The Silent Liver Disease "Nonalcoholic Steatohepatitis (NASH)": Targeting Necroptosis and Ferroptosis by Natural Compounds

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Fig 1

Introduction

NASH is a common chronic liver disease that is closely associated with diabetes and obesity. Human NASH exhibits necrosis and necroinflammation, that may contribute to the disease's etiology. Necroptosis is a key form of programmed cell death that is required to regulate inflammation in many tissues. Ferroptosis is a type of programmed necrosis and identified as an iron- and lipid hydroperoxide-dependent non-apoptotic cell death. AMPK controls fat and glucose metabolism based on the cell's energy level (1).

Pentoxifylline (PTX) is a methylxanthine derivative with strong hemorrhagic characteristics. PTX causes several cellular physiological changes through activation of phosphodiesterase-4 and inhibition of nuclear factor kappa B (NF-κB) & modulation of cytokines (2).

Kaempferol (KP) is a naturally occurring flavonoid. This molecule has antioxidant and anti-inflammatory activities and the ability to limit tumor growth. KP can protect normal liver cells against cytotoxicity, ROS production, and DNA damage caused by H_2O_2 (3).

Fenugreek extract (FE), *Trigonella foenum - graecum* L., has anti-hyperlipidemic and anti-hyperglycemic effects. Diosgenin (DSG), is the active compound of the hydrolyzed fraction of fenugreek extract (FE). Numerous animal studies also show that FE possesses hepatoprotective properties (4).

Materials and Methods

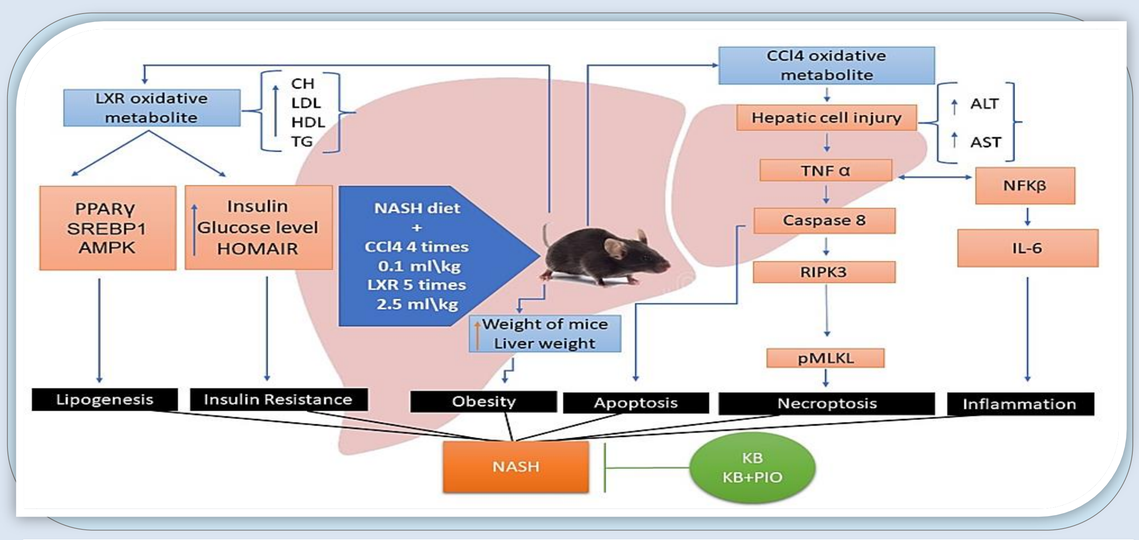
NASH Induction: Mice were fed a high-fat diet (20% protein, 60% fat, and 20% carbohydrate equivalent to 3.6 Kcal/g). Then, mice were taken four intraperitoneal (Ip) injections of 0.1 mL/kg/bw of CCl4 to develop NASH on 14th, 17th, 21st, and 24th days from the beginning of experiment. Whereas liver X receptor (LXR) agonist was administered five times (I.p.) on 20th - 24th days at a dose of 2.5 mL/kg.

Animal Groups

Mice were divided randomly according to treatments (n=8) and all treatments were given orally for four weeks after induction of NASH and parallel with the NASH protocol.

Control: Mice received DMSO with a normal chow diet.
NASH: mice maintained on NASH protocol for four weeks.
PTX: 100 mg/kg/ daily
KP: 40 mg/kg/ daily
PTX+KP: PTX (100 mg/kg) plus KP (40 mg/kg) daily.
PIO: Pioglitazone was given (50 mg/kg) daily
KP+PIO: KP (40 mg/kg) plus PIO(50 mg/kg)
FE : fenugreek extract (2.6 mg/ kg) daily
LD-1 : DSG (1 mg/kg) low dose.
MD-5 : DSG (5 mg/kg) medium dose
HD-10: DSG (10 mg/kg) high dose.

Serum liver indices, lipid profile, glucose, insulin, and Homeostasis model assessment (HOMA) of insulin resistance (IR) were determined. Liver histopathology, liver gene expression of adenosine monophosphate (AMP)-activated protein kinase (AMPK), Farnesoid X receptor (FXR) and retinoic acid receptor (RAR), PPAR γ, SREBP1, and pMLKL Protein expression of caspase-8, RIPK3 and phospho-acetyl CoA carboxylase (p-ACC) were evaluated.



Effect of Kaempferol alone and in combination with pioglitazone against NASH-induced mice; ALT, alanine aminotransferase; AST; aspartate aminotransferase; IL-6, interleukin 6; CCL, Carbon tetrachloride; TNF-α, tumor necrosis factor-alpha; RIPK3, Circulating receptor-interacting protein kinase; PPAR γ, Peroxisome proliferator-activated receptor-gamma-γ; AMPK, adenosine-monophosphate activated protein kinase; SREBP-1, sterol regulatory element-binding protein-1; LXR, liver X receptor; NF-κB, Nuclear factor-kappa B; MLKL, mixed lineage kinase domain-like protein; KP, Kaempferol; LDL, Low-density lipoprotein HDL, High-density lipoprotein, TG, Triglyceride; CH, Cholesterol

Results

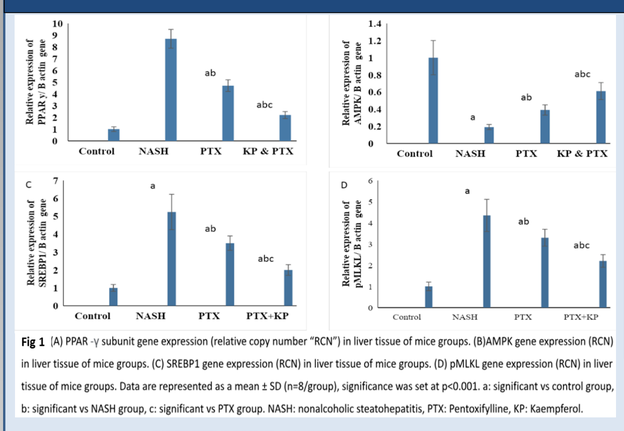


Fig 1 (A) PPAR -γ subunit gene expression (relative copy number "RCN") in liver tissue of mice groups. (B)AMPK gene expression (RCN) in liver tissue of mice groups. (C) SREBP1 gene expression (RCN) in liver tissue of mice groups. (D) pMLKL gene expression (RCN) in liver tissue of mice groups. Data are represented as a mean ± SD (n=8/group), significance was set at p<0.001. a: significant vs control group, b: significant vs NASH group, c: significant vs PTX group. NASH: nonalcoholic steatohepatitis, PTX: Pentoxifylline, KP: Kaempferol.

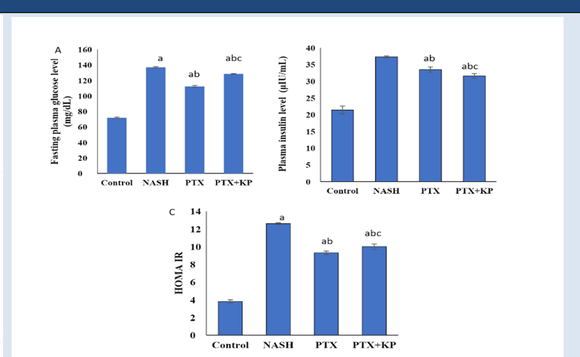


Fig. 2 A) Fasting plasma glucose level in mice groups. (B) Plasma insulin level in mice groups. (C) HOMA-IR in mice groups. Data are represented as a mean ± SD (n=8/group), significance was set at p<0.05. a: significant vs control group, b: significant vs NASH group, c: significant vs PTX group. NASH: nonalcoholic steatohepatitis, PTX: Pentoxifylline, KP: Kamepferol.

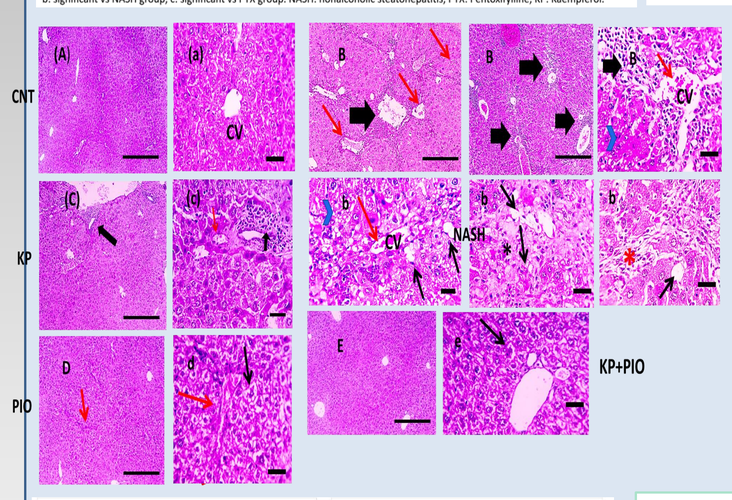


Fig. 3 : A: microscopic pictures of H&E-stained liver sections in treated groups (a) show normal hepatocytes arranged in radiating plates around a central vein (CV) with normal sinusoids (s) in control –ve group. (b) Meanwhile, liver sections from NASH group show distended sinusoids with leukocytes (thick arrows) around congested central vein (red arrow) with prominent centrilobular macro-vesicular (thin black arrow) to few micro-vesicular steatosis (arrowheads) in hepatocytes. Portal (black asterisk) and pericellular (red asterisk) fibrosis are seen. (c) PIO stained liver sections showed milder pathological changes in the treated groups with single drug including: mildly congested blood vessels (red arrow) with mild hydropic degeneration (thin black arrow) in hepatocytes in (d) KP treated mice liver sections showed mild congestion (red arrow), mild portal fibrosis with few leukocytic cells infiltration (thick arrows) and mild hydropic degeneration in hepatocytes (thin black arrow) and (e) KP+PIO-stained liver sections show the mildest pathological changes in the treated groups with drug combinations including: very mildly congested blood vessels (red arrows) and accidental lobular inflammation (arrowheads) in group. Low magnification X: 100 bar 100, high magnification X: 400 bar 50. (F) Statistical analysis of histopathological lesional scores in H&E-stained hepatic sections showing significantly higher scores in NASH group when compared with control group. Significant reduction of hepatic lesional scores is seen in treated group with KP+PIO when compared with NASH group. Stars mean significant when p <

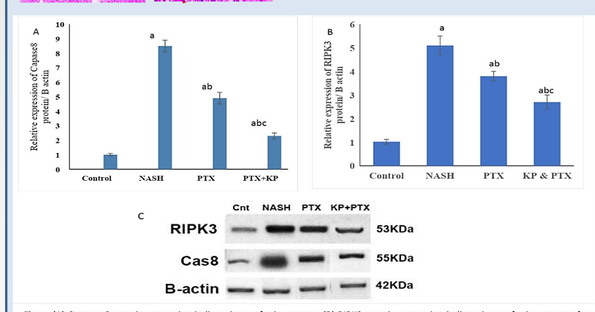


Fig. 4 (A) Caspase 8 protein expression in liver tissue of mice groups. (B) RIBK3 protein expression in liver tissue of mice groups after treatment. (C) Western blot analysis showing protein expression of RIPK, Caspase 8 and β-actin in different experimental groups. Data are represented as a mean ± SD (n=8/group), significance was set at p<0.05. a: significant vs control group, b: significant vs NASH group, c: significant vs PTX group. NASH: nonalcoholic steatohepatitis, PTX: Pentoxifylline, KP: Kaempferol.

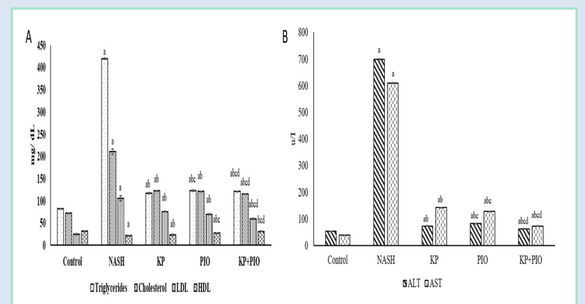


Fig. 5. (A) serum liver enzymes ALT and AST levels in mice groups. (B) Plasma lipid profile in mice groups. Data are represented as a mean ± SD (n=8/group), significance was set at p<0.05. a: significant vs control group, b: significant vs NASH group, c: significant vs KP group, d: significant vs PIO group

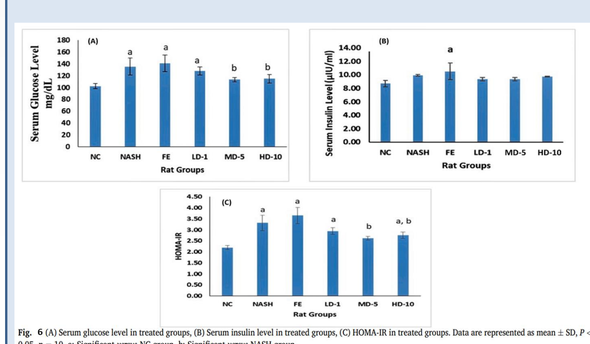


Fig. 6 (A) Serum glucose level in treated groups, (B) Serum insulin level in treated groups, (C) HOMA-IR in treated groups. Data are represented as mean ± SD, $P < 0.05$, $n = 10$. a: Significant *versus* NC group. b: Significant *versus* NASH group.

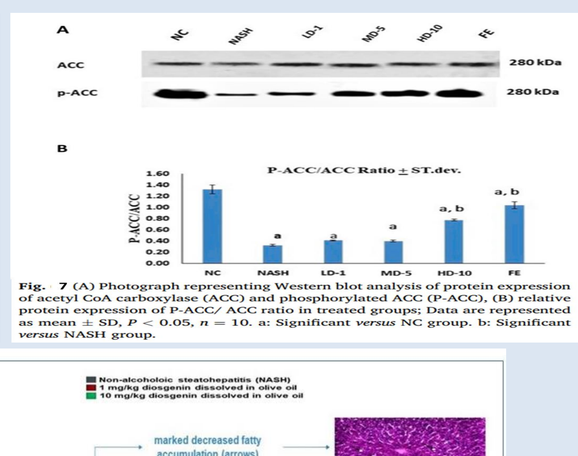
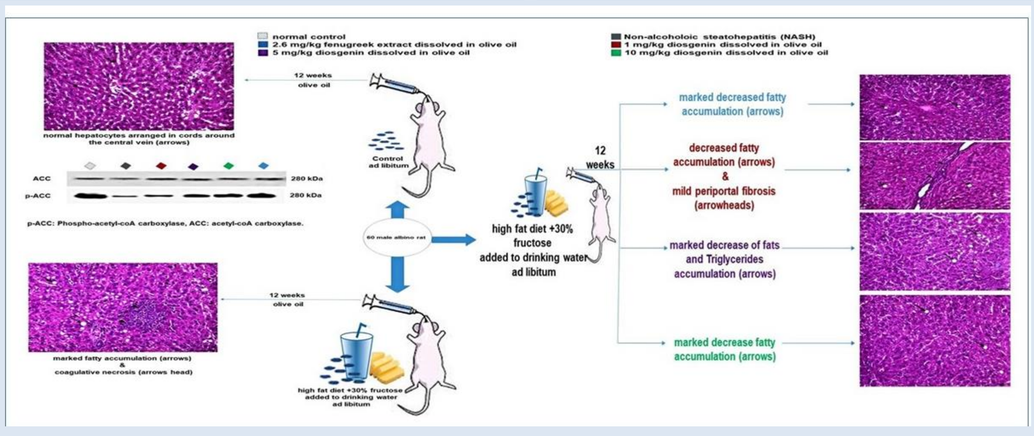


Fig. 7 (A) Photograph representing Western blot analysis of protein expression of acetyl CoA carboxylase (ACC) and phosphorylated ACC (P-ACC), (B) relative protein expression of P-ACC/ ACC ratio in treated groups; Data are represented as mean ± SD, $P < 0.05$, $n = 10$. a: Significant *versus* NC group. b: Significant *versus* NASH group.



Conclusions

- **The present study indicated a new mechanism by which kaempferol, a natural flavonoid, and pioglitazone are effective in the NASH treatment and its prevention through down regulation of Caspase-8, pMLKL, and RIPK3 mediated apoptosis and necroptosis and suppression of proinflammatory cytokines as well as KP and PIO acts through AMPK and PPAR pathways and suppress the fats accumulation in the liver and decreased resistance of insulin and blood glucose level.**
- **The current study also proved that pentoxifylline, alone or in association with Kaempferol, is effective in treating and preventing NASH by down-regulating caspase 8, pMLKL and RIPK3 which stimulate apoptosis and necroptosis pathways as well as acting through the decrease of lipogenesis genes such as AMPK and SREBP-1; all alleviates NASH.**
- **DSG could be considered one of the fenugreek seed constituents that contributed to the hepato-protective effect of FE. FE and DSG affected several hepatic pathways including, activation of AMPK signaling, upregulation of FXR & down-regulation of RAR, and inhibition of acetyl CoA carboxylase thus inhibiting lipid accumulation in hepatocytes.**

References

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