALP) are capable of changing their oxidative and reductive status in the light of changing pro- and anti-oxidant signals which in turn helps them to regulate metabolic gene regulatory responses in diabetic hepatic cells [147–148]. The expression of number of target proteins and/or enzymes change under the transcriptional control of Nrf2 and NF-κB. Therefore, in the current study level of oxidative stress was assessed in liver homogenate suspected of having DH i.e. NAFLD by measuring enzymatic antioxidant markers: “SOD, CAT and GPx”; and non-enzymatic markers: “LPO, GSH and HMG-CoA/Mevalonate ratio” [149]. The cellular enzymatic antioxidant mechanisms initially use SOD to detoxify superoxide radicals by producing H_2O_2, which is decomposed to water by the mutual activity of both CAT and GPx [150–151]. It was observed that activity of SOD decreased reflecting that oxidative stress was induced in the rat liver as a result of 9 week of feeding HF-HFr diet in which STZ was injected after 4 weeks of feeding (Figure.7). Whereas, the activities of CAT and GPx the two most important antioxidant enzymes very interestingly

increased in DM Control as compare to Control rats. CAT activity increased in HF and FO treated groups as compare to control however, treatment of rats with CF, EF and DM-Glic decreased their activity (Figure.7).

On the other hand GPx activity decreased across all the treatment groups rather its activity was comparable to the one observed in control rats. The plausible explanation for the aforementioned observation can be referred to the ones cited by Stevens, M. J. et.al and Foyer, C. H. et.al [152–153]. Their finding and findings from other studies identified that the changes in the cellular redox potential regulate both the presence and activities of CAT and GPx [154–155]. CAT and GPx's apparent enzymatic inactivity or silencing could be attributed to decrease in protein expression or mRNA levels or decrease in its phosphorylation. Or it could be over phosphorylation causing ubiquitination of these enzymes resulting in subsequent degradation [156–157]. This call for further exploring the activity of CA seed oil and it fractions in diabetic rats in the context of measuring activity, quantity and expression (mRNA) of hepatic CAT and GPx in combination with their phosphorylation status and comparing it with the hepatic tissue's oxidation state.

Lipid peroxidation reduction is imperative for not only normal cellular functioning rather, also for the integrity of cell membrane [158–159]; since intact membrane is crucial for functioning of insulin receptors which are membrane-spanning receptors [160]. Increased LPO was witnessed in DM-C rats which upon treatment with gliclazide, EF, CF, FO and HF improved in descending order (Figure.6). This improvement mitigated inflammatory and oxidative stress which reflected in the antioxidant and pro-inflammatory markers measured in this study. Furthermore, a principal non-protein thiol GSH is also known as the master antioxidant molecule. Its imbalance has been implicated in number of metabolic, inflammatory and infectious conditions [161]. The intracellular redox state can be predicted by measuring the ratio of reduced glutathione and oxidized glutathione (i.e. GSH/GSSG) [162]. Toxic GSSG is converted to non-toxic GSH by enzyme glutathione reductase (GSR) [162]. The GSH conjugation with pro-oxidant and xenobiotic stresses is facilitated by the enzyme glutathione transferase (GST). Levels of GSH were significantly decreased in DM Control rats due to its engagement in GST-catalyzed conjugation with STZ. However, treatment improved the levels of GSH in all the treatment groups reflecting availability of GSH to mitigate hyperglycemia induced oxidative and inflammatory stress [163–164].

A growing body of evidence cited over the last ten years shows that liver cells are protected from oxidative stress and progression of DH can be slowed down via the activation of Nrf2/Keap1/HO-1 pathway [165–168]. The HO-1 is one of the important antioxidant enzyme regulated by Nrf2/ARE signaling [168]. It has been shown in a study that mice deficient in Nrf2 gene lack adaptability to hepatic oxidative stress due to high fat diet and initial hepatic insult can progress to develop NAFLD [169–170]. The results of the said study did demonstrated hyperglycemic, oxidant and inflammatory insult in T2DM rat developed by using HF-HFr diet and STZ. However, intervention with seed oil and its fractionated portions attenuated the oxidative, inflammatory and apoptotic stress by regulating the Keap1/HO-1/Nrf2 pathway and downregulating NF-κB and Bax levels (Figure.8 and 11).

The low expression of Nrf2 and an increase in expression of Keap1 in the diabetic hepatic tissue corresponded to previously cited results [171–172]. The increase expression of Keap1, a negative regulator of Nrf2 is an indication that less of Nrf2 translocates in the nucleus to activate and recruit antioxidant genes [173]. However, the administration of HF, CF, DM Glic and FO decreased the mRNA level of Keap1 and EF behave contrary to other groups (Figure.11). The data obtained on the Nrf2 mRNA showed that the EF, CF, FO and DM Glic showed the increment in antioxidant potential by facilitating the translocation of Nrf2 in the nucleus and activating downstream antioxidant pathway. The key enzyme in the catabolic pathway of heme-HO-1, is also known to exhibit strong antioxidant and anti-inflammatory properties [174–175]. It is regulated by transcription factor Nrf2. The overexpression and induction of HO-1 in number of mouse model has demonstrated its ameliorative effect in NAFLD and similarly increased HO-1 has also shown to alleviate stresses caused by steatosis and fibrosis [176–179]. The increased expression of HO-1 in our treatment groups are in agreement to previously cited evidences (Figure.11). Very interestingly, in EF group there is simultaneous increase in Nrf2, Keap1 and HO-1 the oddity being the parallel increase in both Nrf2 and Keap1. Whereas, NF-κB measured by ELISA decreased and the expression levels of IL-1β, TNF-α and COX-1decreased in all the treatment group. Again EF group showed bizarre increase in IL-1β and COX-1 (Figure.8 and 9)

Nrf2 besides attenuating the oxidative stress has been shown to suppress pro-inflammatory NF-κB and cytokines (TNF-α, IL-1β). It is known that pro-inflammatory markers negatively impacts glucose tolerance. For example cytokine TNF-α stimulates glucose production and decreases uptake of glucose in peripheral tissues whereas [180], IL-1β is responsible for loss of pancreatic □-cell mass [181] and is known to impairs insulin signaling mechanism in liver tissue [182]. Therefore, suppressing oxidative and inflammatory stress in DM can protect against glucose intolerance, cell loss, hyperglycemia and hepatic injury. Thus supporting the notion that both the delay in the onset and progression of DH cannot be solely attributed to adaptive response of Nrf2 rather therapeutic activation of Nrf2 expression and simultaneous repression of inflammatory transcription factor NF-κB and its downstream cytokines IL-1β and TNF-α—also plays significant role [183–184].

Consequently, the levels of anti-apoptotic Bcl-2 increased in only CF treated rats whereas; pro-apoptotic Bax expression level decreased across all the treatment group except EF in comparison to DM Control rats (Figure.10). These observation clearly highlighted the putative activity of CA seed oil in mitigating release of cytochrome c into the cytosol and caspase-3 initiated execution of DNA fragmentation which precludes programmed cell death. These activities were not mitigated by upregulation of expression of Bcl2 rather; it was more likley due to Bax. This explanation could have been supported by simultaneous measurement of cytochrome c and caspase-3 [185–187].

5 CONCLUSION

In our study despite the short duration of treatment (4 weeks) with seed oil and its soluble fractions —the promising anti-hyperglycemic, antioxidant and anti-inflammatory effects were observed. Mitigation of inflammation was similar with not much tissue specificity [previous work, 70]. However, the antioxidant

effects specially the expression of HO-1 and Keap1/Nrf2 were tissue specific [previous work, 70]. The expression levels in kidneys were idiosyncratically opposite to what has been observed in the hepatic tissue [previous work reference, 70]. In either case the seed's oil and it's all three soluble fraction dampen and reverse the deleterious perturbation of hyperglycemia induced inflammation and oxidation in both the kidneys and liver to near normal constitutive levels. The findings of this study gives credibility to what has been previously reported about the ethno-therapeutic potentials of *C. anthelminticum.*

The findings of this study identifies the gap and gives confident for further exploration of DM models developed chronically; as a means for appropriate representation of the pathophysiology of diabetes and DH. The phytochemical analysis of oil and it soluble fractions using GC-MS and FTIR showed that the oil is rich in fatty acid and terpenoids which corroborated its anti-diabetic, anti-inflammatory and antioxidant potential (unpublished work). Some of the chemicals found in the oil like caryophyllene have insulinotropic effect and 11, 14-Eicosadienoic acid have anti-amylase activity [188–190]. Number of *in-silico* methods inclusive of network pharmacology, molecular docking and stimulation can be used to explore multi-targeted and multi-mechanistic potential of either the individual phytochemical or combination of it. Moreover, much benefits can be derived from it by synthesizing metal or metal-oxide nanoparticles using green approach.

Declarations

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Institutional Review Board Statement: This study was approved by the Ethical Review Board for Animal Research and Ethics, Dow University of Health Sciences, Pakistan Approval number: AR.IRB-21/DUHS/Approval/2021/037. All the methods used in this study are in compliance with ARRIVE guidelines and Organization of Economic Co-Operation and Development (OECD) guideline 420 for testing chemicals.

Author Contribution

Dr Nida Baig Project Administration, Methodology, Investigation, Writing- Original draft preparation. Dr Shamim Akhtar Conceptualization, Supervision, Writing-Reviewing and Editing, Misbah Anwar Investigation, Data curation, Sabahat Aziz Software, Visualization, Ambreen Rafique Validation, Data Curation, Aliza Mansoor Software, Visualization.

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Figures

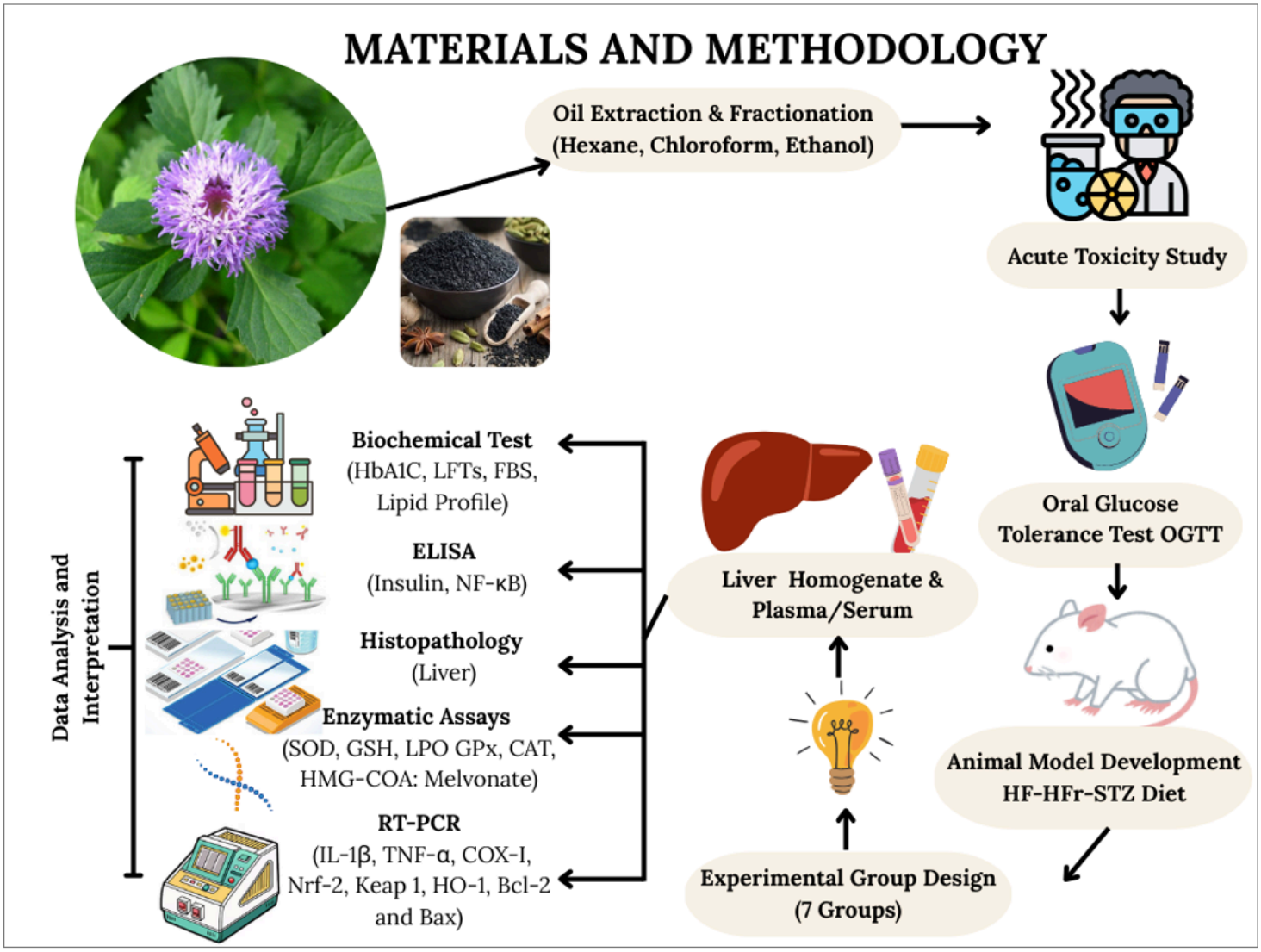


Figure 1

Graphical Presentation of the Experimental Work

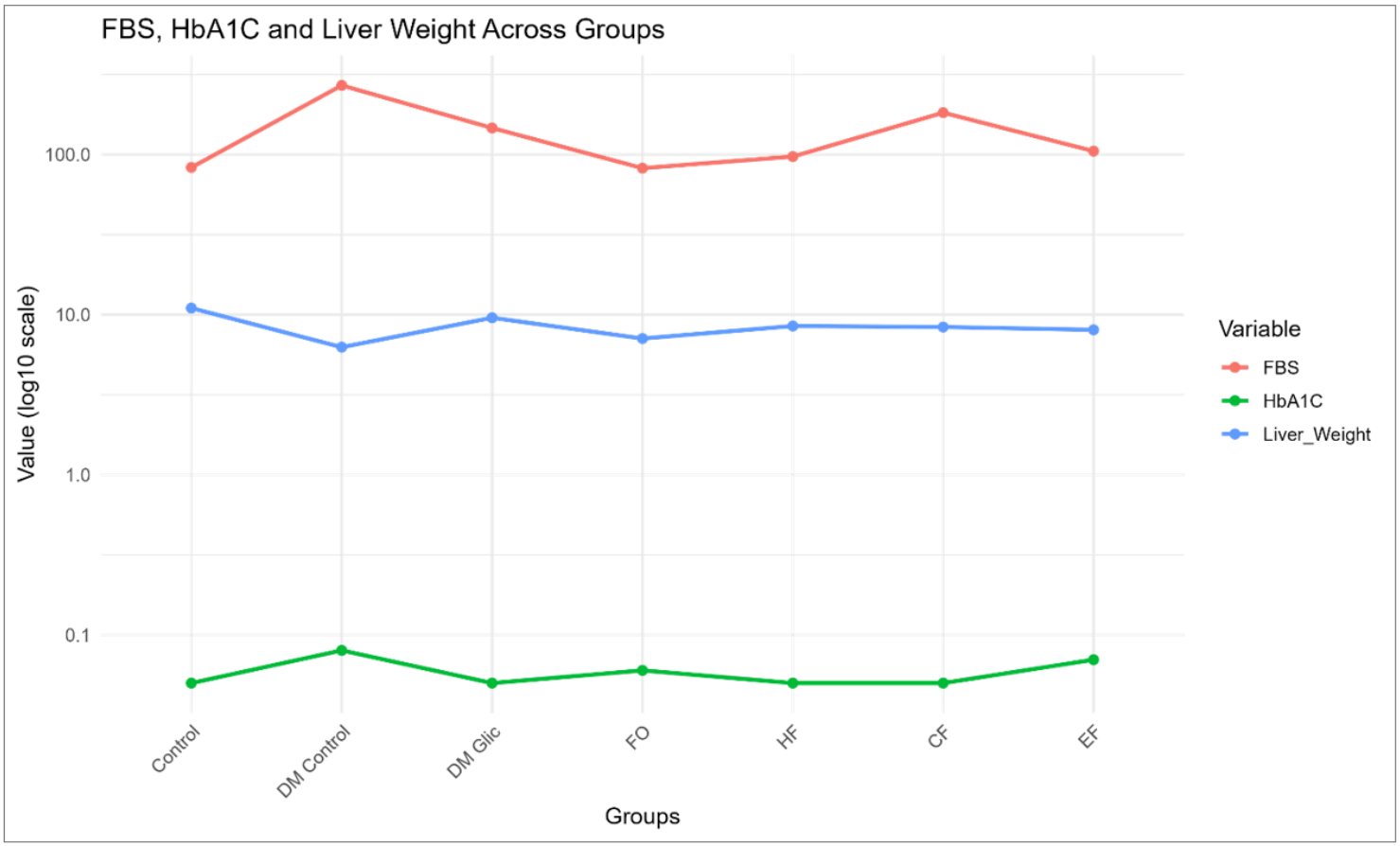


Figure 2

Depicts the liver weight (blue), HbA1c (green) and FBS (red) results. The data are expressed as the mean ± S.E.M (the values are presented in log-scale). Statistical analysis was carried out using the One-way ANOVA followed by LSD multiple comparison post-hoc test (significant difference p ≤ 0.05).

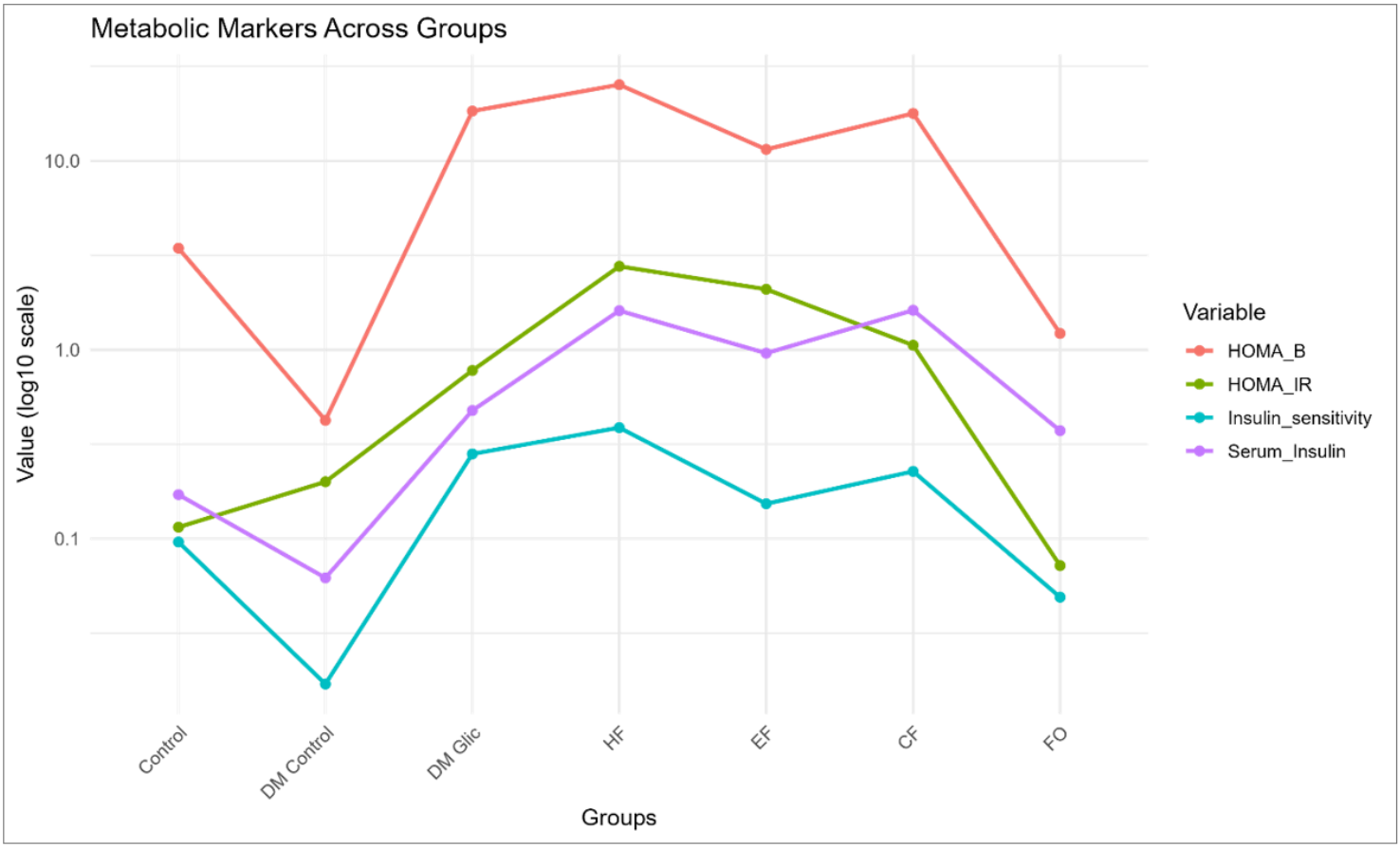


Figure 3

Show the results of serum insulin (purple), IS (blue), HOMA-β (red) and HOMA-IR (green). Statistical analysis was carried out using the One-way ANOVA followed by LSD multiple comparison post-hoc test. The significance difference between DM Control, DM Glic and treatment groups (i.e. FO, HF, CF and CF) when compared with Control and DM Control were observed ($p < 0.05$).

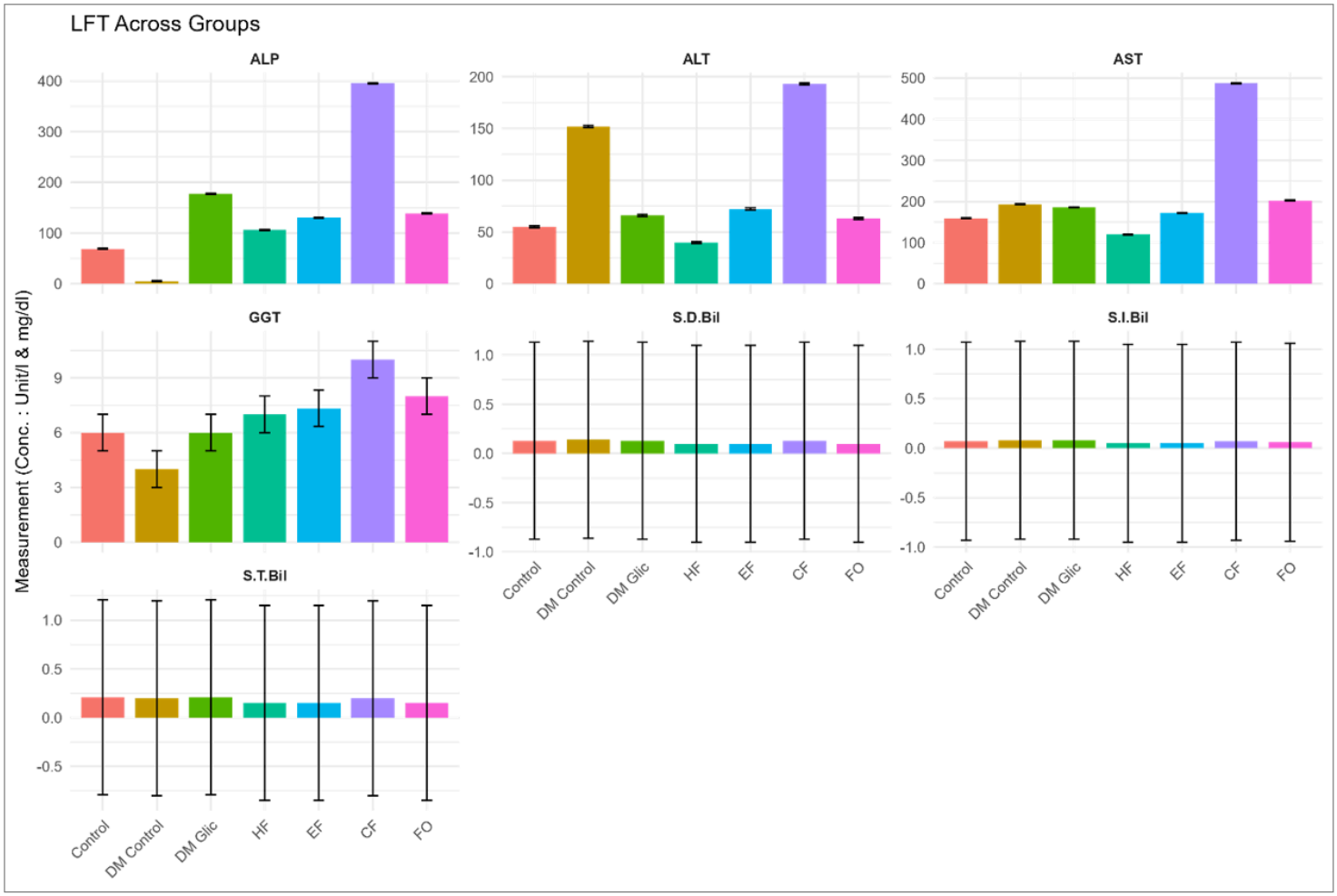


Figure 4

Illustrates the liver function parameters i.e. the serum total, direct and indirect bilirubin along with the enzymes ALT, AST, ALP and GGT. The data are expressed as the mean ± S.E.M. Significant difference (p 0.05) in liver enzymes were observed.

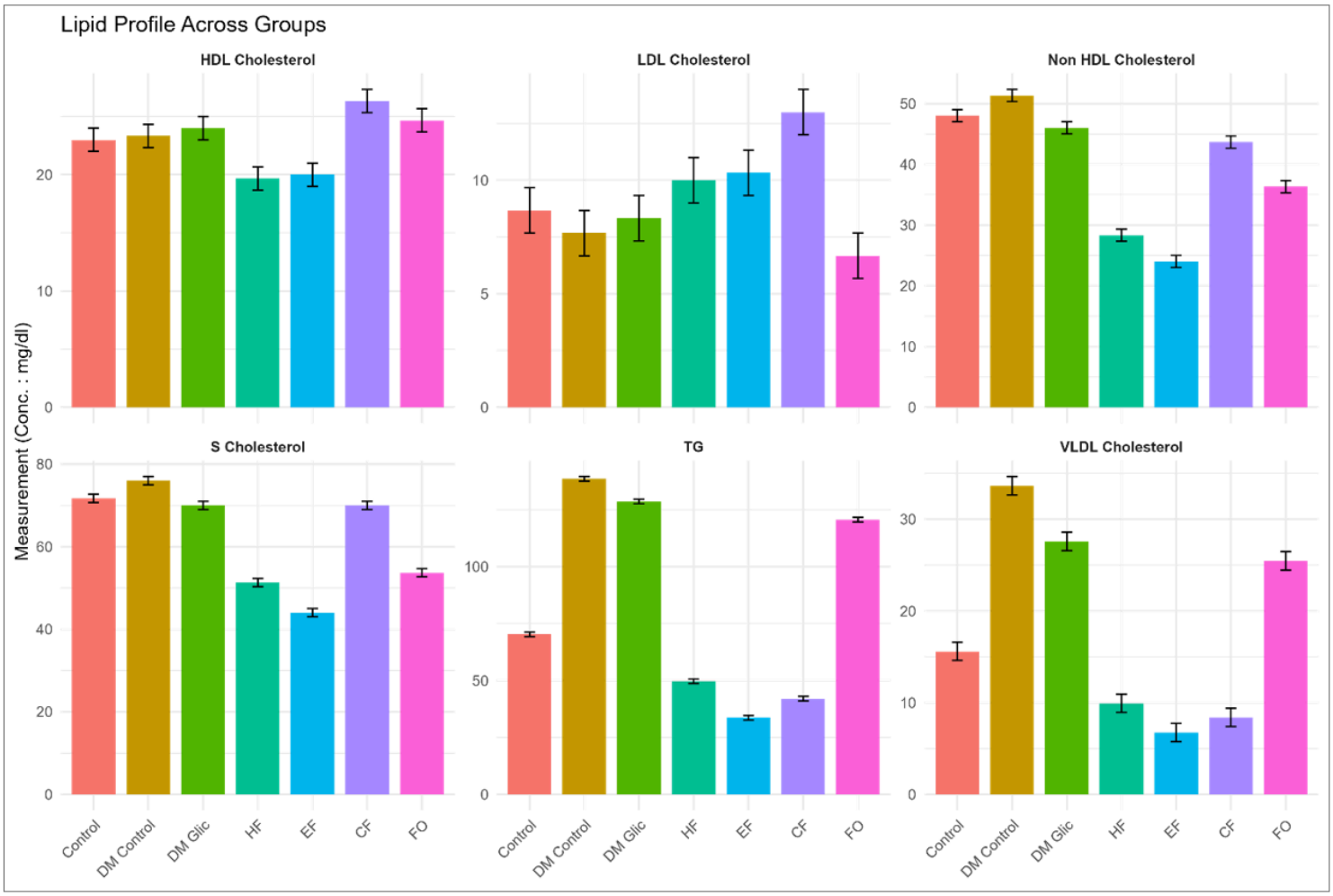


Figure 5

Presents lipid Profile of the Control, DM Control, DM Glic and CA seed's FO and its HF, CF and EF soluble fraction treated rats. The data are expressed as the mean ± S.E.M. The significant difference (p < 0.05) was observed in Serum cholesterol between Control and FO, HF & EF group as well as between DM Control and FO, HF & EF groups. Difference in VLDL and TG was seen between DM Control and Control, HF, CF and EF treatment groups; as well. LDL-cholesterol decreased in FO group only. No significant differences were observed in HDL-cholesterol. Similarly non-HDL cholesterol were decreased between Control & DM Control group and FO, HF and EF treatment groups significantly (p < 0.05).

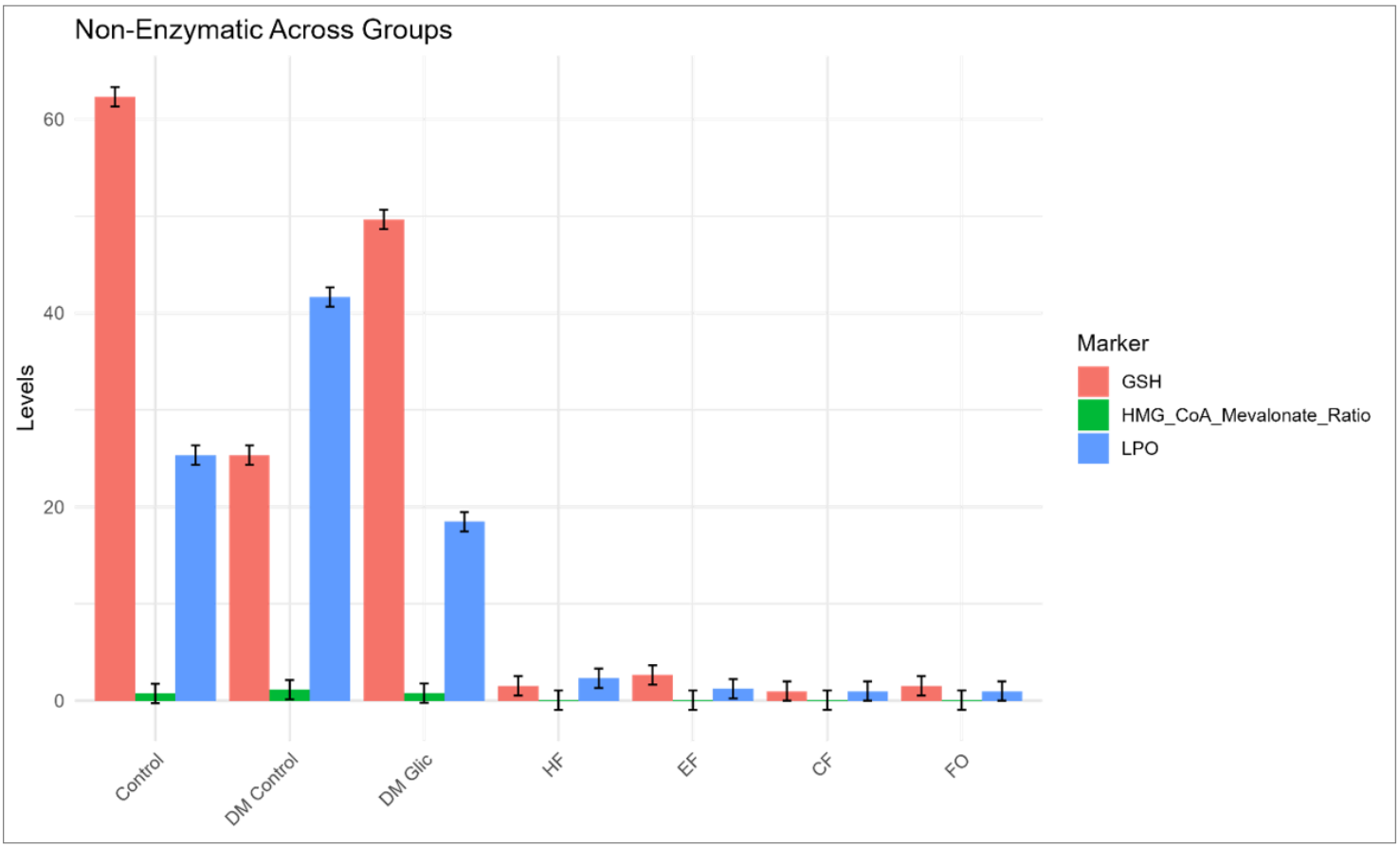


Figure 6

Figures show non-enzymatic markers: GSH (Red), HMG-CoA/Mevalonate Ratio (Green) and LPO (Blue); respectively in the liver homogenate. ANOVA and LSD multiple comparison post-hoc test was employed to analyze the data generated in all the study groups. $p \leq 0.05$ is statistically considered significant while comparing the difference between control group and all the other study groups.

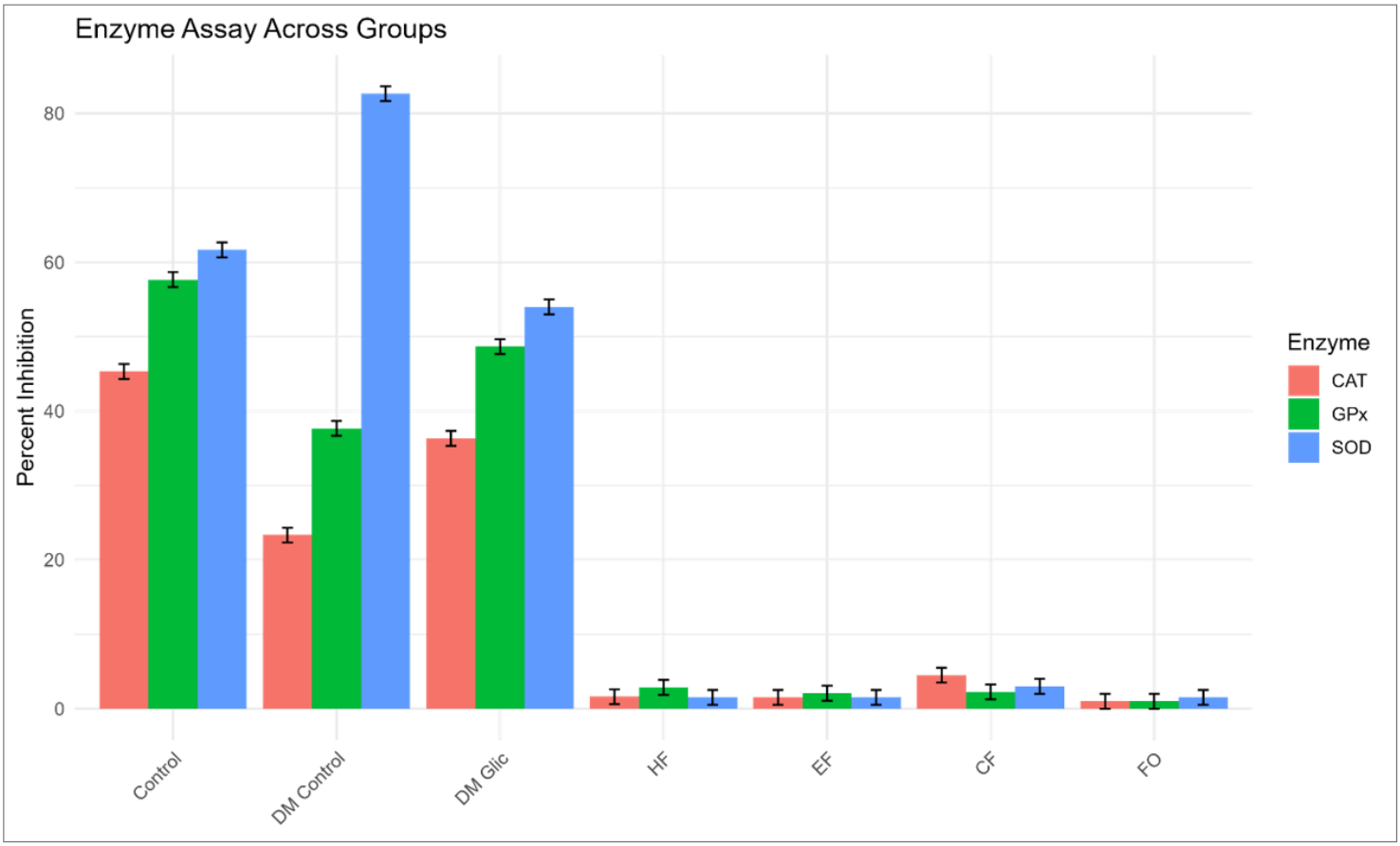


Figure 7

The figure illustrates the enzymatic antioxidant markers: CAT (Red), SOD (Blue) and GPX (Green). The activity of these enzymatic markers were measured in liver homogenate of all the study groups using *in-vitro* assays. The data obtained from the assays are shown as percent inhibition and was analyzed using ANOVA followed by LSD multiple comparison post-hoc test. Statistically significant difference (i.e. p 0.05) was observed between Control versus all the other studied groups.

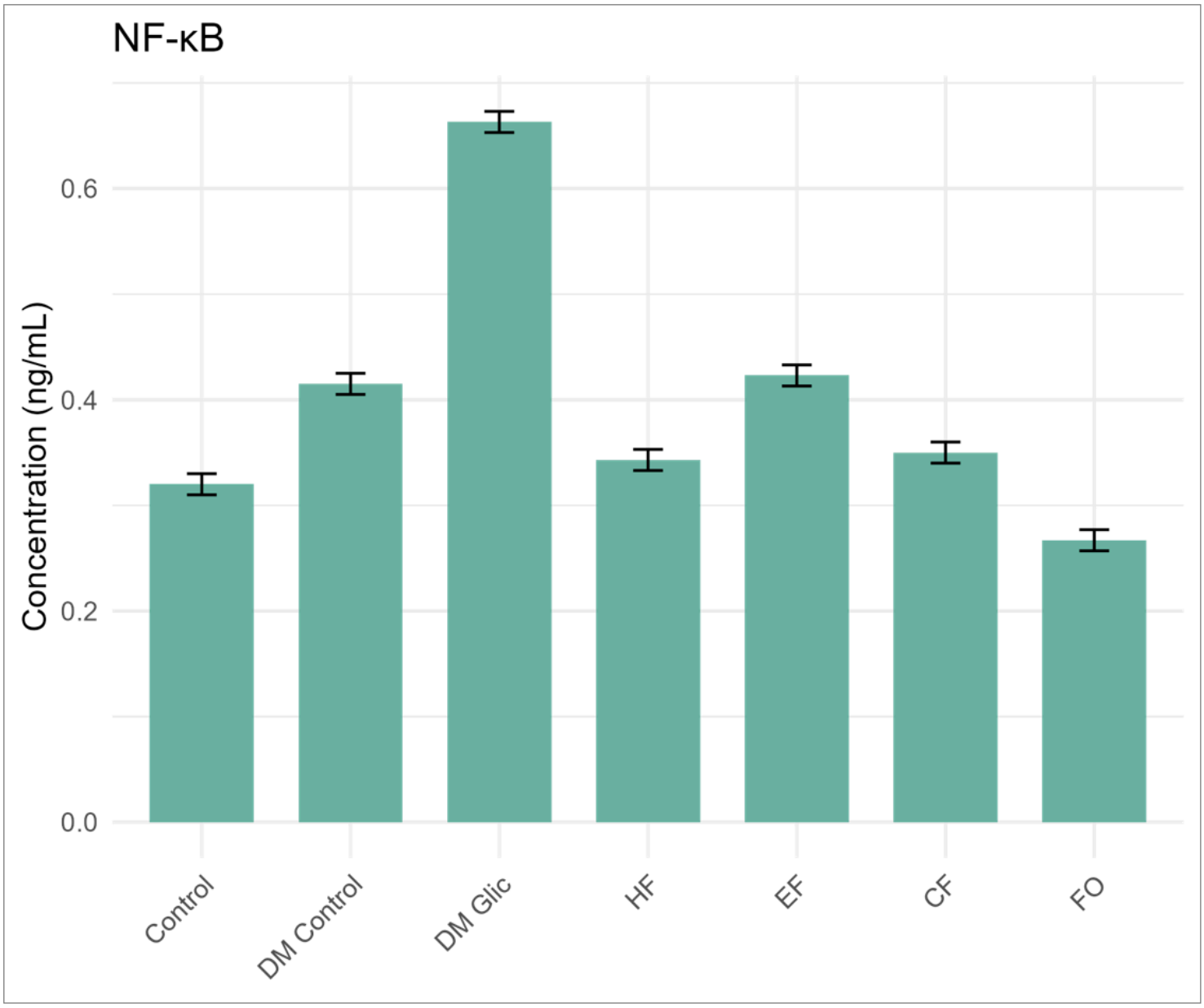


Figure 8

Presents the pro-inflammatory transcription factor: (NF-κB). It was measured in liver homogenate by ELISA. The levels in the all the study groups were analyzed using one-way ANOVA and LSD multiple comparison post hoc test and presented as mean ± S.E.M. The significant difference (p 0.05) between control and DM Control and all the treatment groups are were observed.



Figure 9

Bar graph and Heatmap presents the pro-inflammatory markers measured in liver homogenate. In the bar graph IL-1β, TNF-α & COX-1 are presented as blue, dark red and yellow graphs; respectively. There expression (i.e. mRNA) were measured by qRT-PCR. The data from all the study groups were expressed as the mean ± S.E.M and analyzed using one-way ANOVA and LSD multiple comparison post hoc test. p 0.05 is considered as the significant difference. The columns in the Heatmap presents the experimental conditions (i.e. experimental groups), the rows represents the pro-inflammatory genes and level of gene expression is depicted by the color (the scale on the right side of the Heatmap). Varying intensity of red parallels the level of expression; red being the highest, pink and light pink being moderate to low levels of expression; respectively.

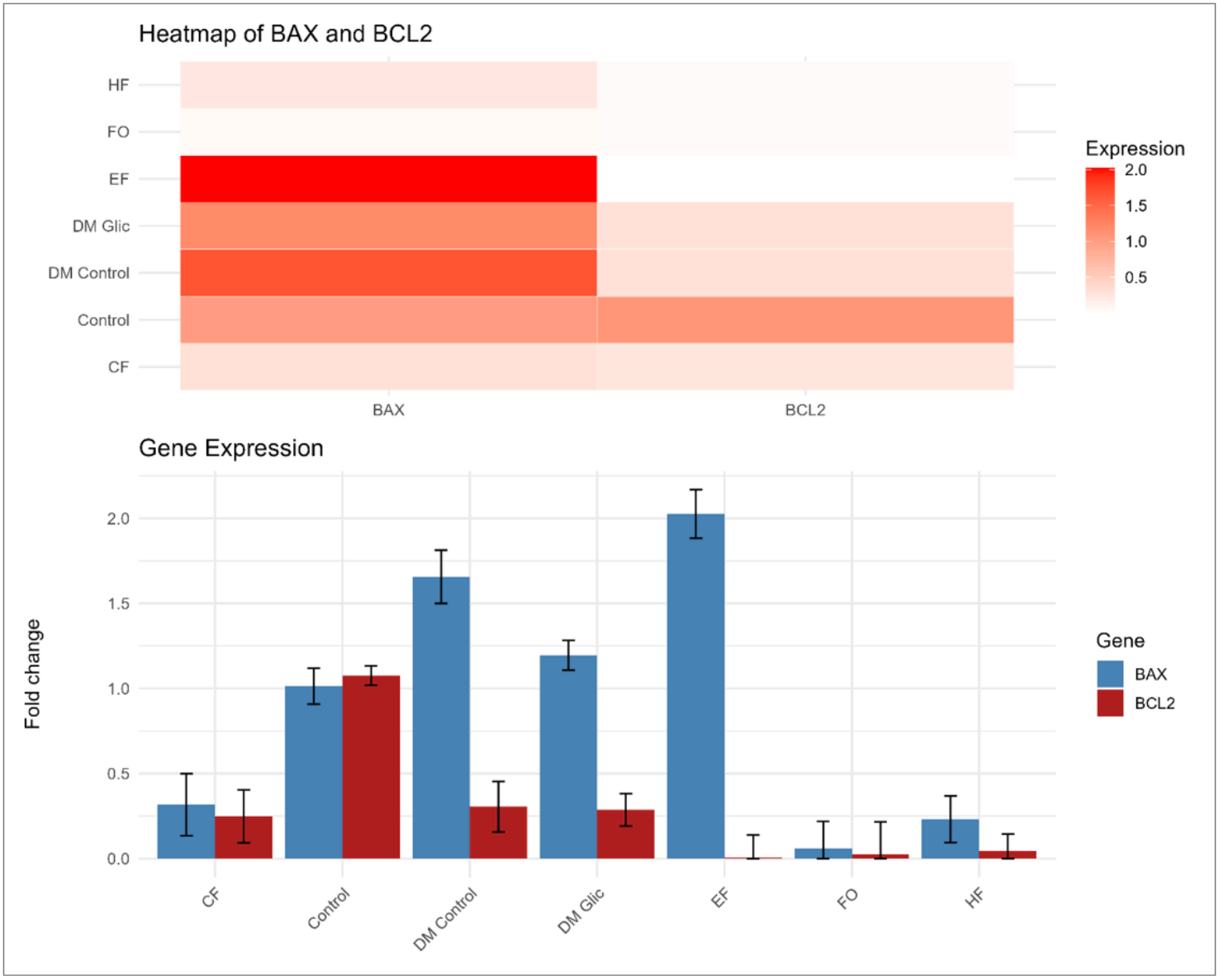


Figure 10

Bar graph and Heatmap presents the anti-apoptotic markers measured in liver homogenate. In the bar graph Bcl2 and Bax are presented as dark red and blue graphs; respectively. There expression (i.e. mRNA) were measured by qRT-PCR. The data from all the study groups were expressed as the mean ± S.E.M and analyzed using one-way ANOVA and LSD multiple comparison post hoc test. *p*□ 0.05 is considered as the significant difference. The columns in the Heatmap presents the experimental conditions (i.e. experimental groups), the rows represents the pro-inflammatory genes and level of gene expression is depicted by the color (the scale on the right side of the Heatmap). Varying intensity of red parallels the level of expression; red being the highest, pink and light pink being moderate to low levels of expression; respectively.

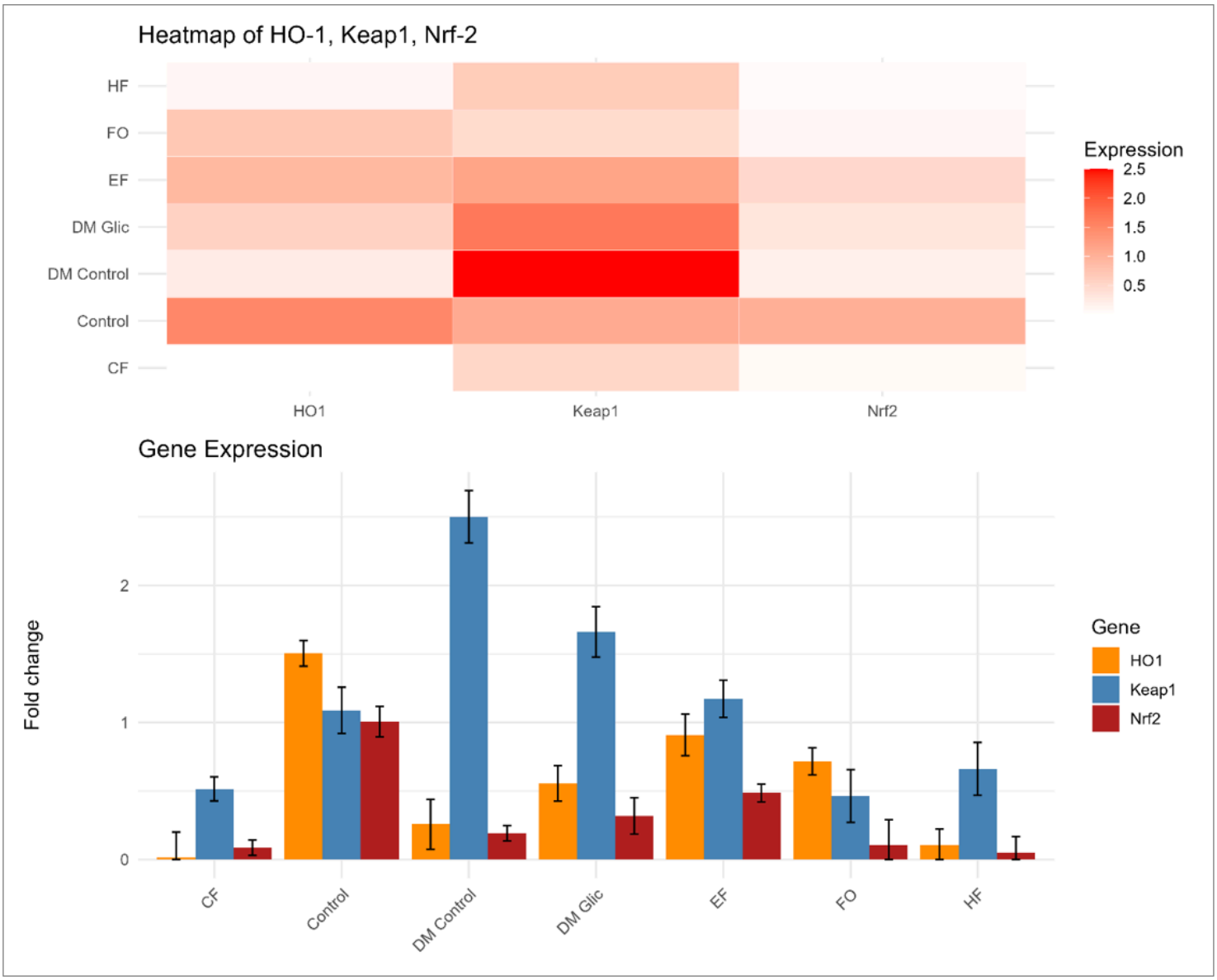


Figure 11

Bar graph and Heatmap presents the antioxidant markers measured in liver homogenate. In the bar graph Nrf2, Keap1 & HO-1 are presented as dark red, blue and yellow graphs; respectively. There expression (i.e. mRNA) were measured by qRT-PCR. The data from all the study groups were expressed as the mean ± S.E.M and analyzed using one-way ANNOVA and LSD multiple comparison post hoc test. *p*□ 0.05 is considered as the significant difference. The columns in the Heatmap presents the experimental conditions (i.e. experimental groups), the rows represents the pro-inflammatory genes and level of gene expression is depicted by the color (the scale on the right side of the Heatmap). Varying intensity of red parallels the level of expression; red being the highest, pink and light pink being moderate to low levels of expression; respectively.

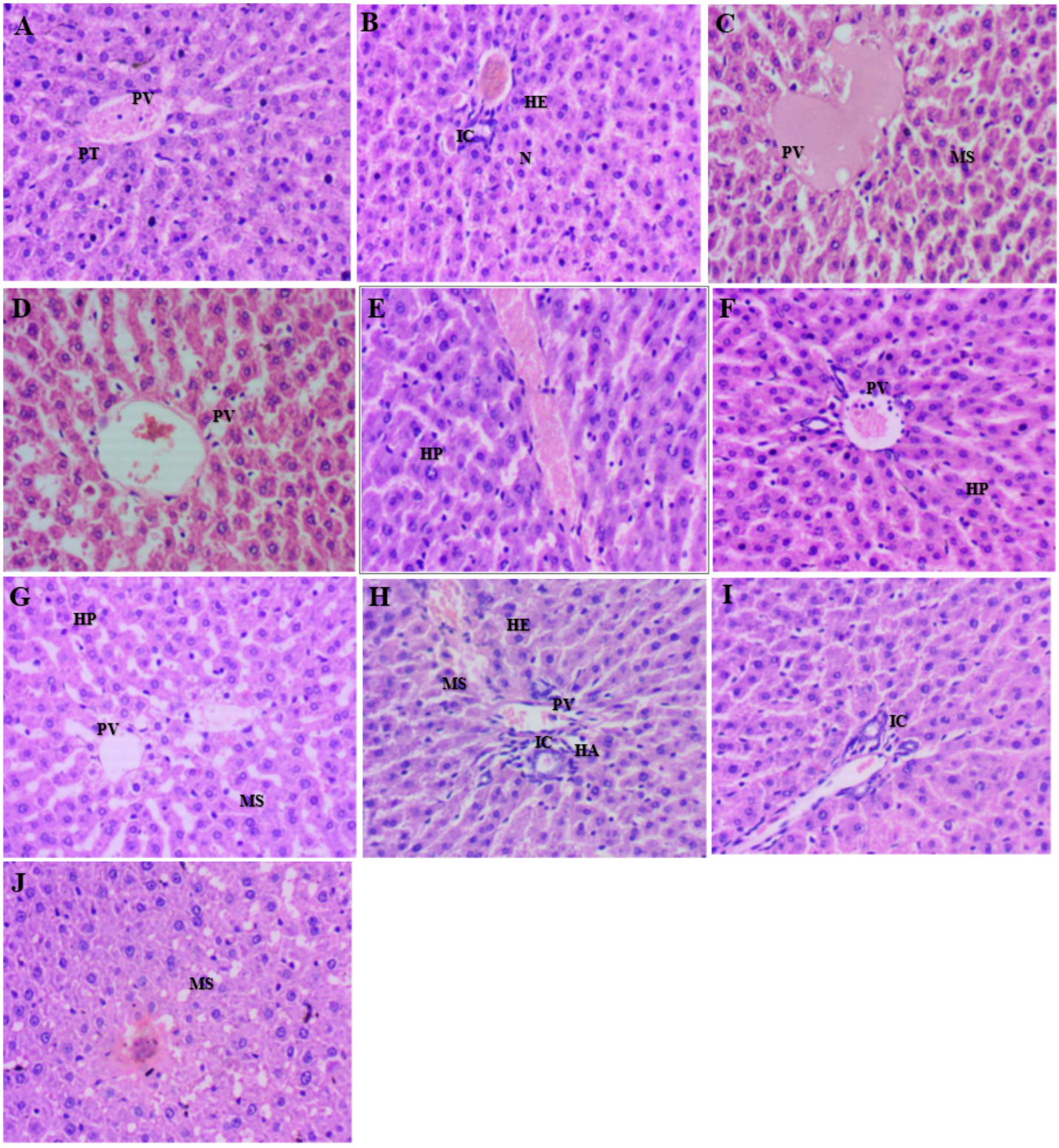


Figure 12

Depicts the photomicrographs of histopathological changes in hepatic tissue of diabetic (HF-HFr-STZ) rats treated with CA's FO and its HF, CF and EF soluble fractions. (A) Shows a photomicrograph of the tissue from the Control (C) liver depicting liver architecture with no perturbations, (B) and (C) depicts congestion of blood with signs of inflammation in the tissue of DM-C group, and attenuation of inflammation in treatment control (DM Glic); respectively. D, E, F, G and H are the photomicrograph of

CA's FO and HF, CF, and EF soluble-treated liver; respectively. Inflammatory and degenerative changes seen in DM-C have attenuated when treated with CA seed oil and its fractions. Thus trying to restore near normal liver cell architecture.