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RESEARCH ARTICLE

Peroxisome Proliferator-Activated Receptor Gamma Agonists Modulate High-Fat Diet- and Carbon Tetrachloride-Induced Non-Alcoholic Fatty Liver Disease Pathophysiology and Transcriptional Expression of Inflammatory Markers in a Murine Model

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A B S T R A C T

The objectives of the present study were to investigate the effect of induced activation of peroxisome proliferator-activated receptor gamma (PPARγ) through ligands, rosiglitazone (RG), kaempferol (KEM) and GW9662 (GW) in pathophysiological alterations and inflammation by regulating the expression of PPARγ-regulated genes in a rat model of nonalcoholic fatty liver disease (NAFLD). Male wistar rats (N=45) were fed high fat diet (HFD; 35%) in combination with single dose of carbon tetrachloride (CCl_4; 0.5ml/kg/intraperitoneally) to induce NAFLD. The effects of synthetic PPARγ agonist; RG (15mg/kg) and PPARγ antagonist; GW (10mg/kg) were evaluated in comparison with putative natural ligand; KEM (12mg/kg). Co-administration of HFD and CCl_4 mimicked NAFLD as evident by elevation of hepatic injury markers and lipid profile in serum. The results of AST/ALT ratio and AST to platelet ratio index indicated NAFLD without advanced liver disease. RG and KEM, in contrast to GW, countered NAFLD-associated effects by ameliorating hepatic injury markers, insulin resistance and lipid profile. KEM and RG treatment increased the expression of IL-33, an anti-inflammatory cytokine, while the expression of TNFα, a pro-inflammatory cytokine was non-significantly decreased. The results suggested that PPARγ activation by agonist ligands might contribute towards amelioration of NAFLD-associated pathophysiology of metabolic syndrome and hepatic inflammation through regulation of IL-33 and TNFα.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is metabolic syndrome that is associated with hepatic steatosis and progresses into nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Ishtiaq *et al.*, 2019; Rashid *et al.*, 2020). The prevalence of NAFLD is steadily increasing parallel to the increasing prevalence of obesity. In recent years, the global prevalence of NAFLD is estimated to be 25.24% and up to 1/3 of NAFLD patients have NASH, which is a hallmark of chronic liver disease (Tsuchida *et al.*, 2018). The pathogenesis of NAFLD is based on a theory of "two hit hypothesis". Accordingly, fat accumulation (steatosis) indicates "first hit", followed by oxidative stress and inflammation, induced by fat deposition that represent the "second hit" (Ishtiaq *et al.*, 2019). In this context, transcriptional expression of immunoregulatory cytokines, particularly interleukin-(IL)-33 (Zhang *et al.*, 2017) and tumor necrosis factor alpha (TNFα) (Connolly *et al.*, 2009) might be critical in modulating the NAFLD pathophysiology. The impaired regulatory functions of IL-33 and TNFα in inflammatory pathways as well as IL-33-mediated expressions of genes of cholesterol transport (Vasanthakumar *et al.*, 2015; Zhang *et al.*, 2017) impart key role in liver immunology and metabolic homeostasis in obesity-associated hepatic inflammation. Thus, the intrahepatic etiologies in pathogenesis of NAFLD are major focal points of anti-NAFLD treatment strategies and might have impact for preventing an emerging menace of health care system in the near future.

Over the past few years, peroxisome proliferator-activated receptor gamma (PPARγ) (an inducible

transcription factor of nuclear receptor superfamily) and PPARγ agonists including rosiglitazone (RG) are approved by FDA for the treatment of insulin resistance and type 2 diabetes (Della-Morte *et al.*, 2014). The PPARγ is recognized as a sensor of intracellular *milieu* that induces genomic effects in accordance to need of cellular and body environment. The PPARγ-based genomic effects include both gene activation and repression depending on the molecular context. Beside high expression in adipose tissue, ligand-induced expression of PPARγ in multiple cells and tissues, notably in the liver, can influence the cellular activities, including metabolic processes (glucose homeostasis and fat metabolism), lipid accumulation and inflammation (Feng *et al.*, 2017; Hussain *et al.*, 2021). Experimental studies (Feng *et al.*, 2017; Bi *et al.*, 2018) demonstrate mechanism of action of PPARγ agonists, particularly rosiglitazone (a potent PPARγ thiazolidinedione agonist), in amelioration of hepatic injury markers, altered lipid profile, glucose dysregulation, and inflammation in different disease models, thereby, underscoring the potential role of PPARγ as therapeutic target for NAFLD.

The modulation of PPARγ-related activities can also be induced by majority of identified plant-based phytoconstituents that selectively transactivate several PPARγ-dependent genes as partial agonists (Wu *et al.*, 2020). The difference between natural products-based phytoconstituents and full agonists (rosiglitazone) lies in binding mode to PPARγ (Wu *et al.*, 2020). One of the herbal interventions of kaempferol (KEM) has been reported for its hypocholesterolemic effects for many hepatic diseases (Xiang *et al.*, 2021). The molecular interaction of KEM with PPARγ has been revealed by molecular dynamic simulation studies that pave a new way for investigation of natural activators of PPARγ (Lokhande *et al.*, 2020a). To date, assessment of preventive/therapeutic potential of PPARγ signaling in diet- and chemical-associated NAFLD model remains elusive. Therefore, the aim of study was to investigate hepatoprotective effects of PPARγ ligands (agonist, antagonist) in high fat diet (HFD)- and carbon tetrachloride (CCl_4)-induced NAFLD in murine model. Additionally, KEM-prompted effects in NAFLD were also explored and compared to PPARγ ligands (agonist and/or antagonist). We hypothesized that ligand-induced activation of PPARγ might ameliorate NAFLD-associated pathophysiological changes, involving altered energy homeostasis, lipid accumulation, insulin resistance, and inflammation by controlling influential checkpoints such as PPARγ-induced expressions of TNFα and IL-33.

MATERIALS AND METHODS

Approval of Ethical Committee and experimental model: The study was approved from Institutional Bioethics Committee (IBC) of University of Agriculture, Faisalabad, Pakistan (1749/ORIC). Experiment was conducted on healthy wistar male albino rats (n=5), weighing 100-150g, and maintained in the animal housing facility at a temperature 25±5°C. All animals were feed *ad libitum* and were observed daily.

Experimental design: After seven days of acclimatization, Forty five male wistar albino rats were divided randomly into nine groups (n = 5). A rat model of NAFLD was established by combination of HFD and CCl_4 as mentioned previously (Kubota *et al.*, 2013; Tsuchida *et al.*, 2018) with slight modifications. In brief, HFD (35%, mixed with normal daily feed *ad libitum*) for four weeks and CCl_4 (0.5ml/kg in olive oil (1:15, v/v), i.p) at once, were administered in combination for induction of NAFLD in all the groups except, Veh, CCl_4, and HFD alone groups. CCl_4 + HFD group was labeled as positive control and compared to other treatment groups. SLM group was administered silymarin (200mg/kg orally); KEM group was administered kaempferol (12mg/kg i.p. in DMSO/0.9% saline (1:6); RG group was treated with PPARγ agonist rosiglitazone (15mg/kg i.p. in DMSO/0.9% saline (1:6); GW group was treated with PPARγ antagonist GW9662 (10mg/kg i.p. in DMSO/0.9% saline (1:6); RG + GW group was treated with both ligands of PPARγ receptor (rosiglitazone and GW9662). All the treatments (RG, SLM, KEM and GW) were given 24h and 1h prior to administration of CCl_4.

Estimation of liver injury biomarkers: Liver injury markers including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), were analyzed in serum by colorimetry method using spectrophotometer (Thermo Scientific Multiskan GO™ with Skanlt software 4.1). AST/ALT ratio, and AST-to-platelets ratio index (APRI) were also calculated.

Estimation of lipid profile: Total triglycerides (TG), total cholesterol (TC) and high-density lipoprotein level (HDL) (Human Diagnostics Worldwide) were determined using colorimetry as above. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were calculated using formulae described by Friedewald *et al*. (Friedewald *et al.*, 1972).

Histological analysis: Preserved liver tissue samples in 10% neutral buffered formalin were processed for histological examination according to previously described method (Murtaza *et al.*, 2021).

Glucose tolerance test and insulin resistance: The blood glucose level was determined by method described by Marques *et al*. (Marques *et al.*, 2016). In brief, animals were administered orally glucose solution (2g/kg body weight) and blood droplets from tail vein were collected at various intervals (0, 15, 30, 60, 90, 120 min). Glucose level was measured with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK). Serum insulin level was checked by using commercially available kit: Rat INS (Insulin) ELISA Kit, ElabScience®, catalogue no: E-EL-R3034. The homeostasis model assessment for insulin resistance (HOMA-IR) values were calculated as follows: HOMA-IR=fasting serum glucose (mmol/L)×fasting serum insulin (μU/mL)/22.5.

Gene expression analysis by quantitative reverse transcription PCR (qRT-PCR): Quantitative RT-PCR

analysis was performed for determining the expression of genes using primers (Table 1). Total RNA from liver tissue was extracted by Trizole method, followed by reverse transcription using kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). Real time PCR was carried out in duplicates by using intercalating dye Maxima SYBR Green/ROX-based PCR master-mix (Thermo scientific), following the manufacturer's instructions. The product specificity was checked by melting curve analysis after each amplification. ΔCq values (where Cq is quantification cycle) were calculated using *gapdh* as a reference gene to determine relative mRNA expression levels.

Statistical Analysis: Results are represented as Mean ± S.D and statistical significance was assessed by one-way analysis of variance (ANOVA), two-way ANOVA, and Kruskal-Wallis H test, where appropriate. Statistical significance for multiple comparison was analyzed by Tukey's post hoc test. Whereas, significant effects between two groups was determined by unpaired student's t-test. P-value less than 0.05 was considered significant. All data were analyzed by using GraphPad Prism® version 6.01.

RESULTS

RG and KEM attenuated NAFLD-associated hepatic damage: The results of liver weight, liver to body weight ratio and histopathological analysis suggested induction of NAFLD following HFD and CCl_4 combined administration. In particular, the results revealed that CCl_4 alone, at the administered dose, did not affect liver weight but, a mild to moderate degree of congestion was seen in the liver of CCl_4-administered rats. A pronounced elevation in liver weight was observed by HFD alone and HFD + CCl_4 groups that was considerably declined in SLM, KEM and RG treatment groups. Moreover, KEM showed a more non-significant decrease in liver weight than SLM when compared to RG (Fig. 1A & 1B). Histolopathological examination showed distinct fatty changes in HFD alone group (Fig. 1C) that were more severe in form of hepatocellular ballooning, vascular congestion, diminished sinusoidal spaces and coalescence of vacuoles (fat vacuoles/globules) in HFD + CCl_4 group (Fig. 1C). However, SLM, KEM and RG treatments indicated relatively mild degree of vascular congestion, improved sinusoidal spaces and low vacuolation (Fig. 1C).

Ameliorative effects of RG and KEM on NAFLD-associated elevation of hepatic injury biomarkers: Rats treated with HFD and CCl_4 alone did not produce any significant change in liver injury markers ($P>0.05$ *vs* Veh) while the combination of HFD and CCl_4 presented a marked increase in ALT ($P<0.01$), AST ($P<0.01$), LDH ($P<0.05$), and ALP ($P<0.05$) levels *vs* Veh (Fig. 2A-2D, respectively). Elevated levels of NAFLD-associated hepatic injury markers were significantly lowered by RG, KEM and SLM except GW alone or RG+GW. AST/ALT ratio was also calculated that showed a non-significance ($P>0.05$) difference among groups (Fig. 2E), while, overall values of all groups remained less than one that is an indication of NAFLD without advanced liver disease. Significantly high values of APRI in HFD+CCl_4 treatment groups indicated succesful induction of NAFLD. While there was non-significant decrease in APRI values in SLM and KEM groups. A significant decrease in APRI values was observed in PPAR agonist RG treated group (Fig. 2F).

RG and KEM alleviate NAFLD-associated increased lipid profile: Dysregulated levels of TG, TC, LDL, VLDL and HDL were seen in HFD+CCl_4 group *vs* Veh group (Fig. 4A-4E). As expected, CCl_4 alone group did not show increase in lipid profile markers. HFD alone and HFD+CCl_4 groups exhibited high levels of TG, TC, VLDL and LDL (Fig. 3A, 3B, 3D, 3E) whereas HDL levels in treated groups remained relatively lower as compared to Veh group (Fig. 3C). Our results showed an improved lipid profile by treatment groups SLM, KEM and RG by mitigating HFD+CCl_4-dependent elevation of TG, TC, LDL and VLDL and improving HDL levels. GW did not show any effect on HFD+CCl_4-associated dysregulated lipid profile, except HDL levels which were decreased in GW treated group.

Table 1: Primers for specific gene expression by real time polymerase chain reactions (RT-qPCR)

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
Rat-PPARγ	GCGAGGGCGATCTTGACA	ATGCGGATGGCCACCTCTTT
Rat-IL-33	GGTGCTACTCCGTTACTACGA	GCGGAGAGACATCACCCTTT
Rat-TNF-α	CCTCTGCGGGACACTCATTT	CCACCTCCTTTGAAGCCACT
Rat-GAPDH	ATGACTCTCCCACGGCAAG	TACTCAGCACCAGCATCACC

Table 2: Hypoglycemic effect to oral glucose tolerance test (OGTT) analysis of peroxisome proliferator-activated receptor gamma (PPARγ) agonist ligands in high-fat diet (HFD)- and carbon tetrachloride (CCl_4)-induced non-alcoholic fatty liver disease (NAFLD) model

Time intervals / Groups	0 min	15 min	30 min	60 min	90 min	120 min	Overall summary of *p-value*
Veh	99±4.0	135±10.5	138±9.0	139±4.0	120±2.0	100±8.0	*p>0.05*
CCl_4	102.5±1.5	157.67±14.2	162.67±16.9	135±17.8	118.5±3.5	111±2.6	*p>0.05*
HFD	107±2.0	178±34.6	163±7.0	148.3±20.7	126.3±17.6	135.67±14.0	*
HFD+ CCl_4	109±9.5	185±12.7	188.67±31.1	139±15.2	111.25±21.9	138.25±24.1	****
SLM+ HFD+ CCl_4	101±7.9	144.75±15.9	150.33±8.5	142.75±20.5	122.33±9.1	105±10.6	XXX
KEM+ HFD+ CCl_4	97.3±6.4	160.25±20.6	175±29.2	161±24.8	116.75±16.5	104±16.5	X
RG+ HFD+ CCl_4	98±12.1	149±23.3	148±6.2	157±11.7	139±5.6	112.25±11.8	X
GW+ HFD+ CCl_4	101.75±11.3	166.75±20.0	172.5±21.1	154.25±17.8	147.5±28.8	121±7.0	*p>0.05*
RG+GW+ HFD+ CCl_4	86±6.6	165±10.1	169±11.5	151.3±22.5	131±28.8	125.3±34.6	*p>0.05*

All data were expressed as Mean±S.D., *n = 5*; *: statistical difference of $P<0.05$; · ****; statistical difference of $P<0.0001$ when compared to Veh group; X: statistical difference of $P<0.05$, XX: statistical difference of $P<0.01$, XXX: statistical difference of $P<0.001$, when compared to CCl_4 + HFD group.

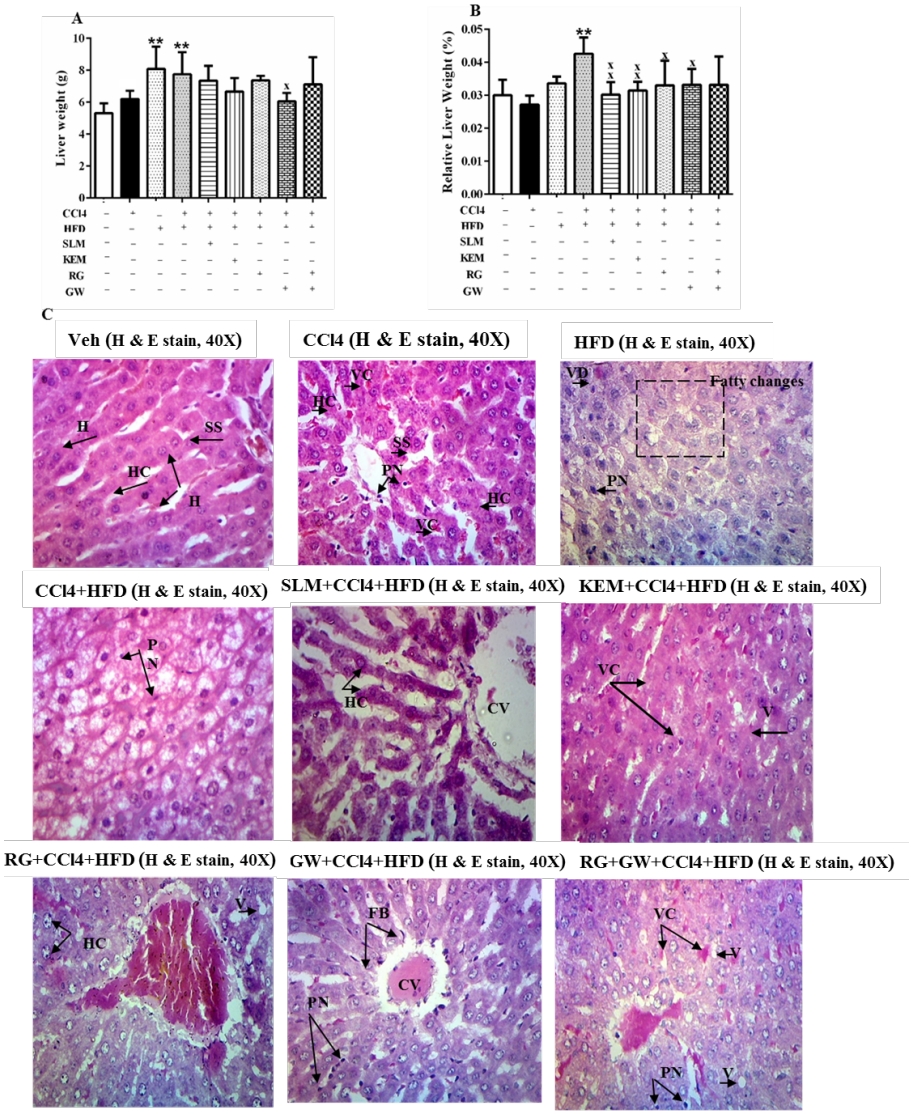

Fig 1: Rosiglitazone (RG) and kaempferol (KEM) attenuated non-alcoholic fatty liver disease (NAFLD)-associated hepatic damage. **(A)** Liver weight (g); **(B)** Relative liver weight (%); **(C)** Photomicrographs of liver tissue of experimental groups (labelled). All data were expressed as Mean±S.D., *n = 5*; *: statistical difference of P<0.05, **: statistical difference of P<0.01 ***: statistical difference of P<0.001;****; statistical difference of P<0.0001 when compared to Veh group; X: statistical difference of P<0.05, XX: statistical difference of P<0.01, XXX: statistical difference of P<0.001, XXXX: statistical difference of P<0.0001 when compared to CCl_4 + HFD group. CCl_4, carbon tetrachloride; HFD, high fat diet; SLM, silymarin; KEM, kaempferol; RG, rosiglitazone; GW, GW9662. CV, central vein; FB, fibroblasts; H, hepatocytes; HC, hepatic cords; PN, pyknotic nuclei; SS, sinusoidal spaces, VD, vascular degeneration; V, vacuoles (H & E stain, 40X).

RG and KEM prevented glucose intolerance and insulin resistance in HFD- and CCl_4-induced NAFLD model: Glucose intolerance was evaluated by oral glucose tolerance test (OGTT). OGTT results revealed a pronounced change in blood glucose levels with the time. In particular, an increased value of blood glucose in HFD alone (P<0.05) and HFD+CCl_4 (P<0.0001) groups was noted during time intervals (0, 15, 30, 60, 90, 120 mins) in comparison to Veh group. However, RG (P<0.05), KEM (P<0.05) and SLM (P<0.001) treatments significantly improved elevation of glucose levels in blood. GW and RG+GW groups did not show any hypoglycemic response when compared to elevated blood glucose levels of HFD+CCl_4 group (Table 2). Insulin resistance was determined by homeostatic assessment model for insulin resistance (HOMA-IR). The results show high blood glucose and insulin levels in HFD- and CCl_4 treated group suggesting development of insulin resistance consistent with elevated HOMA-IR values (Fig. 4A-4C). SLM, KEM and RG treatment significantly lowered the HFD- and CCl_4-dependent rise in fasting glucose, insulin and HOMA-IR. GW and RG+GW treatment did not impart any significant difference on levels of insulin and fasting glucose (Fig. 4A-4C).

RG and KEM induced effects on transcriptional expression of PPARγ, IL-33 and TNFα in HFD- and CCl_4-associated NAFLD: The mRNA levels of PPARγ were increased in HFD alone group whereas no significant rise in PPARγ expression was observed in CCl_4 alone or in HFD+CCl_4 combination group (Fig. 5A). SLM and KEM groups showed significantly higher PPARγ mRNA levels while no significant change was observed in RG, GW alone or in combination groups. The expression of IL-33, a PPARγ regulated gene, was decreased in HFD+CCl_4 group (Fig. 5B). Interestingly, IL-33 mRNA levels were significantly increased in SLM, KEM and RG group. Moreover, KEM and RG treatments presented a comparable increased expression of IL-33. GW alone group did not show change in expression of IL-33. The results of TNFα mRNA levels showed significant rise in TNFα expression in HFD alone and HFD+CCl_4 group. CCl_4 alone did not affect mRNA expression of TNFα. RG, KEM and SLM treatment lowered the HFD+CCl_4-dependent rise in TNFα expression (Fig. 5C).

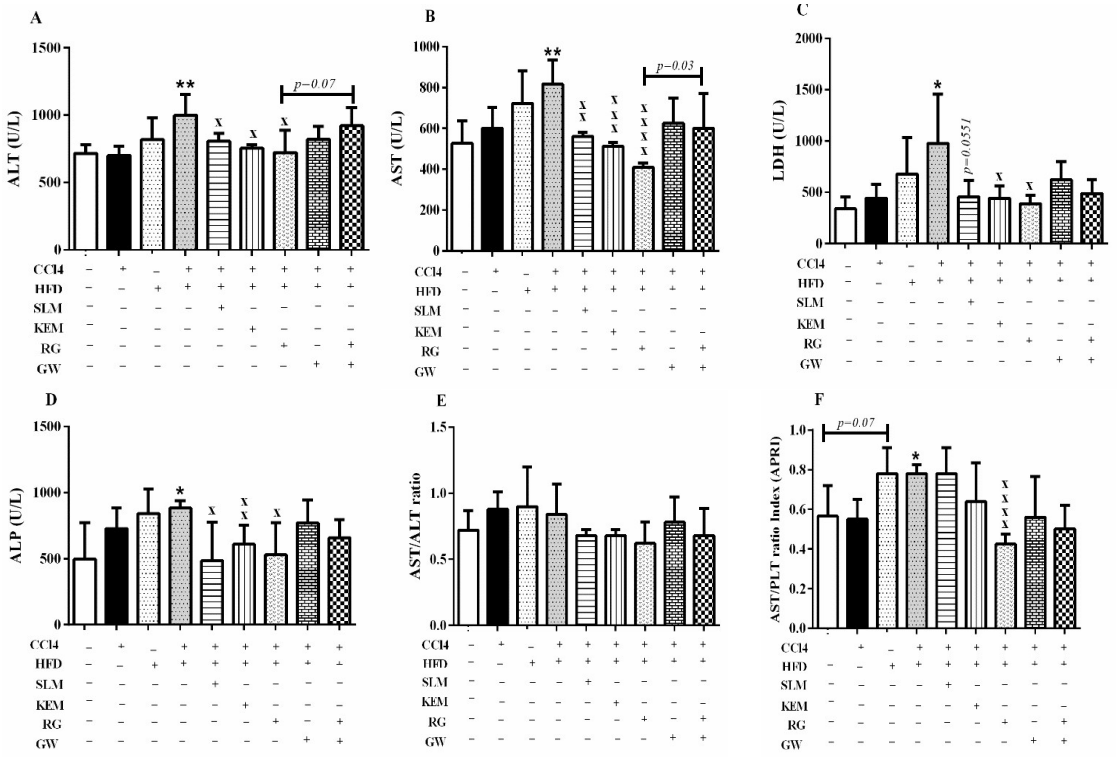

Fig 2: Ameliorative effects of rosiglitazone (RG) and kaempferol (KEM) on non-alcoholic fatty liver disease (NAFLD)-associated elevation of hepatic injury biomarkers. **(A)** Alanine aminotransferase levels, ALT; **(B)** aspartate aminotransferase, AST; **(C)** lactate dehydrogenase, LDH; **(D)** alkaline phosphatase, ALP; **(E)** AST/ALT ratio; **(F)** AST/PLT ratio index, APRI. All data were expressed as Mean±S.D., *n = 5*; *: statistical difference of P<0.05, **: statistical difference of P<0.01 ***: statistical difference of P<0.001; ****; statistical difference of P<0.0001 when compared to Veh group; X: statistical difference of P<0.05, XX: statistical difference of P<0.01, XXX: statistical difference of P<0.001, XXXX: statistical difference of P<0.0001 when compared to CCl_4 + HFD group. CCl_4, carbon tetrachloride; HFD, high fat diet; SLM, silymarin; KEM, kaempferol; RG, rosiglitazone; GW, GW9662.

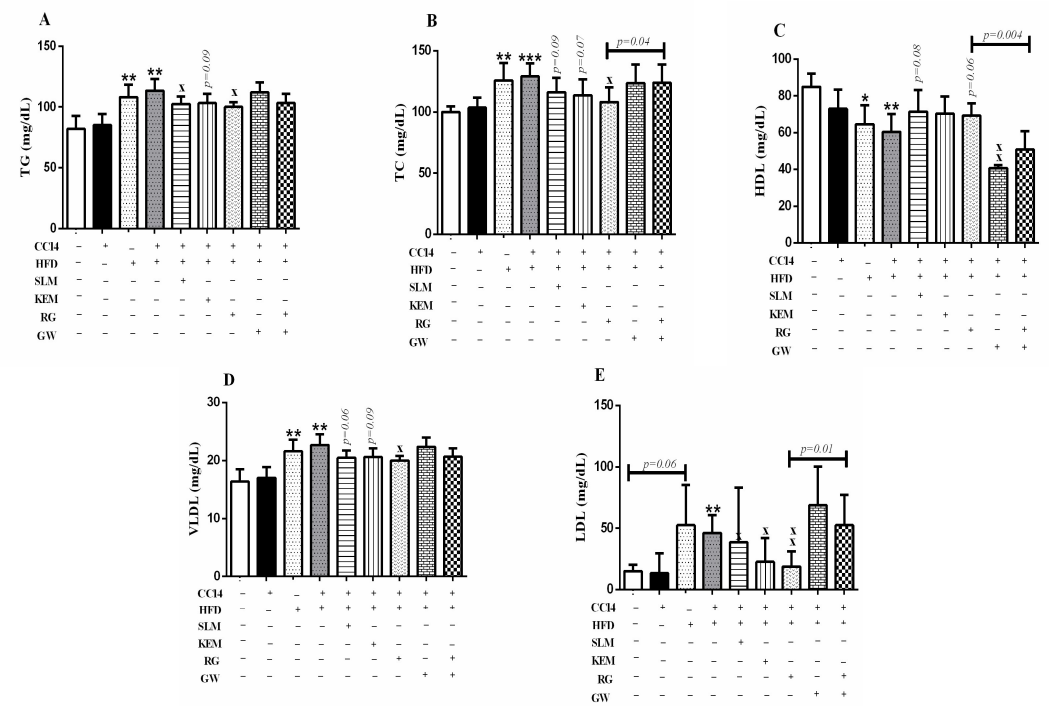

Fig 3: Rosiglitazone (RG) and kaempferol (KEM) alleviated non-alcoholic fatty liver disease (NAFLD)-associated increased lipid profile. **(A)** Triglyceride levels, TG; **(B)** total cholesterol, TC; **(C)** high-density lipoprotein, HDL; **(D)** very low-density lipoprotein, VLDL; **(E)** low-density lipoprotein, LDL. All data were expressed as Mean±S.D., *n = 5*; *: statistical difference of P<0.05, **: statistical difference of P<0.01 ***: statistical difference of P<0.001; ****; statistical difference of P<0.0001 when compared to Veh group; X: statistical difference of P<0.05, XX: statistical difference of P<0.01, XXX: statistical difference of P<0.001, XXXX: statistical difference of P<0.0001 when compared to CCl_4 + HFD group. CCl_4, carbon tetrachloride; HFD, high fat diet; SLM, silymarin; KEM, kaempferol; RG, rosiglitazone; GW, GW9662.

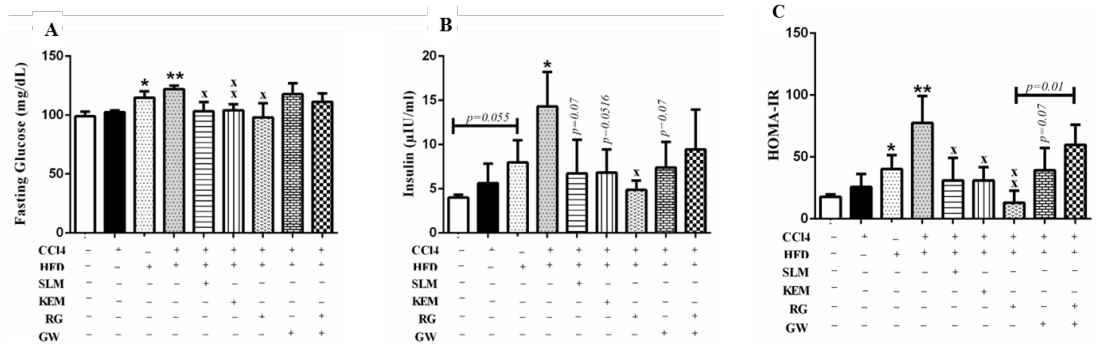

Fig 4: Rosiglitazone (RG) and kaempferol (KEM) prevented glucose intolerance and insulin resistance in high-fat diet (HFD)- and carbon tetrachloride (CCl_4)-induced non-alcoholic fatty liver disease NAFLD model. **(A)** Blood fasting glucose level (mg/dL); **(B)** insulin level (µIU/ml); **(C)** HOMA-IR. All data were expressed as Mean±S.D., *n = 5*; *: statistical difference of P<0.05, **: statistical difference of P<0.01 ***: statistical difference of P<0.001; ****; statistical difference of P<0.0001 when compared to Veh group; X: statistical difference of P<0.05, XX: statistical difference of P<0.01, XXX: statistical difference of P<0.001, XXXX: statistical difference of P<0.0001 when compared to CCl_4 + HFD group. CCl_4, carbon tetrachloride; HFD, high fat diet; SLM, silymarin; KEM, kaempferol; RG, rosiglitazone; GW, GW9662.

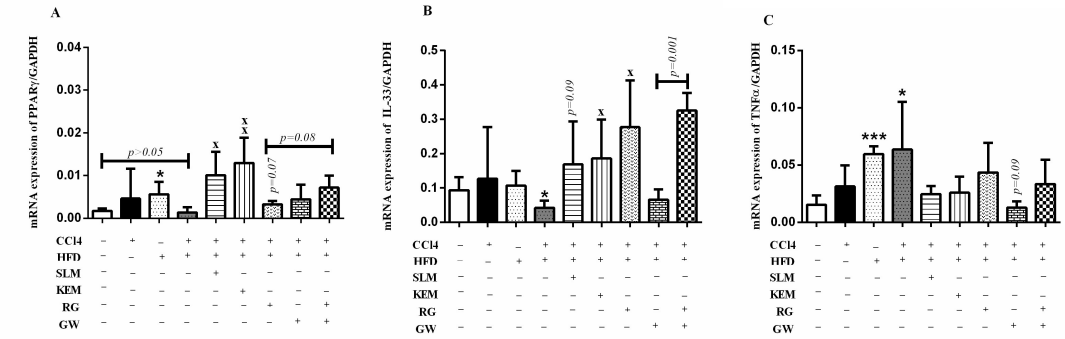

Fig 5: Rosiglitazone (RG) and kaempferol (KEM) induced effects on mRNA expression of peroxisome proliferator-activated receptor gamma (PPARγ), interleukin (IL)-33 and tumour necrosis factor alpha (TNFα) in high-fat diet (HFD)- and carbon tetrachloride (CCl_4)-associated non-alcoholic fatty liver disease (NAFLD). **(A)** mRNA expression of PPARγ; **(B)** mRNA expression of IL-33; **(C)** mRNA expression of TNFα. All data were expressed as Mean±S.D.; *: statistical difference of P<0.05, **: statistical difference of P<0.01 ***: statistical difference of P<0.001; ****; statistical difference of P<0.0001 when compared to Veh group; X: statistical difference of P<0.05, XX: statistical difference of P<0.01, XXX: statistical difference of P<0.001, XXXX: statistical difference of P<0.0001 when compared to CCl_4 + HFD group. CCl_4, carbon tetrachloride; HFD, high fat diet; SLM, silymarin; KEM, kaempferol; RG, rosiglitazone; GW, GW9662

DISCUSSION

Metabolic dysregulation results in a wide range of hepatic diseases ranging from nonalcoholic fatty liver (NAFL), characterized by steatosis with mild inflammation in 5% of hepatocytes, to NASH that is characterized by steatosis with inflammation (hepatitis) (Lindenmeyer *et al.*, 2018). Therefore, determinants of NAFLD are central in pathophysiology that interconnect NAFLD with the higher risk of disease progression over the time. In this study, we found that co-administration of CCl_4 and HFD prompts a wide spectrum of metabolic changes that are associated with pathophysiology of NAFLD. Moreover, PPARγ signaling plays an important role in alleviating NAFLD-associated hepatic damage, dysregulated metabolic parameters and inflammation, as evidenced by expression of inflammatory markers.

The development of potential therapeutic strategies against pathogenesis of NAFLD and NAFLD-associated progression into hepatic diseases necessitate effective animal models. Indeed, previous studies have established NAFLD model with combination of CCl_4 and metabolic injuries for NAFLD-associated progression of NASH, fibrosis and HCC, but require a prolong experimentation along with costly drug response (Tsuchida *et al.*, 2018). We investigated early onset of HFD- and CCl_4-associated NAFLD in male wistar rats with short timeline and lessened the dosing interval of treatments. In consistent with previous studies (Tan *et al.*, 2013; Tanaka *et al.*, 2020), we administered HFD for four weeks to experimental animals to induce obesity-related metabolic injuries. A low dose administration of CCl_4, concomitantly with HFD, accelerated metabolic injuries to investigate pathophysiology of NAFLD (Kubota *et al.*, 2013).

NAFLD-associated high values of liver injury markers reflect a diagnostic approach of hepatic damage (Amirinejad *et al.*, 2020). Additionally, AST/ALT ratio and APRI validate the NAFLD as the non-invasive biomarkers. The values of non-invasive biomarkers <1 are an indication of NAFLD without advanced liver disease (Harrison *et al.*, 2008). Moreover, hepatic steatosis as an evidence of histological findings is most common prognostic implication of NAFLD that is often accompanied with acute hepatic dysfunction (YERIAN, 2011). HFD- and CCl_4-induced hepatocellular inflammation is a predictor of NAFLD (Allman *et al.*, 2010). Specific recruitment of monocytes followed by its differentiation initiates inflammatory events during a liver injury (Lefere *et al.*, 2020). Connolly and colleagues

(Connolly *et al.*, 2009) reported the potential of dendritic cells (25% of hepatic leukocyte) to mediate inflammation via TNFα-dependent pathway in inflammatory *milieu* of fibrotic murine model. Moreover, expression and release of IL-33 from various type of cells, including hepatocytes, hepatic stellate cells and macrophages exhibit IL-33-associated autocrine and paracrine-signaling in acute hepatitis (Arshad *et al.*, 2012).

Inflammation-prompted insulin resistance is one of the key determinants of NAFLD and reflects hyperinsulinemia and hyperglycemia in NAFLD (Ishtiaq *et al.*, 2019). These findings support the observation of present research that HFD+CCl_4 administration contributes to NAFLD via insulin resistance. Insulin resistance augments influx of free fatty acids in the liver by hindering lipolysis and provoking lipogenesis in adipose tissue, and subsequently enhances the vulnerability of the liver to damaging insults (Golabi *et al.*, 2019). Our results presented considerable effects of PPARγ agonist, RG as well as KEM and SLM in improving NAFLD-associated insulin resistance and dyslipidemia that were in accordance with key role of PPARγ in increasing insulin sensitivity by promoting storage of fatty acids as triglycerides and inhibiting ectopic fat accumulation (Ishtiaq *et al.*, 2019).

Dysregulated lipid profile as a characteristic of diet-induced obesity (Nakano *et al.*, 2020). This was evident in present study with increased levels of TG, TC, VLDL and LDL along with low levels of HDL, following administration of HFD and HFD+CCl_4. An approach to combat exacerbated lipid levels involve regulation of lipid homeostasis at cellular level (Ishtiaq *et al.*, 2019). For instance, induced expression of PPARγ may help to improve elevated levels of lipid profile (Yan *et al.*, 2017). The findings of present study are consistent with the role of PPARγ signaling in regulation of altered lipid profile. Amelioration of HFD+CCl_4–associated altered lipid profile (levels of TC, TG, LDL and VLDL) with improved levels of HDL was evident in PPARγ agonist, SLM and KEM.

The safety profile of rosiglitazone has been implicated for its clinical use (Ratziu *et al.*, 2010) as well as further preclinical studies of different liver disease models. Therefore, PPARγ agonists have attained significant attention as a promising therapeutic approach in NAFLD-associated liver pathophysiology. The natural ligands of PPARγ can also be focused in terms of safety profile. Recently, efficacy studies of plant-based phytoconstituents have indicated KEM as one of the putative natural activators of PPARγ (Lokhande *et al.*, 2020b; Lokhande *et al.*, 2020a). In accordance with previous studies (Ishtiaq *et al.*, 2019; Shirvani *et al.*, 2021), the current study demonstrated improvement in NAFLD-associated insulin resistance, lipid profile, hyperglycemia, hyperinsulinemia, and hepatic injury. Anti-inflammatory activity of RG and KEM might be due to PPARγ-regulated increased expression of anti-inflammatory cytokine IL-33 and decreased expression of proinflammatory cytokine TNFα. Further studies are needed to see whether the observed transcriptional expression of IL-33 and TNFα progress to protein level. Also the binding potential of KEM to PPARγ and its affinity may be studied through *in vitro* experimentation.

Conclusions: The combination of HFD and CCl_4 can exacerbate liver injury more than individual administration. The high values of aminotransferases levels, lipid profile, histopathological changes, insulin resistance and importantly, high mRNA expression of TNFα and a low mRNA expression of IL-33 and PPARγ are implicated in pathophysiology of NAFLD. PPARγ signaling evidenced the improvement in NAFLD-associated insulin resistance, lipid profile, hyperglycemia, hyperinsulinemia, and hepatic injury. Anti-inflammatory activity of RG (PPARγ agonist) and KEM (putative PPARγ agonist) might be due to PPARγ-regulated increased expression of anti-inflammatory cytokine IL-33 and decreased expression of proinflammatory cytokine TNFα. Our findings provide mechanistic insights for the implication of PPARγ, the role of PPARγ ligands and therapeutic perspectives in NAFLD. In addition, the findings of present research about putative role of PPARγ ligands-regulated expressions of genes in therapeutic applications of NAFLD pathophysiology needs confirmation by western blot analysis and protein expression level. It might be helpful for the scientific community to understand the emerging role of PPARγ-associated high value targets and signaling pathways in NAFLD pathophysiology.

Author's contribution: SMI and JAK conceived and designed the study. SMI executed the experiment. JAK, FM and MS analyzed the data. SMI and JAK wrote the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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