ejpmr, 2015,2(3), 706-718

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

ETHNOPHARMACOLOGICAL APPROACH OF HEDERAGENIN ATED FROM S*APINDUS TRIFOLIATUS* L. ON NON ALCOHOLIC FATTY LIVER DISEASE (NAFLD) ASSOCIATED WITH TYPE 2 DIABETES IN C57BL/6 MICE

Vinitha Lakshmi[1*], Dhamotharan. R[1], Kalyan kumar. G[2]

[1]Post-Graduate Research Center, Department of Plant Biology and Biotechnology, Presidency College, Chennai- 600005, India.

[2]Department of Pharmacology, Vanta Bioscience (Kemin Industries South Asia), Gummidipundi, Chennai, Tamilnadu-601201, India.

Article Received on 18/03/2015 **Article Revised on 10/04/2015** **Article Accepted on 02/05/2015**

***Correspondence for Author**

Dr. Vinitha Lakshmi.

Post-Graduate Research Center, Department of Plant Biology and Biotechnology, Presidency College, Chennai- 600005, India.

ABSTRACT

Ethnopharmacological relevance: Hederagenin, a saponin extracted from fruits of S*apindus trifoliatus* is used as astringent, thermogenic, anti-inflammatory, analgesic, antifungal, antiviral, anti helmintic and tonic in action. NAFLD is a liver disorder characterised by fat accumulation and is treated only at the advanced stages of cirhossis. The cure of it still remains untold. Whereas the current approach with traditional medicine has found it successful in reducing the liver enlargement and removes the accumulated fat indicating the right choice of medicine for NAFLD. **Aim of the study:** The present study investigated the activity of Hederagenin on non alcoholic fatty liver disease (NAFLD) associated with type 2 diabetes in murine model which is a novel approach. **Materials and methods:** Male C57BL/6 mice (6–8 weeks of age) were fed with high fat diet for eight weeks and then dosed with Hederagenin at doses of 100, 300 and 500 mg/kg p.o., metformin for 350mg/kg p.o. Normal control animals were fed with normal standard pellet diet whereas the positive control group along with treatment group still maintained the high fat diet. At the end of the experimental period, the final body weight and feed intake were taken and whole blood was collected by retro orbital sinus punctures. Plasma/serum was obtained by cold centrifugation (4°C) till analysis. Later animals were sacrificed by cervical dislocation and, liver were excised and stored at - 80°C. **Results:** Hederagenin significantly

suppressed lipid accumulation in the liver, reduced biochemical, insulin resistance, and lipid in HFD-fed C57BL/6 mice. These findings may provide molecular evidence for the use of Hederagenin as a therapy in the management of fatty liver and obesity-relative disorders. **Conclusion:** These findings may exhibited the use of Hederagenin as a therapy in the management of fatty liver and obesity-relative disorders.

KEYWORDS: NAFLD, NASH, Hederagenin, C57BL/6 mice, HFD fed mice, Type 2 diabetes.

INTRODUCTION

NAFLD is a common liver disorder that is strongly associated with insulin resistance and Type 2 diabetes and its characterised by fat accumulation in the liver.(Vijay viswanathan et al., 1968; Viswanathan et al., 2009). Both the pathogenesis appears to involve complex interactions between genetic and environmental factors (Shahid Ahmed et al., 2010). Recent reports suggest Type 2 diabetes mellitus, obesity and dyslipidemia often coexists with NAFLD. In particular, hyperlipidemia and insulin resistance importantly contribute to the initiation and progression of NAFLD. Recent clinical studies also shows that NAFLD is one of the main common liver diseases that lead to the liver cirrhosis and hepatocellular carcinoma. The prevalence of NAFLD has been reported to be in the range of 15-20% in the general population, whereas in Type 2 diabetic population the prevalence was as high as 50-75% (Bedgoni et al., 2005)

However, the molecular mechanisms responsible for progression of NAFLD have not been fully understood (Yu and Ginsberg, 2005) Further, intestinal cholesterol absorption is elevated in those with Type 2 diabetic patients with coronary heart diseases nd low cholesterol absorption associates with fewer recurrent cardiovascular events. (Federico, 2008).

In United States, NASH is considered to be the 3rd most common liver disease after hepatitis C and alcoholic fatty liver. Global prevalence on NASH is 10-24% amongst general populace but increases to 25–75% in obese diabetic individuals (Farrell and Larter, 2006; Browning and Horton, 2004).

Much of the increased prevalence of NAFLD is driven by obesity. However, high rates of NAFLD in relatively normal weight people (by Western standards) from the Indian

subcontinent and Southeast Asia suggest that, even in the absence of obesity, insulin resistance leads to hepatic fat accumulation. Indeed, patients with total lipodystrophy, who have no adipose tissue, have severe insulin resistance with marked hepatic steatosis (Menaka et al.,2012). Studies of the molecular basis of NAFLD have largely focused on triglyceride (TG), the major lipid stored in hepatocytes. Although much is known about the regulation of hepatic TG synthesis, secretion, and storage, much less is known about the role of TG and/or its precursors in stimulating the inflammatory changes needed for the progression of steatosis to steato hepatitis (Petta, 2009).

MATERIALS AND METHODS

Hederagenin isolation

Fruits of *Sapindus trifoliatus* (L.), (family, Sapindaceae) were collected from PERD Centre, Ahmedabad, and Gujarat, India. The plant was authenticated by comparing with the herbarium voucher specimen deposited at Presidency College, University of Madras, Herbarium (Specimen No. PBPB-41). The plant was air dried in shade, powdered mechanically and stored in airtight containers. The extract was filtered, pooled and the solvent was removed under reduced pressure at 50 ± 60°C using rotary flash evaporator and the yield was 7.6g, 0.76%w/w of hederagenin. The structure was confirmed by IR, 1H NMR and mass spectral studies.

Animals

Male C57BL/6 mice (6–8 weeks of age) were purchased from Biogen Laboratory animal facility, Bangalore, India. They were housed and maintained in clean polypropylene cages and fed with either low fat diet or high fat diet and water *ad libitum*. This study was performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee (Approval No. 971/BC/06/CPCSEA). All animals were housed 2/cage and kept in the animal house for one week for proper acclimatization before starting the experiment under controlled conditions of illumination (12 h light/12 h darkness) and temperature ranging 20-25°C. They were housed under the above laboratory conditions, maintained on high fat diet (% w/w) (standard diet -87.7; pork diet – 10; cholesterol -2; bile salts- 0.3) and water.

Reagents and instrument required

The chemical and drugs used in the study were Hederagenin, Glucose, Hep G2 cell lines, trypsin, EDTA, carboxymethyl cellulose (CMC), metformin, isoflurane (anaesthetic agent)

catalase, superoxide dismutase (SOD) hydrogen peroxide, formaldehyde eosin, hematoxylin was purchased from Sigma–Aldrich, St. Louis, MO, USA. TNF-α ELISA kit (Ray Biotech Inc., Norcross, USA), Animal restrainer (e.g., Broom restraint, Plas Labs), Micrometer, glucometer (Accu-Check, Roche, Germany) and Advia (Hematology analyzer), hematoxylin and eosin stains, semi automated microtome, Disterene phthalate xylene, sodium chloride solution, Tris hydrochloride, thio barbituric acid.

Experimental design

Male C57BL/6 mice were randomly divided into six groups; 6 mice each.

- Group 1 - Normal group and was maintained on normal mouse chow diet throughout the experiment (sixteen weeks).
- Group 2 - NAFLD group received distilled water (1 ml/kg/day, p.o.),
- Group 3 received Hederagenin (100 mg/kg/day, p.o.),
- Group 4 received Hederagenin (300 mg/kg/day, p.o.),
- Group 5 received Hederagenin (500 mg/kg/day, p.o.),
- Group 6 received Metformin (350mg/kg/day, p.o.).

The groups 2 to 6 were maintained on a HFD containing 87.7% standard diet (w/w), 10% pork fat (w/w), 2% cholesterol (w/w) and 0.3% bile salts (w/w) (Pan *et al*., 2006) for eight weeks. For an additional eight weeks, HFD was given in addition to the following treatment regimens. At the end of the experimental period, the final body weight of overnight fasted each animal was recorded and were given mild Isofurane anesthesia and whole blood was collected by retro orbital sinus puncture in EDTA coated vials. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later animals were sacrificed by cervical dislocation and, liver were excised and stored at - 80°C (Cryo Scientific Ltd., India) for further evaluations

Assessment of biochemical parameters in plasma

After the last drug treatment, animals were fasted overnight. Blood samples were collected by retro orbital method and blood samples were centrifuged for 5 min at 4000 rpm. The collected plasma were estimated for fasting blood glucose and insulin (ELIZA), alanine transaminase (ALT) and aspartic transaminase (AST). Serum total cholesterol and triglycerides were determined using (Flex reagent) by performing in Dimension Xpand Plus, Siemens India. Insulin was also estimated using ultra sensitive mouse insulin kit (Catlog-90080). Insulin resistance were calculated using

HOMA-IR index = [fasting glucose (mmol/L)×fasting insulin (µU/ml)]/22.5)

Estimation of TNF α were done using Biotrak ELISA system (RPN2718),

Assessment of Liver Triglycerides

The liver was rapidly removed and hepatic triglycerides were measured from these tissues (50 mg) according to methods modified from (Folch *et al*., 1957).Briefly, snap frozen liver kept at −80 °C was homogenized and extracted with chloroform/methanol (2:1 v/v) solution. The lipid extraction was considered complete when the minced liver tissue settled on the bottom of the vial after vortexing. The lower organic phase was separated in a separate tube and dried using nitrogen evaporator. This can also be stored at -20°C until analysis. At the time of immediate analysis, the dried sample was resolubilized in 2-propanol containing 10% Triton X-100. Hepatic triglyceride levels were determined by Bioassay enzymatic kits

Assessment of Liver Tissue Histopathology

Liver tissue samples were fixed at 10% formalin and embedded in paraffin. Sections measuring 5µm thickness were cut and stained with H&E stain. All histological examinations were performed by an experienced pathologist who was blinded to the experiment groups. Histopathological changes were assessed by a semi quantitative method according to standards proposed by Dixon *et al*., (2004).

Statistical Analysis

The values are expressed as Mean ± SE. The Graphs were generated using GraphPad Prism® (Version 5). Statistical analysis was undertaken using One-Way ANOVA with Dunnett's post-test using GraphPad Prism®. The results were considered significant when $P \leq 0.05$.

RESULTS

Effect of Treatment on Body weight, Feed intake and Liver Index

The animals fed with HFD diet for 6 weeks showed significant increase in body weight compared with diet fed animals ($P < 0.05$). Following treatment, there was significant reduction ($P < 0.01$) in body weight gain and cumulative feed intake ($P < 0.05$) in Group V compared with Group II and no changes in body weight gain and feed intake was observed in other treatment groups [Table. 1]. A significant increase in the body weight and the liver index was observed in the HFD-fed mice as compared to normal mice ($P \leq 0.05$). The % increase in the body weight was significantly reduced in Metformin 350mg/kg group, Hederagenin (300 mg/kg/day, p.o.) group and Hederagenin (200 mg/kg/day, p.o.) as

compared to NAFLD group (P≤0.05). Liver index was also improved in Metformin 350mg/kg group and in the Hederagenin (300 mg/kg/day and 200 mg/kg/day, p.o.) as compared to NAFLD group (P≤0.05). No significant differences in daily food and water intake were observed among the groups over the experimental period.

Liver enzyme activities

Feeding with a HFD for sixteen weeks induced a significant increase in serum activities of AST and ALT in mice as compared to the normal group (P≤0.05). All the treatment regimens significantly decreased the elevated activities of AST and ALT as compared to NAFLD group (P≤0.05), (Table 2).

Serum total cholesterol, Triglycerides and hepatic triglycerides

Table 2 shows a significant elevation in serum total cholesterol and triglyceride levels in HFD-fed mice as compared to normal mice (P≤0.05). The elevation in serum total cholesterol was significantly ameliorated only by treatment with Hederagenin. The elevation in serum triglycerides level was markedly attenuated by treatment with Hederagenin. In addition, hepatic triglyceride level was significantly higher in NAFLD group as compared to normal group (P≤0.05).Treatment with Hederagenin significantly reduced the high hepatic triglyceride level as compared to NAFLD group.

Fasting blood glucose, insulin and HOMR-IR index

A significant increase in fasting blood glucose, fasting insulin and HOMA-IR index was observed in mice with NAFLD as compared to normal mice (P≤0.05, Table 3). Hederagenin was the sole treatment that could decrease the elevated fasting blood glucose significantly as compared to the NAFLD group. However, fasting insulin was reduced significantly as compared to NAFLD group. All the implemented pharmacological agents significantly reduced the elevated HOMA-IR index as compared to NAFLD group (P≤0.05), (Table 3)

The inflammatory cytokine, TNF-α

Serum TNF-α level was significantly increased in NAFLD mice as compared to normal mice (1351±121 versus 3214±321, P≤0.05, (Fig. 5.2. A and B). Hederagenin could significantly attenuate this increase (P≤0.05,) and lower down the inflammatory cytokine levels. (Fig.1)

Histopathological examination

In the present study, the NAFLD group showed significant changes in the liver histology. Liver samples from the NAFLD group stained with Hematoxylin & Eosin or Masson's trichrome stain showed diffuse macrovesicular steatosis and multifocal portal inflammation as well as hepatocellular fibrosis. Liver samples from the metformin showed moderate portal fibrosis. The liver in the Hederagenin group showed only mild fatty change with few inflammatory cell infiltrations along with mild portal fibrosis.

The degree of steatosis, lobular inflammation and fibrosis in the NAFLD group was significantly higher than in the normal group. All the treatment regimens, with the exception of Hederagenin, significantly reduced the degree of steatosis; the steatosis score in Hederagenin was significantly lower than that observed in metformin group. However, the Hederagenin (500mg/kg) was the sole group that showed a significant decrease in the liver lobular inflammation. Finally, fibrosis was attenuated in all treatment groups, except the Hederagenin (100mg/kg) group. The fibrosis score in Hederagenin (500mg/kg) group was significantly lower than that recorded in metformin group (350mg/kg) (Fig.2)

Table 1 Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.), on body weight, feed intake and liver index in the experimental groups.

Groups	Increase in body weight (g)	Final body weight (g)	Cumulative feed intake (g)	Liver index (%)
Normal	33.3 ± 1.1	31.28±1.21	84.91±4.68	4.16±0.06
NAFLD	44.1 ± 1.0	44.1±3.16$^{\$\$}$	46.23±2.40	7.26±0.27 [a]
Hederagenin (100 mg/kg/day)	40.1 ± 0.9	39.92±3.21	45.75±2.68	6.04±0.31 [b]
Hederagenin (300 mg/kg/day)	39.2 ± 1.4	36.92±1.48	42.27±2.48	5.70±0.19 [b]
Hederagenin (500 mg/kg/day)	38.2 ± 1.2	34.60±1.34*	23.18±1.32*	5.12±0.19 [b]
Metformin (350 mg/kg/day)	36.3 ± 1.3	36.96±1.04*	24.99±1.33*	4.81±0.19 [b]

NAFLD: nonalcoholic fatty liver disease, **HFD:** high-fat diet. Results are expressed as Mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. [a] P≤0.05 versus normal group. [b] P≤0.05 versus NAFLD group, n=6.

Table 2 Effect of Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.), and their combinations on serum AST, ALT, TC TG and as well hepatic triglycerides level in the experimental groups

Groups	AST (IU/L)	ALT (IU/L)	TC (mg/dl)	Serum TG (mg/dl)	Hepatic TG (mg/dl)
Normal control	41±4	39±5	64±5	162±10	11±0.3
NAFLD control	93±7[a]	74±5[a]	120±8[a]	231±10[a]	15±0.3[a]
Hederagenin(100mg/kg)	72±8[b]	69±3	100±4[b]	195±10[b]	12±0.3[b]
Hederagenin(300mg/kg)	67±8[b]	58±3[b]	97±4[b]	170±10[b]	13±0.3[b]
Hederagenin(500mg/kg)	55±8[b]	48±3[b]	86±4[b]	163±10[b]	11±0.3[b]
Metformin (350mg/kg)	49±8[b]	44±3[b]	70±4[b]	156±10[b]	10±0.3[b]

NAFLD: nonalcoholic fatty liver disease, **HFD:** high-fat diet, **AST:** Aspartate aminotransferase, **ALT:** Alanine aminotransferase, **TC:** total cholesterol. **TG:** triglycerides. Results are expressed as Mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons.[a] P≤0.05 versus normal group. [b] P≤0.05 versus NAFLD group, n=6.

Table 3: Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.) on fasting blood glucose, fasting insulin and HOMA-IR index in the experimental groups.

Groups	fasting blood Glucose (mg/dl)	fasting insulin (µU/ml)	Homo- IR index
Normal control	71±2	13±1	2.2±0.17
NAFLD control	89±3[a]	24±4[a]	4.9±0.5[a]
Hederagenin (100mg/kg)	80±4[b]	21±3[b]	3.7±0.5[b]
Hederagenin(300mg/kg)	79±4[b]	20±8[b]	3.3±0.5[b]
Hederagenin(500mg/kg)	77±4[b]	16±5[b]	2.9±0.5[b]
Metformin (350mg/kg)	70±7[b]	14±6[b]	2.4±0.5[b]

NAFLD: nonalcoholic fatty liver disease, **HFD:** high-fat diet, **HOMA-IR index**: Homeostatic Model Assessment–Insulin Resistance index. **HOMA-IR index** = [fasting glucose (mMol/L)×fasting insulin (µU/ml)] / 22.5). Results are expressed as Mean±S.E.M. and analyzed using one way ANOVA followed by Bonferroni's test for multiple Comparisons. [a] P≤0.05 versus normal group, [b] P≤0.05 versus NAFLD group, n=6.

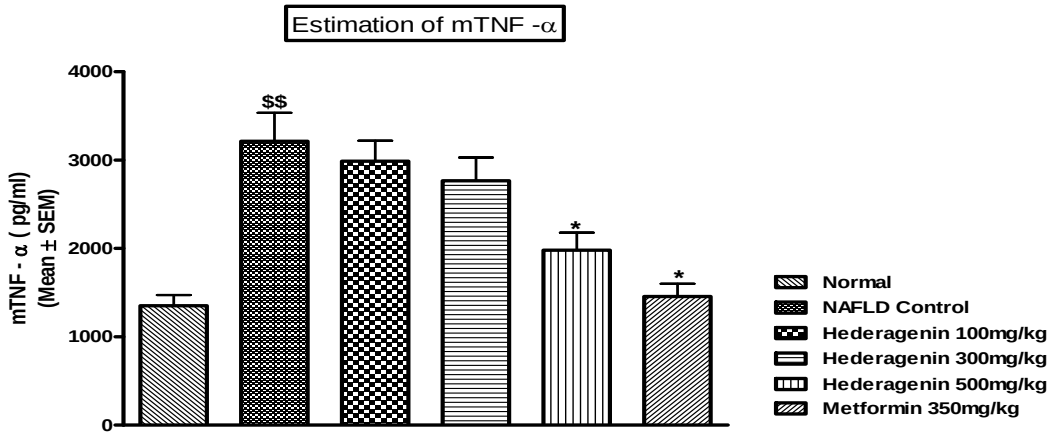
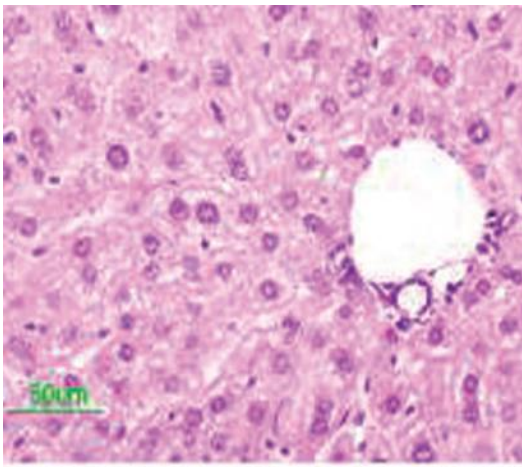
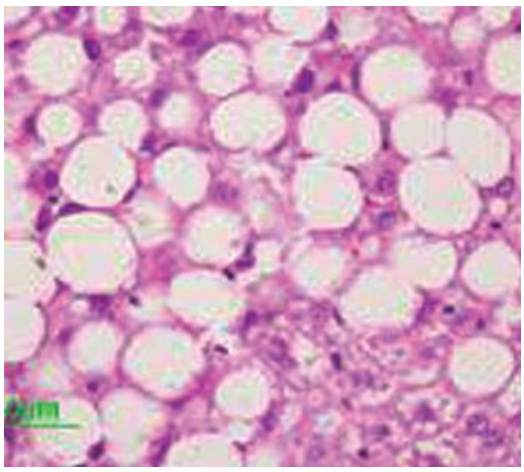


Figure 1: Estimation of Tumour Necrosis Factor Alpha TNF-α mouse, Biotrak ELISA system (RPN2718

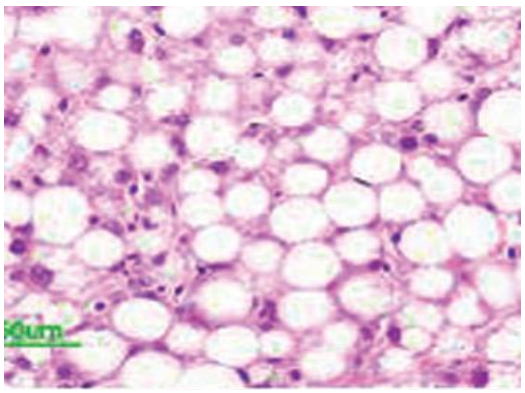
Results are expressed as mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. $$P≤0.05 versus normal group, *P≤0.05 versus NAFLD group.



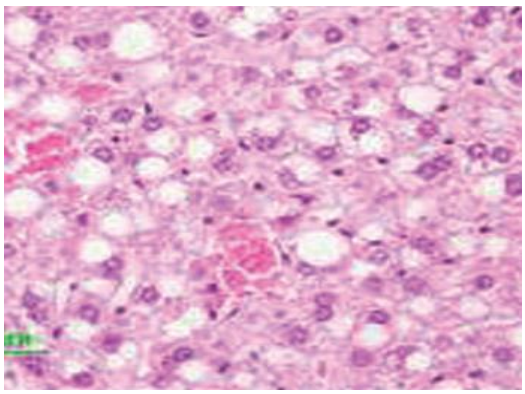
Group I [Normal control ;A],



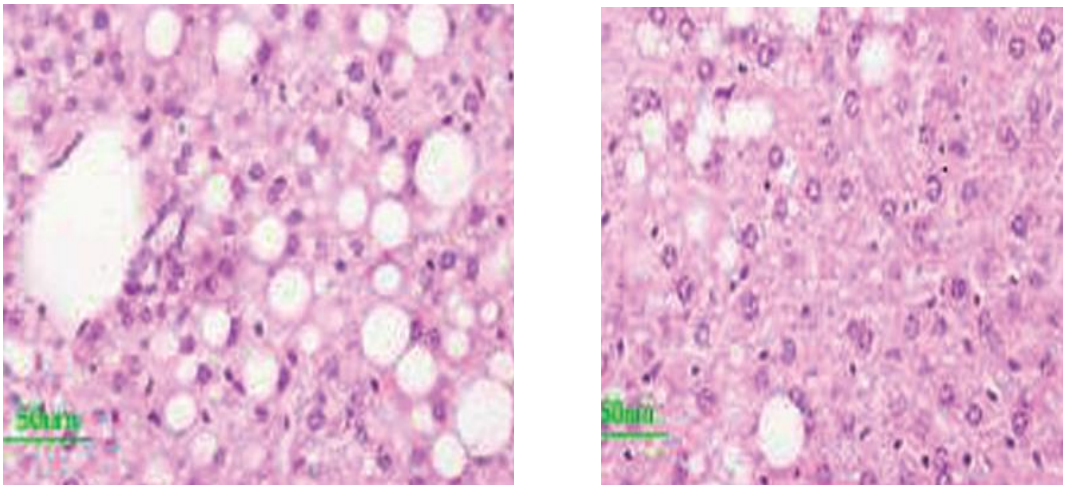
Group II [NAFLD control; B]



Group III [Hederagenin 100mg/kg; C]



Group IV [Hederagenin 300mg/kg; D]



Group IV [Hederagenin 500mg/kg; D] **Group V [Metformin 350mg/kg; E]**

Figure 2 Effect of Hederagenin on histological changes in liver at magnification of x200.

Histology of the liver sections stained with hematoxylin and eosin (H&E) stain. Microscopic picture of sections of liver from normal group showing normal liver tissues with normal hepatic cords, portal area and hepatic lobules. Liver sections of NAFLD group show diffuse marked macrovesicular steatosis, multifocal portal inflammation and marked fibrosis. Liver sections from Metformin group show moderate local extensive steatosis with mild ballooning degeneration. Liver sections from Hederagenin 500mg/kg group show moderate fatty change, few inflammatory cells infiltrations together with mild portal fibrosis. Liver sections from Hederagenin 300mg/kg group group show mild steatosis, moderate steatohepatitis (H&E stain X100).

DISCUSSION

The study focused on the effect of Hederagenin and metformin in high fat diet-induced NAFLD in C57BL/6 mice. The NAFLD animals developed marked obesity, hyperglycemia, and fatty liver. Histopathological examination of the livers of NAFLD control mice revealed moderate to severe steatosis, lobular inflammation and developed typical histopathologic non-alcoholic steatohepatitis lesions. There was severe hepatic fat accumulation along with increased liver weight; however, this was accompanied with only mild elevation of liver specific enzymes. A lack of correlation between the degree of NAFLD and levels of liver enzymes is not surprising, since in a clinical situation the liver enzyme levels do not readily correlate with severity of hepatic steatosis. These observed features are similar to the pathological features of human NAFLD. In the NAFLD control group, the animals showed significant reduction in plasma TG with corresponding increase in liver TG levels as

compared with normal controls. Though such a reduction seems contrary with human metabolic syndrome condition, it has already been reported by other researchers.

In response to sudden excessive fat ingestion when the plasma lipid level exceeds oxidative capacity of energy requiring tissues like skeletal muscle, the liver acts as an effective buffer organ to avoid accumulation of circulating lipid and starts taking up lipid from plasma to store as TG. Excessive stored TG in hepatocytes is the hallmark of NAFLD which is strongly associated with hyperinsulinemia and hyperglycemia. (Shuqin Zheng *et al*., 2008).

In addition, significant elevations in total cholesterol and triglycerides in the sera of mice were observed in the current study. In agreement, a significant elevation in serum triglycerides or both serum total cholesterol and triglycerides was observed after HFD feeding. Further, the current study revealed that NAFLD mice showed high hepatic triglycerides level and this extends findings of others. Further, a significant increase in HOMA-IR index was observed in NAFLD group as compared to the normal group. These results are supported by previous studies. This indicates that mice with NAFLD suffer from high insulin resistance and thus, insulin resistance plays an important role in the development of fatty liver.

In the current study, serum level of TNF-α was significantly increased by HFD feeding and this finding came on line with those obtained previously (Yalniz *et al*., 2007). Data in this study showed that Hederagenin, induced a significant reduction in liver index and activities of AST and ALT. Consistently, ALT activity was reduced by Hederagenin in mice maintained on a diet-induced non-alcoholic steatohepatitis (Fujita *et al*., 2007) and HFD-fed mice. Hederagenin significantly lowered HFD-induced hypertriglyceridemia; whereas, the Hederagenin significantly lessened the hypercholesterolemia. These results agreed with those reported by Xu *et al*., 2008. Metformin or Hederagenin, significantly decreased the HOMA-IR index. Consistently, the HOMA-IR index decreased significantly in mice with HFD-induced NAFLD and in obese rats after treatment with metformin. Recently, metformin was shown to significantly reduce plasma insulin and the HOMA-IR and diabetic mice.

CONCLUSION

Hederagenin isolated from S*apindus trifoliatus* L. Demonstrated the reduction of fatty liver, therby preventing the liver from cirrhosis and further damage. This experiment also indicates that the drug equals the antidiabetic compound metformin by reducing glucose levels to an

extent, thereby stating antidiabetes associated with non alcoholic fatty liver disease may be managed together with Hederagenin.

REFERENCES

1. Bedgoni G, Miglioli L, Masuti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence and risk factors for non alcoholic fatty liver disease. The Dionysos nutrition and liver study. Hepatology, 2005; 42:44-52.
2. Browning, J.D., and Horton, J.D. Is the slippery slope from steatosis to steato hepatitis paved with triglyceride or cholesterol. J. Clin. Invest. 2004; (114): 147–152.
3. Dixon, J.B., Bhathal, P.S., Hughes, N.R., O'Brien, P.E., Nonalcoholic fatty liver disease: improvement in liver histological analysis with weight loss. Hepatology 2004; (39):1647–1654.
4. Farrell, G.C., and Larter, C.Z. Is the slippery slope from steatosis to steato hepatitis paved with triglyceride or cholesterol. Hepatology 2016; (43): S99–S112.
5. Federico A. Emerging drugs for Non-alcoholic fatty liver disease. Expert Opinion 2008; 13: 145-158.
6. Fujita, K., Yoneda, M., Wada, K., Mawatari, H., Takahashi, H., Kirikoshi, H., Inamori, M., Nozaki, Y., Maeyama, S., Saito, S., Iwasaki, T., Terauchi, Y., Nakajima, A.,Telmisartan, an angiotensin II type 1 receptor blocker, controls progress of nonalcoholic steatohepatitis in rats. Dig. Dis. Sci. 2007; 52:3455–3464.
7. Menaka C. Thounaojama, Ravirajsinh N. Jadejaa, Deven S. Dandekarb, Ranjitsinh V. Devkara, A.V. Ramachandrana. Sida rhomboidea. Roxb extract alleviates pathophysiological changes in experimental in vivo and in vitro models of high fat diet/fatty acid induced non-alcoholic steatohepatitis. Experimental and Toxicologic Pathology 2012; 64,217– 224.
8. Petta S. Non alcoholic fatty liver pathogenesis: The present and future. Dig Liver Dis 2009; (l.4): 615-25.
9. Shahid Ahmed, Syed Azhar Ahmed, Nadir Ali. Freqency of metabolic syndrome in Type 2 diabetes and its relationship with insulin resistance., J Ayub Med Coll Abbottabad 2010; (22) 1-7.
10. Shuqin Zheng, Lizbeth Hoos, John Cook, Glen Tetzloff, Harry Davis Jr.,Margaret van Heek, Joyce J. Hwa . Ezetimibe improves high fat and cholesterol diet-induced non-alcoholic fatty liver disease in mice European Journal of Pharmacology 2008; 584: 118–124.

11. Tumor necrosis factor (mouse), Biotrak ELISA system code: RPN2718; GE Amersham
12. Vishwanath M. Sardesai and Joan A. Manning. The determination of triglycerides in plasma and tissues. Clnical chemistry 1968; 14 (2) :156-161.
13. Vijay viswanathan, Mahesh Kadiri, Srikanth Medimpudi, Satyavani Kumpatla. Association of Non alcoholic fatty liver disease with diabetic microvascular and macrovascular complications in South Indian diabetic subjects. International journal of Diabetes in Developing countries 2010; 30 (4) : 208-212
14. Xu, P., Zhang, X., Li, Y., Yu, C., Xu, L., Xu, G. Research on the protection effect of pioglitazone for non-alcoholic fatty liver disease (NAFLD) in rats. J. Zhejiang Univ. Sci. B, 2008; 7: 627–633.
15. Yalniz,M., Bahcecioglu, I.H., Kuzu, N., Celebi, S., Ataseven, H., Ustundag, B., Ozercan, I.H., Sahin, K.. Amelioration of steatohepatitis with pentoxifylline in a novel non alcoholic steato hepatitis model induced by high-fat diet. Dig. Dis. Sci. 2007; 52: 2380–2386.
16. Yu Y.H. and Ginsberg, H.N. Is the slippery slope from steatosis to steato hepatitis paved with triglyceride or cholesterol. Circ. Res. 2005; 96: 1042–1052.