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EFFECT OF *TERMINALIA CHEBULA* FRUIT EXTRACT ON HIGH FAT DIET AND ETHANOL INDUCED INFLAMMATION IN PANCREAS

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ABSTRACT

The effect of hydro alcoholic fruit extract of *Terminalia chebula* (HAETC) on ethanol (EtOH) and high fat diet (HFD) induced changes in pancreas was investigated in rats. Male albino Wistar rats of Group1 and 2 served as control and received normal diet (5% fat). Group 3 and 4 rats were administered orally with EtOH and also fed HFD (25% fat) for 90 days. In addition, groups 2 and 4 rats received 100 mg/Kg body wt of HAETC for the last 60 days. We observed a significant decrease in the activity of serum lipase, amylase, pancreas level of caspase-1 and myeloperoxidase in rats co-administered with HAETC than in EtOH and HFD control rats. The HAETC co-administered rats showed a significant decrease in total cholesterol, triglyceride and LDL levels and maintained the antioxidant status in HFD-EtOH fed rats. Histopathology of liver and pancreas added more evidence for the anti-inflammatory property of *T.chebula*. The study revealed that *T.chebula* fruit extract significantly reduced the inflammation probably by modulating the activity of caspase-1 and maintaining the antioxidant status in pancreas.

KEYWORDS: Antioxidants, ethanol, high fat diet, inflammation, pancreas, *Terminalia chebula.*

INTRODUCTION

Pancreatitis is a heterogeneous and progressive inflammatory disease characterized by severe abdominal pain that occurs when the proteolytic enzymes which digest the food are

preactivated in pancreas itself. Pancreatitis affects 10-15% of world population.[1] Alcohol abuse and biliary tract disorders are the most important causes of pancreatitis.[2] Consumption of drugs azathioprine, sulfonamides and corticosteroids, infection with Mumps virus, Hepatitis virus, Rubellosis, Epstein-Barr virus and Cytomegalovirus are some of the factors predisposed to pancreatitis.[3] Pancreatitis may be acute and is characterized by acute necro-inflammation of the peri-pancreatic tissues which recovers after medication.[4] Chronic pancreatitis is characterized by irreversible destruction of pancreatic cells with progressive loss of both exocrine and endocrine functions.[5] The symptoms of pancreatitis are loss of appetite, vomiting and weight loss. Circulatory shock, cardiac insufficiency, renal and hepatic failure are the major causes of death at the end stages of pancreatitis.

The metabolites of ethanol are toxic to acinar cells and cause preactivation and abnormal secretion of hydrolytic enzymes which digest the own cells of pancreas followed by the production of proinflammatory cytokines. The cytokines IL-1B and IL-18 activated by caspase-1 which itself need to be activated by a multiprotein complex known as inflammosomes.[6]

Treatment is mostly supportive, and until the availability of clinically recommended drugs to reduce inflammation in pancreas is limited.[7] It has been proved that antioxidant supplements can reduce oxidative stress and the related complications of pancreatitis. Ayurvedic medicines are prepared from herbs and other natural products which are biocompatible without much side effects. Administration of medicinal herbs that possess anti-inflammatory and antioxidant properties is a new approach to attenuate inflammation related disorders such as pancreatitis.

Terminalia chebula Retz (Combretaceae), native to Southeast Asia is a popular medicinal plant used in Indian ayurvedic system of medicine.[8] The presence of phytonutrients gallic acid, ellagic acid, chebulagic acid, chebulanin, rutin, casuarinin and quercetin has been shown to possess antioxidant property. *T.Chebula* has been reported to exhibit anticancer, antidiabetic, antibacterial, gastroprotective, hepatoprotective and cardioprotective activities.[9-14]

Based on the traditional claims on *T.chebula*, the present study is planned to evaluate whether HAETC could modulate the inflammatory changes in the pancreas of experimental animals

treated with EtOH and HFD. In the present study, we used EtOH and HFD to induce pancreatitis in rats as HFD potentiates the toxic effects of ethanol on pancreas.

MATERIALS AND METHODS

Plant material and extraction procedure

The dried fruits of *T.Chebula* were purchased from local market at Chennai. The fruits were authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, Chennai (Voucher. No. PARC/2013/2102). The fruits of *T.chebula* were air dried and powdered using domestic grinder. The HAETC was prepared by soaking 100 gm of *T.chebula* fruit powder in 250 ml of 70% ethanol (v/v) for 3-5 days with intermediate shaking and filtered. The filtrate was evaporated to dryness using rotary evaporator. The concentrate was lyophilized and used for further study.

Animals and diet composition

Male albino Wistar rats weighing 175-200 g were kept in air-conditioned room where temperature and artificial light were controlled (20º C, 24 h circadian cycle: 12 h light, 12 h dark). Rats were housed in polypropylene cages with free access to drinking water and food. They were fed standard rat chow obtained from M/S: Provimi Animal Nutrition India Pvt Ltd., Bangalore, India. Animals were fed HFD which consisted of 23.4% wheat, 23.4% yellow corn, 19.5% milk powder, 15.6% crude coconut oil, 15.6% pork lard, 1% calcium chloride, 0.5% Vitamin B_{12} and 1% common salt. The energy given by the normal diet is 3.43 Kcal/g and the HFD along with EtOH is 5.24 Kcal/g.

Experimental protocol

After 7 days of acclimatization period, the rats were divided into 4 groups. Group 1 and 2 rats were fed standard rat chow for 14 weeks. Group 3 and 4 rats were fed HFD containing 25% fat and administered 1.1 ml of 20% EtOH per 100 g of body wt daily for the period of 90 days by intra gastric intubation. In addition, group 4 rats were co-administered orally with 100 mg/ kg body wt of HAETC for the last 60 days of the experimental period. The study protocol was approved by the Institutional Animal Ethics Committee (XIII/VELS/COL/24A/CPCSEA/IAEC/23.9.11).

Sample collection

After 90 days, rats were fasted overnight and anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/Kg body weight) and killed by cervical decapitation. Blood

was collected and serum separated was stored at 4ºC until analysis. A portion of liver and pancreas were removed quickly and fixed in 10% formaldehyde for histological examination.

Tissue homogenate preparation

Immediately after the animal sacrifice, pancreas was removed and washed in ice cold saline and kept at -20 ºC. It was homogenized in 0.1 M Tris HCl buffer, pH 7.4 and centrifuged at low speed to remove any cell debris. The supernatant was used for the determination of myeloperoxidase, reduced glutathione (GSH), lipid peroxides (TBARS) and caspase-1 activity.

Biochemical investigations

Determination of serum amylase activity

Method of Gomori was used to determine the activity of amylase.[15] The method was based on the activity of enzyme on substrate starch by lugol's Iodine solution. The color intensity was measured spectrophotometrically at 640nm and the enzyme activity was expressed as IU/L.

Determination of serum lipase activity

The activity of serum lipase was measured by the method of Lowry and Tinsely.[16] The serum was added in 25ml olive oil/ triton X 100 emulsion as substrate to initiate the lipolysis reaction. Sub samples were taken at predetermined time intervals and used for the assay of liberated free fatty acids spectrophotometrically at 715nm. The activity was expressed as IU/L.

Assay of serum transaminase

Both serum Alanine transaminase (ALT) and Aspartate transaminase (AST) were determined using King's method.[17]

Determination of Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined in aliquots of pancreatic tissue homogenate according to the method of Bradley.[18] The enzyme activity was expressed as units/mg protein.

Estimation of blood lipids

Zak's method was used to determine total cholesterol level (TC).[19] Triglyceride (TG) was estimated by the method of Van Handel and Zilversmit.[20] HDL cholesterol was measured

following the precipitation with heparin and manganese chloride.[21] The dual precipitation method was used for LDL cholesterol estimation.[22] The plasma was incubated with sodium dodecyl sulfate for 2 h and then centrifuged at 10,000g for 10 min at 4˚C. The supernatant contains a mixture of HDL and LDL was used to determine cholesterol level.

Determination of oxidative stress markers

The level of lipid peroxides in pancreas was determined by measuring thiobarbituric acid-reacting substances (TBARS).[23] The value was expressed as nmol/100mg tissue protein. Glutathione (GSH) was determined by the method of Moron, and expressed as mg/g protein.[24] Aliquots of tissue homogenate were mixed with equal volume of ice cold 5% TCA and the precipitated proteins were removed by centrifugation. The supernatant was used for the estimation.

Determination of Caspase-1 activity

Caspase-1 activity was measured according to the method of Thornberry.[25] Briefly the pancreas was homogenized in a lysis buffer (25 mM HEPES (pH 7.5), 1 mM EDTA, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml, 2 mM dithiothreitol) at 5 ml/100 mg of pancreas tissue. Extracts were centrifuged at 15,000 g for 30 min at 4°C, and the supernatant was centrifuged again at 2,00,000 g for 1h at 4°C. The cytosol was used for caspase-1 activity measurements. According to the kit manufacturer instruction (AD20470), the assay was performed. Reactions with enzyme preparation alone, with enzyme mixed with caspase-1 substrate (Ac-YVAD-pNA) or inhibitor (Ac-YVAD-CHO) and with substrate alone were also run as controls. A recombinant caspase-1 enzyme was used as a positive control. The activity was measured by proteolytic cleavage of Ac-YVAD-pNA for 4h at 37°C. The plates were read at 405nm.

Histopathological study

Liver and pancreas samples were fixed in 10% neutral buffered formalin for 24 h. Ultra-thin sections of the tissues were cut from embedded tissue blocks. The sections were then stained with hematoxylin–eosin and observed under light microscope.

Statistical analysis

Data were analyzed by using commercially available statistics software package (SPSS Version 10.0 for Windows). The statistically significant variation among different groups was

determined by applying one-way ANOVA with *post hoc* Bonferroni test and the p value<0.05 was considered significant.

RESULTS AND DISCUSSION

Prolonged ingestion of large amount of alcohol is a major risk factor for the development of chronic pancreatitis.[26] The pancreas metabolizes ethanol both by oxidative and non-oxidative pathways and produces acetaldehyde and fatty acid ethyl esters (FAEE) respectively.[27] Acetaldehyde and FAEEs induce premature release of pancreatic digestive enzymes and destabilize intracellular membranes, which predispose the gland to auto digestion. Alcohol ingestion alone may or may not induce pancreatitis in experimental animals and hence HFD is needed as an additional factor to induce pancreatitis.

Effect of HAETC on pancreatic marker enzymes

Table 1 illustrates the effect of HAETC treatment on the activity of serum amylase and lipase. The serum amylase and lipase levels indicate the functional status of pancreas.[28] Abnormal synthesis and exocytosis of these enzymes from the acinar cells leads to elevated levels in the circulation. EtOH and HFD fed rats showed a significant increase in the serum activity of amylase and lipase. Apte *et al* reported that mRNA and protein level expression of digestive enzymes are increased in alcohol administered rats.[29] The results show that HAETC possess significant protective activity against pancreatic injury caused by EtOH and HFD.

Effect of HAETC on liver marker enzymes

Elevated levels of transaminase are reported not only in liver diseases but also in pancreatitis. EtOH and HFD fed animals showed significant elevation in the level of AST and ALT (Table 1) showing the changes produced in liver. The *T.Chebula* fruit extract significantly reduced these changes. Ethanol is metabolized not only in liver but also in pancreas and the acetaldehyde thus produced further metabolized to form reactive oxygen species which could cause tissue injury. HAETC was found to decrease the levels of these marker enzymes showing the beneficial role of HAETC in treating pancreatitis.

Effect of HAETC on pancreatic myeloperoxidase activity

Myeloperoxidase is the marker of tissue inflammation and expressed in activated neutrophils which invade the site of injury.[30] Myeloperoxidase kills bacteria and pathogens by producing cytotoxic hypochlorous acid from hydrogen peroxide and chloride ion during

neutrophils respiratory burst.[31] We found a significant increase in pancreatic activity of myeloperoxidase in rats received EtOH and HFD (Table 1). The enzyme activity was significantly decreased due to HAETC co-administration showing the anti-inflammatory property of the test drug.

Table 1: Activity levels of serum amylase, lipase, transaminases and pancreatic myeloperoxidase (MPO) in experimental animals.

Groups	Amylase (IU/L)	Lipase (IU/L)	AST (IU/L)	ALT (IU/L)	MPO (Units/mg Protein)
Control	887 ± 91.4	32 ± 4.3	187± 25.1	56 ± 7.1	2.01 ± 0.24
HAETC control	813 ± 92.7^{NS}	30 ± 4.2^{NS}	183± 23.6^{NS}	52 ± 7.0^{NS}	1.96 ± 0.25 ^{NS}
EtOH+ HFD	1732 ± 188.8*	69 ± 7.6*	297 ± 42.2*	72 ± 9.4$^{\#}$	3.96 ± 0.41*
EtOH+ HFD+HAETC (100mg/Kg body wt)	956 ± 115.7*	47 ± 5.0*	220 ± 23.5*	47 ± 5.5*	2.34 ± 0.24*

Values are expressed as mean ± SD for six animals in each group. Control vs HAETC control, Control vs EtOH + HFD, EtOH + HFD vs EtOH + HFD + HAETC. P values:* = 0.000, # = 0.001, NS-Non significant.

Effect of HAETC on lipid profile

The serum lipids were found increased in EtOH & HFD fed rats than in HAETC co-administered rats (Table 2). *T.chebula* possesses saponins, tannins, flavonoids and terpenoids. It has been reported that saponins and tannins decrease cholesterol absorption and increase its excretion through bile.[32] Flavonoids have been reported to have anti-hyperlipidemic properties. HAETC lowers serum cholesterol and triglyceride levels probably by altering synthesis, degradation and elimination of cholesterol. This shows that HAETC has a potent hypolipidemic effect.

Table 2: Effect of HAETC on blood lipid profile in experimental animals

Groups	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL)
Control	98± 12.2	113± 15.6	32± 3.8	22 ± 2.2
HAETC control	96± 13.1^{NS}	109± 13.7^{NS}	35± 4.9^{NS}	20 ± 2.4^{NS}
EtOH+ HFD	187 ± 21.5*	172± 18.1*	17± 2.2*	63 ± 8.6*
EtOH+ HFD+ HAETC (100mg/Kg body wt)	125± 12.8*	137± 19.6$^{@}$	26± 3.5*	46 ± 6.7*

Values are expressed as mean ± SD for six animals in each group. Control vs HAETC control, Control vs EtOH + HFD, EtOH + HFD vs EtOH + HFD + HAETC. P values:* = 0.000, @ = 0.000, NS-Non significant.

Effect of HAETC on lipid peroxidation

The level of lipid peroxidation products in pancreas was given in Table 3. TBARS concentration in pancreas was found to be increased in EtOH and HFD fed rats than in HAETC co-administered rats. Oxygen and nitrogen derived free radicals (ROS) and lipid peroxidation play an important role in the development of local inflammation and systemic complications during pancreatitis.[33, 34] Various natural antioxidants present in *T. chebula* might be accounted for the TBARS reducing effect of HAETC.

Effect of HAETC on reduced glutathione

The level of reduced glutathione (GSH) in pancreas of experimental animals was given in Table 3. Glutathione is a prime antioxidant that protects cells from the harmful effects of ROS.[35] EtOH and HFD received rats showed significant decrease in the level of GSH when compared to HAETC co-administered rats. This implies that the HAETC decreases the level of ROS concentration through its antioxidant activity.

Effect of HAETC on caspase-1 activity

Table 3 shows the level of caspase-1 in pancreas of experimental animals. The activity of caspase-1 was found to be increased in EtOH and HFD fed rats when compared to control rats. Caspase-1 activity was significantly decreased in HAETC co-administered rats.Caspase-1 is a cysteine protease that proteolytically cleaves the precursor forms of proinflammatory cytokines into active mature peptides.[36] Caspase-1 itself needs activation by inflammosomes, a protein platform containing different domains such as CARD and PYD. ASC is a specific procaspase-1 binding partner and its interaction with procaspase-1 induces activation and production of proIL-18 and pro IL-1B and secretion of active IL-18 and IL-1B.The elevated caspase-1 activity in EtOH and HFD fed rats supports the role of caspase-1 in inflammatory changes induced by EtOH and HFD. The anti-inflammatory nature of HAETC is evidenced by the reduced activity of caspase-1 in HAETC co-treated rats.

Table 3: Levels of pancreatic TBARS, reduced Glutathione and caspase-1 activity in experimental animals.

Groups	TBARS	GSH	Caspase-1

	(nM/100mg protein)	(mg/g protein)	(pg/mg protein)
Control	3.45±10.6	13.2±14	10.2±12
HAETC control	2.36±11.9 @	13.9±12.7 NS	9.6±11 NS
EtOH+ HFD	6.42±13.7 *	5.28±11.8 *	80±11.5 *
EtOH+ HFD+ HAETC (100mg/Kg body wt)	3.15±14.2 *	12.4±13.3 $	19±13.1 *

Values are expressed as mean ± SD for six animals in each group. Control vs HAETC control, Control vs EtOH + HFD, EtOH + HFD vs EtOH + HFD + HAETC. P values: NS-non significant, @=0.002, *=0.000, $=0.007.

Effect of HAETC on the histology of liver and pancreas

The histopathology of liver and pancreas of control and experimental rats were shown in Figure 1& 2 respectively. The ultra sections of liver and pancreas of control and drug control rats showed normal architecture. EtOH and HFD fed rats showed significant inflammation, necrosis and extensive fibrosis. HAETC co-administration showed significant reduction in inflammation and necrotic changes.

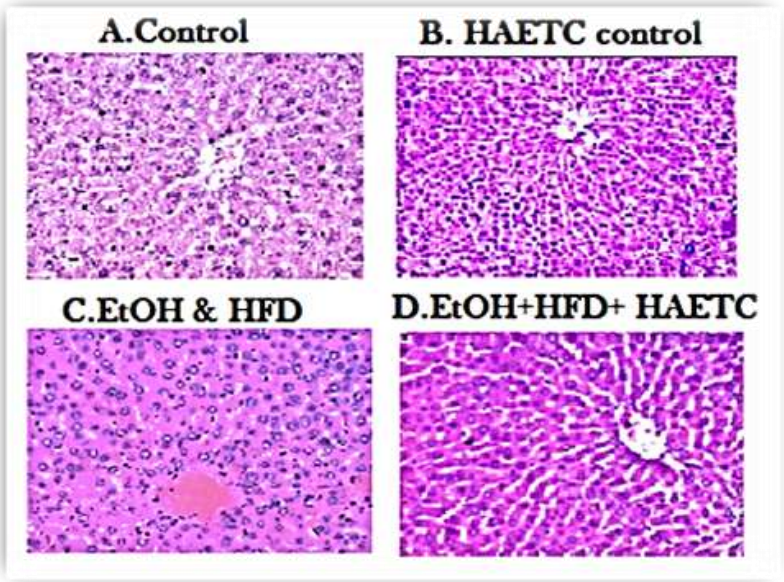

Figure 1. Histopathology of liver. Photomicrograph of liver- Normal tissue architecture in control (A) and No significant pathological alteration in HAETC control rats (B). A high degree of inflammation in EtOH & HFD rats (C). Very mild inflammation in EtOH+HFD and HAETC treated rats (D). (H&E 100x).

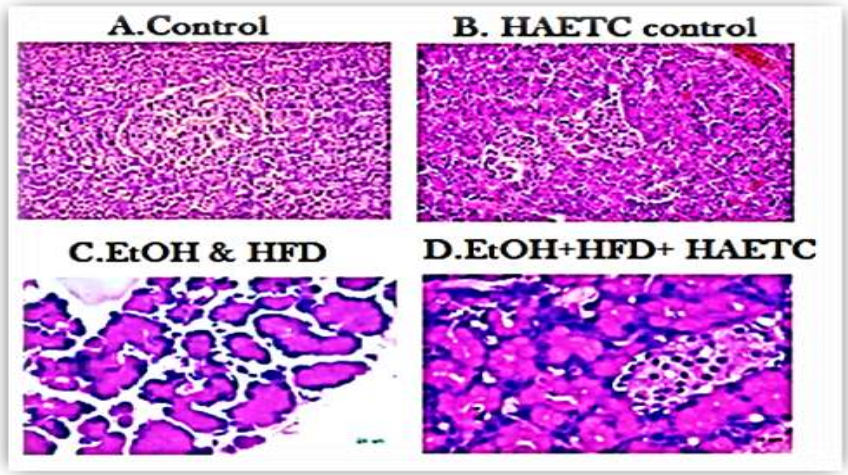

Figure 2. Histopathology of pancreas. Photomicrograph of pancreas- Normal tissue architecture in control (A) and No significant pathological alteration in HAETC control rats (B). A significant inflammation, necrosis and extensive fibrosis in EtOH & HFD rats (C). Very mild inflammation and necrosis in HFD and HAETC treated rats (D). (H&E 100x).

CONCLUSION

The present investigation demonstrates the pancreato protective effect of HAETC in rats administered with EtOH and HFD. HAETC modulates the abnormal changes induced by simultaneous administration of EtOH and HFD probably by modulating the activity of caspase-1 and also by maintaining the antioxidant status. However, further study on the effect of HAETC on the activity of pro-inflammatory cytokines is needed to evaluate the mechanism of action of *T. chebula.*

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