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Hepatoprotective and hypoglycemic effect of lactic acid fermented Indian Gooseberry-Amla beverage on chronic alcohol-induced liver damage and diabetes in rats

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ABSTRACT

Background: Plant-based fermented foods rich in lactic acid bacterial metabolites, antioxidants, and phytochemicals, can promote recovery from ethanol-induced liver damage by restoring liver antioxidant levels and suppressing liver inflammation, and improving certain metabolic disorders such as diabetes mellitus.
Methods: In the present study, the protective effects of nutraceutical-enriched lactic acid-fermented Amla beverage on chronic alcohol-induced biochemical modulations and diabetes in Wistar rats were investigated.
Results: The hepatoprotective studies showed that the fermented beverage was able to reverse the damage caused to the liver with ethanol administration in terms of liver index, liver enzymes (AST, ALT), serum enzymes (g-GT), and serum TG, TCH, hepatic TG, LPO levels, antioxidants (GSH, TSOD, CAT, GSH-Px). Along similar lines, in the hypoglycemic studies, the fermented beverage evidently improved body weight, and fasting blood glucose levels, reducing fasting HbAlc levels, improving C-peptide and GLP-1 levels, and alleviating renal dysfunction and lipid metabolism compared with diabetic rats. All these outcomes were supported by histological observations within the liver and pancreas.
Conclusion: The present study suggests that the consumption of fermented Amla beverage may have a protective effect against chronic alcohol-induced toxicity and diabetes. The effects of fermented Amla beverage may be attributed to antioxidant activity, flavonoids, bioactive compounds produced by LAB and their metabolites, which help to counteract free radicals induced by ethanol and in reducing glucagon levels, enhancing glucose utilization, leading to a decrease in blood glucose. The results show that fermented Amla beverage has positive effects in reducing the detrimental effect of alcohol and diabetes.

Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
g-GT	gamma-glutamyl transferase
LPO	Lipid peroxidation
Liv 52	Liver 52
TG	Triglycerides
TCH	Total cholesterol
GSH	Glutathione
TSOD	Total superoxide dismutase
CAT	Catalase
GSH-Px	Glutathione peroxidase
ROS	Reactive oxygen species
ASH	Alcoholic steatohepatitis
AC	Alcoholic cirrhosis
LAB	Lactic Acid Bacteria
KMS	Potassium meta-bisulphite
HDL	High density lipoproteins, LDL- low density lipoproteins
MDA	Hepatic malondialdehyde
FAB	Fermented alcoholic beverage
NFAB	Non-fermented alcoholic beverage
HMG-CoA reductase	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
HbAlc	glycated hemoglobin
GLP-1	glucagon-like peptide-1

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1. Introduction

Recent lifestyle changes and economic growth have led to the production of many high-fat, high-sugar foods, and increased alcohol consumption due to the pressures of modern life and work. This trend has resulted in a global rise in the incidence of diabetes and hyperglycemia (Duncan et al., 1983). Excessive alcohol consumption causes oxidative stress, which triggers the generation of excessive cellular reactive oxygen species (ROS), leading to liver damage (Galicia-Moreno & Gutiérrez-Reyes, 2014) and can progress to alcoholic cirrhosis (AC), and ultimately resulting in liver necrosis or liver cancer (Guo et al., 2020). Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and disruptions in the metabolism of carbohydrates, fats, and proteins due to defects in insulin secretion, insulin action, or both. Controlling blood glucose effectively is crucial for preventing or reversing diabetic complications and enhancing the quality of life for patients with T2DM (Mirmiran et al., 2014). Unfortunately, current drug therapies for these conditions are costly and have potentially toxic side effects. As a result, the development of functional foods with hepatoprotective and hypoglycemic properties has become an area of recent focus.

In recent times, there is increasing evidence that functional foods and their bioactive compounds can reduce oxidative stress, and alcohol-induced liver disease and can serve as complementary treatments (in combination with conventional drugs) for T2DM (Mirmiran et al., 2014). Studies have revealed that alcohol-related liver disease is linked with alterations in the gut microbiota. Long-term alcohol consumption in the intestinal tract also causes a decrease in functional probiotics and an overgrowth of gram-negative bacteria, which produce endotoxins in the intestinal contents, leading to liver damage (Ran et al., 2021). Pure functional LAB-based lactic acid fermentation can be used to enhance the bioactivity and bioavailability of phytochemicals in plant matrices, while also enriching them with functional bacterial metabolites (Filannino et al., 2013). Lactic acid fermentation plays an important role in the production and preservation of food hydrocolloids and enhances food texture and mouthfeel, with extended shelf life, enhanced flavors, and functional properties providing health benefits and preserving traditional food practices.

Amla, also known as Indian gooseberry (*Emblica officinalis)* is a subtropical fruit belonging to the spurge family and is highly valued for its nutritional and medicinal properties, thanks to its high content of ascorbic acid and total phenols. Amla is particularly rich in phenolics, such as quercetin, β-sitosterol, stigmasterol, and carotenoids, which have been found to have antioxidant, anticarcinogenic, and antimutagenic properties, and can provide defensive functions against cardiovascular diseases (Variya et al., 2016). According to (Dimidi et al., 2019), fermented foods containing lactic acid bacterial metabolites have demonstrated benefits for gastrointestinal health, as well as the potential to improve certain metabolic disorders like diabetes mellitus. Studies have shown that the natural antioxidants, phenols, and flavonoids in fruits and vegetables along with the probiotic lactic acid bacteria can protect liver cells against free radical damage and replenish liver antioxidants caused by ethanol consumption (Zulkawi et al., 2017). Fermented Amla beverage can be utilized to investigate the hepatoprotective and hypo-glycemic properties in alcohol-induced liver-damaged and diabetic rats, respectively. This study assessed the effectiveness of fermented Amla beverages in mitigating chronic alcohol-induced liver damage and hyperglycemia. The findings of this study will establish the potential commercial application of fermented Amla beverages.

2. Material and methods

2.1. Plant material

Amla (Indian gooseberry) [*Emblica officinalis* var. NS6], guava cultivar (*Psidium guajava* var. Punjab Safeda), and ginger (*Zingiber officinale* var. IISR Mahima), were obtained from the Department of Fruit and Vegetable Science, Punjab Agricultural University (PAU), Ludhiana, India. Fully ripe fruits were manually harvested and sorted, and damaged fruits were discarded. The plant materials were washed according to standard protocol (Pandove et al., 2016) by rinsing with sodium hypochlorite solution (0.02%) and then washing with sterile KMS water to remove surface microbes. The materials were stored at -20°C before beverage formulation.

2.2. Beverage formulation and fermentation

Amla beverage was prepared by optimizing the bioprocess parameters using Response Surface Methodology. The optimized parameters included Amla mixture (Amla juice: guava juice: ginger juice at a ratio of 1:1:1.5% v/v), dilution ratio (1:3 with boiled chilled water), spice (salt) concentration (0.6%) for osmotically stable decoction, pasteurization at 82°C for 10-15 seconds, and controlled fermentation at 37°C for 28 hours using a pre-activated (5% v/v) consortia of four LAB1 (*Pediococcus lolii* MH752471), LAB2 (*Lactobacillus plantarum*), LAB3 (*P. acidilactici* strain 5560) and LAB10 (*P. pentosaceous* strain L16) potential lactic acid bacterial strains as a functional starter culture (3.8×10^7 log CFU/ml) for controlled fermentation at 37°C for 28 h.

The lactic acid content, phytochemicals (polyphenols and flavonoids), and free radical scavenging ability (FRAP and DPPH) of the fermented Amla beverage administered to rats are presented in Supplementary data. The conventional methods were employed to determine the total content of phenols and flavonoids. The antioxidant activity was determined as %SA and μM $FeSO_4$ equivalents using the DPPH-SA and FRAP assay, respectively.

2.3. Animal model and treatments

2.3.1. Hepatoprotective activity

Wistar rats weighing 70-100 g were procured from ISF College of Pharmacy, Moga, Punjab, India [Reg.No. No. 816/PO/a/04/CPCSEA]. The rats were kept under standard laboratory conditions at 23°C with 12/12 h light/dark cycles and provided ad libitum, free access to commercial rat food (10 % kcal fat, 30 % kcal proteins, 60 % kcal saccharides) and drinking water for one week prior to the experiment. The study was approved by the Ethics Committee of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India (IAEC approval number: *GADVASU/2020/IAEC/56/14)*. All the experiments were conducted in accordance with the animal care guidelines of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India (Xiang et al., 2012). The study reported is also conducted in accordance to the ARRIVE guidelines. The model of acute alcoholic-induced liver damage was established following the Reference Health Food Inspection and Evaluation Specifications (2003 Edition) guidelines and the method described by (Guo et al., 2020) The rats were randomly assigned to five groups of six each as follows:

1 Normal control group: provided with food and distilled water only,
2 The second group received a daily single oral dose of ethanol (10 ml/kg) for six weeks (Guo et al., 2020).
3 The third, fourth, and fifth groups received standard drug Liv52, non-fermented Amla beverage, and fermented Amla beverage, respectively, at a dose of 10 ml/kg body weight, 2 hours before the administration of ethanol (50% v/v, 10 ml/kg) for six weeks.

2.3.2. Determination of rat liver index

Six hours after the final ethanol challenge, the body weight of each rat was measured. The liver was removed, and its wet weight was weighed. The liver index value was calculated using the following formula: Liver index (%) = Liver weight/ body weight ×100

2.3.3. Liver marker enzymes, TCH, TG and LDLs levels in serum

Liver marker enzymes aspartate and alanine aminotransferase (AST and ALT) were measure by using commercial kits from Sigma Aldrich with a spectrophotometer (UV-2550). Serum TCH (Total cholesterol) and TG (triglycerides) were determined by the commercial assay kits from Sigma Aldrich with a semiautomatic biochemistry analyser (Leidu, RT-9200;USA). The concentration was expressed as $mmolL^{-1}$ serum. Assay kits were employed to detect the levels of TG, glutathione (GSH), malondialdehyde (MDA) as well as the enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in rat liver tissue homogenate, as described by (Guo et al., 2020).

Samples of alcohol and beverages were administered via nasogastric tube. The body weight of rats was weighed and recorded every three days during feeding and the amount of gastric perfusion was adjusted. On the last day, after six hours of ethanol exposure, the rats were sacrificed, and a serum sample was collected for the analysis of ALT, AST, TCH, TG, and LDLs. The livers were rapidly dissected and stored at -80°C until analysis, as per the guidelines for the care and use of animals published by the National Institutes of Health, USA.

2.4. Hypoglycemic activity

The hypoglycemic effect of the fermented beverage was assessed based on the study conducted by (Nayak et al., 2011), whereby the rats were randomly assigned to five groups of six animals each, as follows:

a) Normal control group: provided with food and water only,
b) Diabetic untreated group: provided with food and water only,
c) Diabetic standard group: provided with 2.0 mg/kg/day glibenclamide orally,
d) Diabetic experimental group: provided with 2 ml/kg non-fermented Amla beverage orally from the day of diabetes induction,
e) Diabetic experimental group: provided with 2 ml/kg fermented Amla beverage orally.

All the groups were allowed ad libitum access to food and water. The assessment included measurements of fasting blood glucose, body mass, and the degree of liver degeneration after six weeks. Fasting blood glucose levels of the animals were assessed using the glucometer, which employed the glucose oxidase/peroxidase reaction. Blood for glucose estimation was obtained from the tail veins of the rats.

2.5. Induction of diabetes mellitus

To induce diabetes, adult Wistar rats were fasted overnight and administered 50 mg/kg of Streptozotocin in cold citrate buffer with a pH of 4.5 via intraperitoneal injection. Three days after the injection, rats with a fasting blood glucose level exceeding 200 mg were deemed to be diabetic.

2.6. Measurement of fasting blood glucose, body weight, and blood analysis

During the experimental period, fasting blood glucose (FBG) and body weight were regularly monitored once a week. The FBG measurement was taken using a one-touch glucose auto-analyzer after 12 hours of fasting. The measurement was taken between 8:30 A.M. and 9:30 A.M. from the tail vein, using the second drop of blood after cleaning the tail vein with an ethyl alcohol cotton swab and removing the first drop of blood. Blood was also drawn from the abdominal aorta and allowed to coagulate on ice and subjected to centrifugation at 3500 rpm for 10 minutes at 4°C. The resulting serum and plasma aliquots were stored at -80°C for subsequent analysis. ELISA kits were used to test for glycated hemoglobin (HbAlc), C-peptide, and GLP-1, following the manufacturer's recommendations.

2.7. Histopathological study

Each treatment group was subjected to the excision of a pancreatic tissue section and rat liver section from the left lobe, which was then immediately fixed in a 10% neutral buffered formaldehyde solution (Xiang et al., 2012). After fixation, serial sections of 5 μm were obtained by embedding tissues in paraffin. These sections were then subjected to Hematoxylin-Eosin (H&E) staining and the samples were photographed using a microscope.

2.8. Statistical analysis

The data is presented as mean ± standard deviation (SD). Statistical analysis was conducted using Origin Pro 2021 and SPSS (version 16.0, SAS Institute Inc., USA). One-way ANOVA was used to determine the significance of differences, and Tukey's post hoc test was used for pairwise comparisons. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. The effect of fermented Amla beverage on animal growth performance and liver index

The study aimed to assess the protective effects of lactic acid fermented Amla beverage against chronic alcohol-induced liver damage in rats. Table 1 presents a comparison of the initial and final body weights, liver weights, and liver index of control and experimental rats. The experimental groups' body weights did not differ significantly. However, chronic ethanol administration caused a significant increase in liver weight and liver index compared to the control group ($p<0.01$), indicating alcohol-induced fatty liver. Administration of Liv 52, NFAB, and FAB (10 ml/kg) with alcohol significantly reduced liver weight by 1.25%, 1.21%, and 1.24%, respectively ($p<0.01$), and liver index decreased 1.23%, 1.19%, and 1.23%, respectively ($p<0.05$) compared to ethanol-treated rats. This suggests that functional lactic acid fermented Amla beverage can be an effective alternative to the hepatoprotective drug Liv52 for restoring liver function by increasing antioxidant levels. Flavonoids of the fermented beverage because of their strong antioxidant properties can scavenge free radicals and reactive oxygen species that cause oxidative stress and damage liver cells. Oxidative stress is implicated in the development and progression of various liver diseases, including non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, and viral hepatitis. By reducing oxidative stress, flavonoids can help protect liver cells from damage and inflammation. Many liver diseases involve inflammation as a central

Table 1
Effect of non-fermented and lactic acid fermented Amla beverage on the body weight, liver weight and liver index.

Groups	Body weight (g)			
	Initial	Final	Liver weight (g)	Liver index (×100)
Control	69.83 ±8.90	85.83 ±8.91	2.22±0.01^{c}	2.62±0.27 ^{b}
Ethanol	70.66 ±10.55	86.74 ±10.55	2.72±0.04 ^{a*}	3.17±0.41 ^{a*}
Ethanol + Liv52	71.00 ±7.01	85.00 ±7.01	2.16±0.04 $^{d\#}$	2.56±0.18 $^{b\#}$
Ethanol+NFAB	70.00 ±8.09	84.18 ±3.64	2.23±0.03 $^{b\#}$	2.65±0.14 $^{b\#}$
Ethanol+FAB	70.51 ±9.41	85.50 ±6.86	2.18±0.04 $^{d\#}$	2.57±0.25 $^{b\#}$

Values are mean ± SD (n=6).
* Indicate significant difference $p\leq0.05$ compared with control group.
$^{\#}$ Indicate significant difference $p\leq0.05$ compared with ethanol group.
Liv52: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

component of the disease process. Flavonoids have been shown to possess anti-inflammatory properties by modulating various inflammatory pathways and reducing the production of pro-inflammatory cytokines. By suppressing inflammation, flavonoids can potentially mitigate liver injury and contribute to liver health (Guo et al., 2020). The gut microbiota plays a crucial role in liver health through the gut-liver axis, which involves the bidirectional communication between the gut and the liver. By promoting a balanced gut microbiota, and metabolites such as short-chain fatty acids (SCFAs) and other bioactive compounds through fermentation of dietary fibers. functional LAB can indirectly impact liver function and reduce the risk of liver diseases (Sun et al., 2022). Similar results were obtained regarding the protective effect of anthocyanin-rich black rice extract on chronic ethanol-induced hepatomegaly in male or female Wistar rats (Hou et al., 2010).

3.2. The effect of fermented Amla beverage on the rat liver function

Chronic alcohol consumption can lead to liver damage, as reflected by elevated levels of the transaminase enzymes AST and ALT in the serum. The liver metabolizes most of the consumed alcohol through enzymatic activity, but excessive consumption can cause AST and ALT to leak into the bloodstream (Kasdallah-Grissa et al., 2007), serving as early indicators of liver damage (Fig. 1). However, co-administration of Liv52 (a standard drug), NFAB, and FAB with ethanol led to reductions of these markers by 1.3%, 1.13%, and 1.21% in AST, and by 1.59%, 1.45%, and 1.51% in ALT, respectively. These findings suggest that both the fermented and unfermented Amla beverage have hepatoprotective effects.

Excessive alcohol consumption leads to the formation of liver fat. Organic acids have been found to promote appetite, inhibit glycolysis, and improve fatty acid utilization by reducing hepatic HMG-CoA reductase levels, which are responsible for cholesterol synthesis. Due to its organic acidity and higher levels of polyphenols and flavonoids from lactic acid fermentation, the fermented Amla beverage has more profound effects on serum TCH and TG and liver TG. Oxidative stress disrupts the balance between ROS production and antioxidant defenses, causing tissue damage that leads to various pathological conditions. The high levels of polyphenols and flavonoids in beverages may explain their effects on serum TCH and TG, as well as hepatic TG.

In addition, FAB was found to reduce serum levels of TCH and TG, as well as hepatic TG levels. Fig. 2 depicts the serum levels of triglycerides and total cholesterol, while Fig. 3(a) presents the hepatic TG levels. Chronic alcohol consumption can lead to abnormal lipid metabolism, resulting in increased triglyceride synthesis, reduced lipoprotein synthesis and secretion, and decreased fat transport. Ethanol intake caused a significant increase in serum TG, TCH, hepatic TG, HDL, and LDL levels ($p<0.01$). Both unfermented and fermented Amla beverages mitigated these adverse effects and restored lipid metabolism. Co-administration of Liv52, NFAB, and FAB (maximum dose) significantly reduced serum TG levels ($p<0.05$) compared to ethanol-treated rats. Liv52 and FAB treatments also significantly decreased TCH levels ($p<0.05$). The liver generates excess ROS through alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and alcohol metabolism, overwhelming the body's antioxidant defenses and resulting in oxidative stress and cell damage (Guo, Mao et al. 2020). Amla beverage's protective effects against chronic alcohol-induced damage were evaluated by measuring GSH, SOD, CAT, GSH-Px, and MDA (lipid peroxidation).

Glutathione (GSH), one of the most abundant intracellular thiol-based antioxidants, plays a crucial role in maintaining cell integrity by neutralizing or reducing ROS (Nordberg & Arnér, 2001). Chronic ethanol consumption has been reported to reduce the rate of glutathione synthesis in the liver, leading to chronic ethanol toxicity (Loguercio et al., 1997). In the present study, as shown in Fig. 3(b), rats treated with chronic ethanol had a 32% reduction in hepatic GSH content ($p<0.01$). However, fermented Amla beverage (10 ml/kg) significantly ($p<0.01$) restored rat liver GSH levels, which were comparable to those restored by the standard drug Liv52.

Additionally, the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) work together to defend against ROS (Cederbaum et al., 2009). Chronic alcohol consumption has been shown to decrease T-SOD activity ($p<0.01$), which has also been reported (Hou et al., 2010; Polavarapu et al., 1998). However, in the present study, the T-SOD activity in rats was significantly improved ($p<0.01$) with the consumption of fermented Amla beverage (10 ml/kg) (Table 2).

Furthermore, glutathione peroxidase (GSH-Px), besides reducing hydrogen peroxide to water, is also responsible for converting lipid hydroperoxides into their corresponding alcohols. Thus, this enzyme is essential in protecting tissues from oxidative damage due to lipid peroxidation (Mallikarjuna et al., 2007). During chronic ethanol exposure, GSH-Px activity in liver cells is significantly reduced ($p<0.01$), leading to disruption of glutathione homeostasis and ultimately damaging the liver. Depletion of its co-substrate (GSH) or inactivation by free radicals during chronic ethanol exposure could be the reason for reduced GSH-Px activity. Decreased T-SOD activity may also contribute to the reduced activity of GSH-Px, given their interrelationship in detoxifying toxic

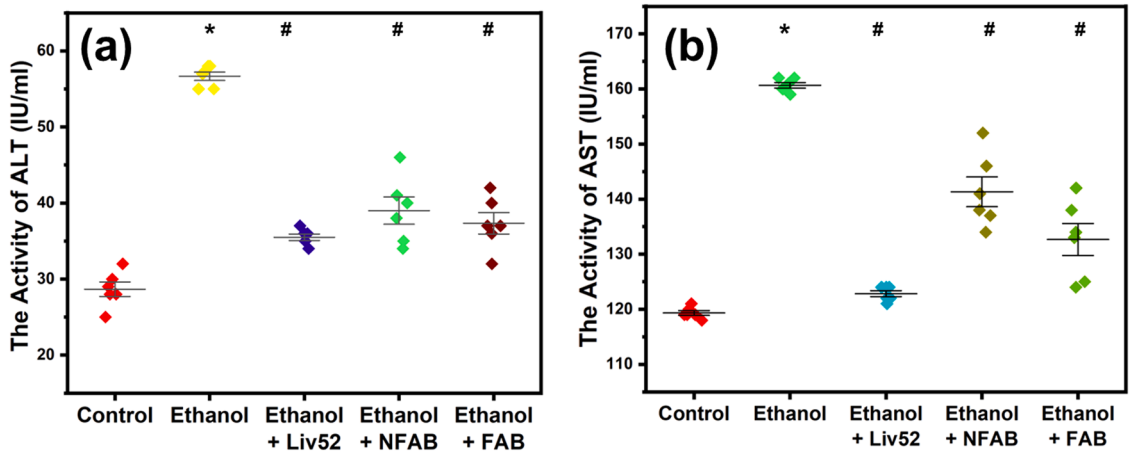

Fig. 1. Effect of non-fermented and lactic acid fermented Amla beverage on the serum (a) ALT and (b) AST.
Values are mean ± SD (n = 6).
* Indicate significant difference $p\leq0.05$compared with control group.
# Indicate significant difference $p\leq0.05$ compared with ethanol group.
Liv52: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

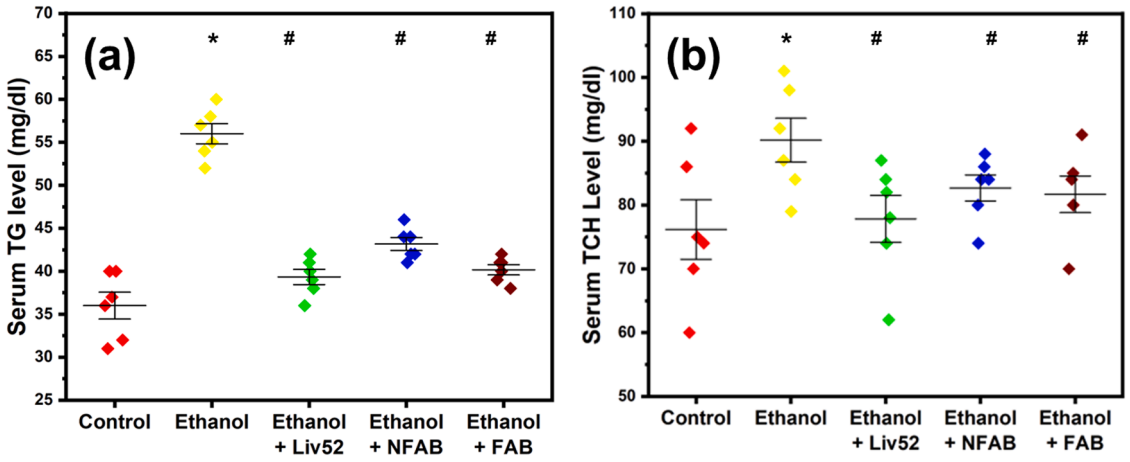

Fig. 2. Effect of non-fermented and lactic acid fermented *Amla* beverage on the serum (a) TG and (b) TCH levels.
Values are mean ± SD (n=6).
* Indicate significant difference p≤0.05compared with control group.
# Indicate significant difference p≤0.05 compared with ethanol group.
Liv52: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.
TG: triglycerides, TCH-total cholesterol.

radicals (Rao et al., 1990). In the present study, Liv52, NFAB, and FAB (10 ml/kg) co-treatment concomitantly increased GSH-Px activity, probably due to the higher T-SOD activity and GSH mirroring together (Table 2).

CAT is an antioxidant enzyme that primarily resides in peroxisomes and neutralizes hydrogen peroxide by catalyzing a reaction between two hydrogen peroxide molecules or by promoting hydrogen peroxide's interaction with hydrogen donors and then converting it to water (Cederbaum et al., 2009). In the present study, Liv52, NFAB, and FAB (10 ml/kg) were found to significantly increase CAT activity in rats with chronic alcohol consumption ($p<0.05$). Among the treatments, FAB (10 ml/kg) had a more pronounced effect against the reduction of CAT activity, probably owing to its higher phenolic and flavonoid contents and stronger capacity for scavenging free radicals ($p<0.01$).

Hepatic lipid peroxidation, indicated by an increase in hepatic malondialdehyde (MDA) levels (Fig. 3(c)), is elevated in chronic ethanol administration ($p<0.01$). Ethanol administration, whether chronic or acute, can trigger fatty liver, which is a precursor to more severe and often irreversible liver lesions. The breakdown of ethanol in the liver generates acetaldehyde or oxygen radicals that are believed to initiate lipid peroxidation and contribute to the development of fatty liver (Noh et al., 2011; Polavarapu et al., 1998). Pre-treatment of rats with Liv52, NFAB, and FAB stalks (10 ml/kg) significantly reduced the formation of MDA in livers ($p<0.05$).

3.3. Histopathological evaluation of alcoholic rat liver

The liver structure in normal control rats (Fig. 4) was normal with no apparent fat vesicles, necrosis, or inflammation in liver cells. On the other hand, chronic ethanol administration resulted in hepatic lobular inflammation, hepatocyte enlargement, small cell gaps, and numerous small fat cavities (Fig. 4). However, rats pre-treated with Liv52 showed significantly reduced alcohol-induced liver damage, with no visible inflammatory cells or fat vesicles (Fig. 4).

In this study, administration of NFAB and FAB (10 ml/kg) was found to alleviate the pathologic damage to the liver (Fig. 4). Hepatocytes showed no inflammation, and their cellular structure remained intact without fat vesicles. The liver's histology showed significant changes due to alcohol, possibly caused by fat accumulation, oxidative stress, and lipid peroxidation leading to cell damage. Administration of Amla beverage showed a protective effect against chronic liver damage when combined with NFAB and FAB (10 ml/kg). Further immunochemical studies are needed to understand the molecular mechanisms underlying the beverage's protective effect.

3.4. Fasting blood glucose levels of diabetic rats

On the 42nd day of the experiment, the fasting blood glucose levels of the rats were assessed as an important indicator of their diabetic status. Results showed that there was a significant reduction in fasting blood glucose levels in diabetic experimental animals treated with FAB and diabetic standard group (treated with hypoglycemic drug, glibenclamide) compared to untreated diabetic animals. In diabetic experimental animals (NFAB and FAB), there was a decrease of 39.01% and 41.05%, respectively, in fasting blood glucose levels from day 3 to day 42. The diabetic standard group showed a reduction in fasting blood sugar from 247.66 mg/dl to 142.83 mg/dl. However, there was no decrease in fasting blood glucose levels observed in untreated diabetic animals (Fig. 5). These results suggest that the hypoglycemic effect of fermented Amla beverage is equivalent to that of the reference oral hypoglycemic drug, glibenclamide. The constituents of the FAB may have reduced glucose levels by promoting insulin secretion or increasing insulin receptor sensitivity. Studies on fermented noni juice have also demonstrated its hypoglycemic and hepatoprotective properties in diabetic rats (Nayak et al., 2011).

3.5. Body mass variation and blood analysis in diabetic animals

During the latter 21 days of the treatment period, diabetic experimental (NFAB, FAB) and diabetic standard group animals showed a significant increase in body mass of 6.07%, 7.6%, and 8.4%, respectively, while the diabetic untreated animals experienced a decrease in body mass (82.16g, 2.62%) over the 42-day treatment period (Fig. 6) ($p<0.05$). Specifically, animals treated with FAB exhibited a 7.6% increase in body mass during the final 21 days of treatment, while untreated diabetic animals showed a 2.62% decrease in body mass during the same period. Notably, the untreated diabetic animals consumed more water compared to the fermented Amla beverage-treated rats.

Table 3 indicates that at the conclusion of the experiment, the rats in the diabetic group had significantly higher levels of glycosylated hemoglobin (HbAlc), and significantly lower levels of C-peptide and GLP-1 compared to the rats in the normal control group ($p<0.05$). However, the groups treated with the standard drug glibenclamide and fermented Amla beverage exhibited decreased HbAlc and increased C-peptide and

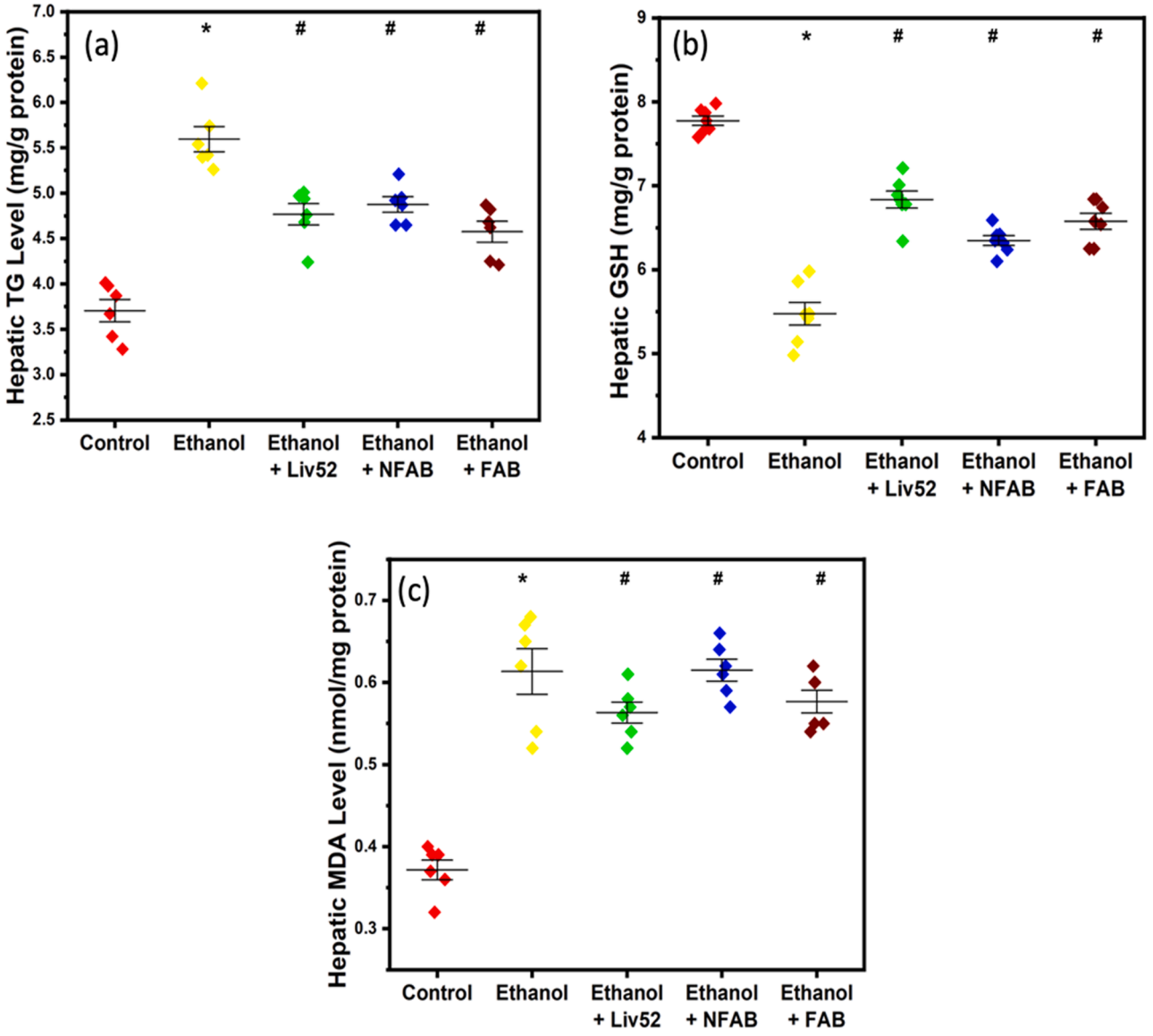

Fig. 3. Effect of non-fermented and lactic acid fermented Amla beverage on the hepatic (a) TG level (b) GSH level (c) LPO level.
Values are mean ± SD (n=6).
* Indicate significant difference p≤0.05compared with control group.
# Indicate significant difference p≤0.05 compared with ethanol group.

Table 2
Effect of non-fermented and lactic acid fermented Amla beverage on the hepatic antioxidant system (mean ±SD, n= 6).

Groups	T-SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/g protein)
Control	159.83±5.90	1685.83±5.20	262±0.01
Ethanol	101.66±4.55*	1286.74±9.55*	221±0.04*
Ethanol + Liv52	134.00±5.01*	1475.00±2.01#	246±0.04#
Ethanol+NFAB	148.25±2.09#	1544.18±4.62#	253±0.03#
Ethanol+FAB	141.51±6.41#	1585.50±6.86#	258±0.04#

Values are mean ± SD (n=6).
* Indicate significant difference p≤0.05 compared with control group.
* Indicate significant difference p≤0.05 compared with ethanol group.
Liv52: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

GLP-1 levels compared to the diabetic group (p<0.05). The fermented Amla beverage was particularly effective in reducing fasting HbAlc levels, improving C-peptide and GLP-1 levels, alleviating renal dysfunction and lipid metabolism, and most importantly, improving the function of β- cells based on histological changes in the pancreas of diabetic rats (Fig. 6). The anti-inflammatory effects of lactic acid bacteria aid in the treatment of the low-grade state of inflammation (Spiller, 2005), which is common in diabetes (Fehervari, 2012). These findings that consuming Amla beverage may help prevent or delay the onset of hyperglycemia in type 2 diabetic rats.

3.6. *Histological analysis of diabetic rats*

The pancreatic histology is displayed in Fig. 7, where normal rat islets exhibited typical cellular features and pancreatic islet diameter (Fig. 7A), unlike the other four groups. In contrast, the islets of diabetic rats had irregular structures with reduced volume and fewer islet cells. These islets were shrunken with atypical cellular changes such as mild hyperchromasia, coarse chromatin, and pyknosis (Fig. 7B).

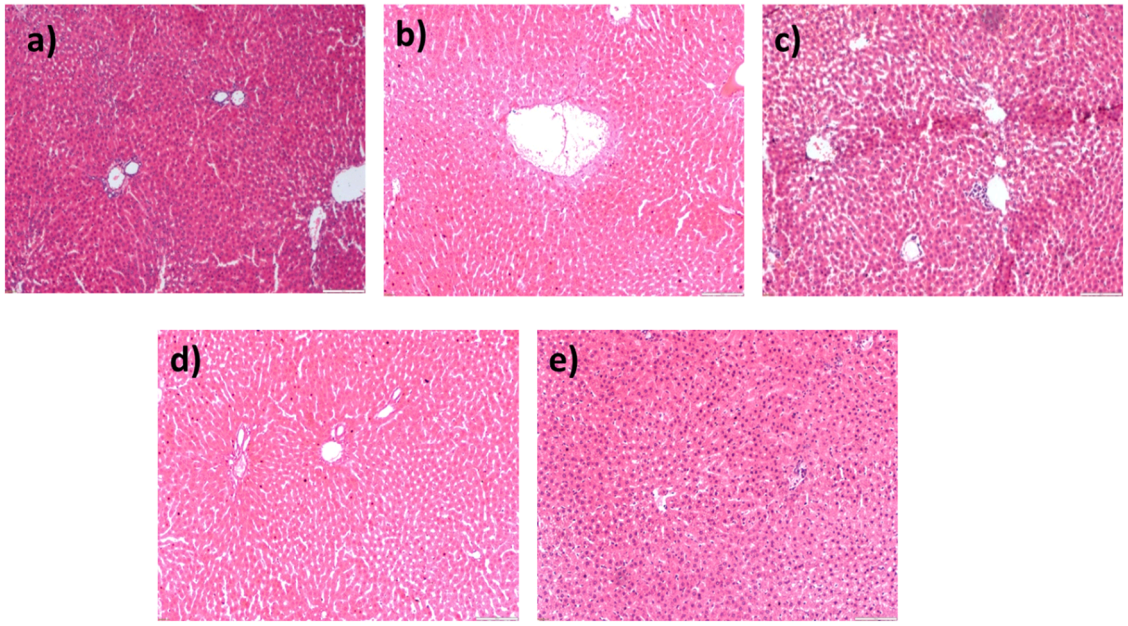

Fig. 4. Representative photomicrographs of livers in control and Amla beverage treated experimental alcoholic rats (H&E stain) (a) Normal control rat liver, (b) Ethanol treated rat liver, (c) Ethanol + Liv52, (d) Ethanol + NFAB and (e) Ethanol + FAB.
DI- diabetes induced.
PDI- postdiabetic induction.
PTI- posttreatment initiation.
Glibenclamide: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

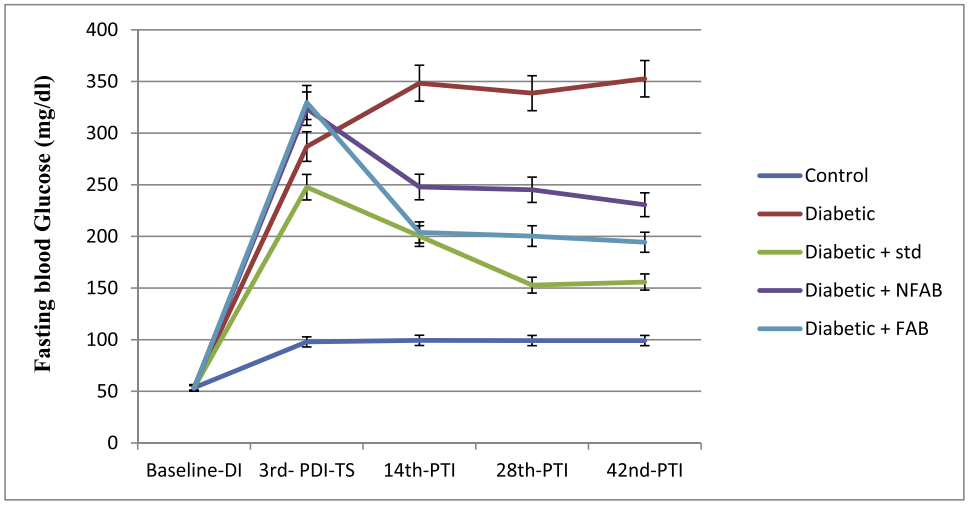

Fig. 5. Fasting blood glucose of normal control, diabetic untreated, diabetic standard, diabetic experimental (with NFAB and FAB) at basal level (before treatment) and during 42 days of treatment (n=6) each column represents mean ±SE.
DI- diabetes induced.
PDI- postdiabetic induction.
PTI- posttreatment initiation.
Glibenclamide: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

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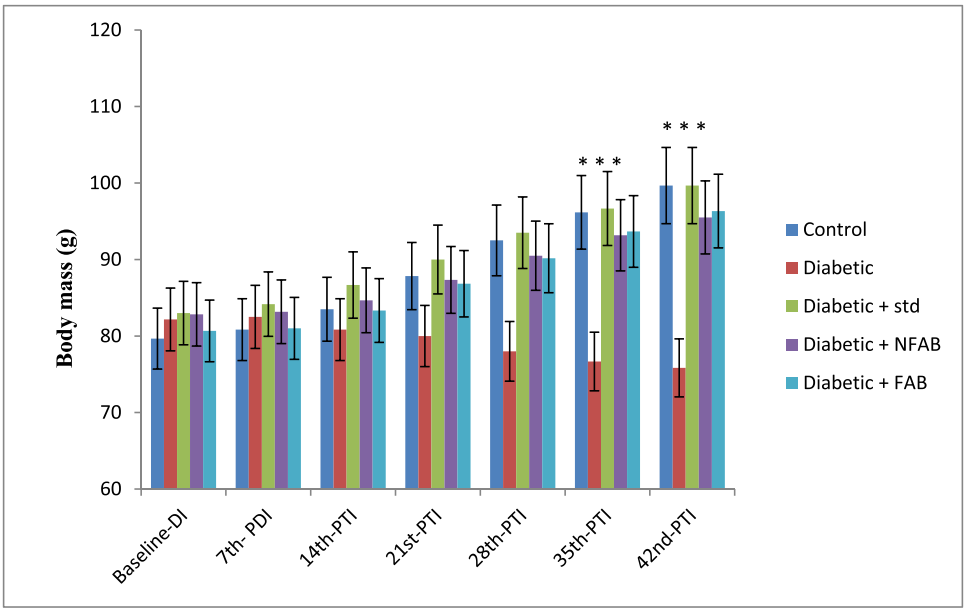
Glibenclamide (Fig. 7C), fermented Amla, and non-fermented Amla beverages (Figures 7D and E) treatments reversed the islet irregularity, making the structure like that of control groups. The findings indicate that fermented Amla beverage may enhance islet regeneration in the pancreas, restoring normal cellular size with hyperplasia in diabetic rats. According to (Cani et al., 2009), manipulation of gut flora has been linked to the release of GLP-1. This change in the microbiota of the gut stimulates L-cells to secrete GLP-1, which can help enhance metabolic health and guard against diabetes.

The liver tissue obtained from untreated animals showed significant fatty degeneration on histological analysis (Fig. 8B) compared to the normal control (Fig. 8A) and diabetic experimental animals treated with lactic acid fermented Amla beverage (Fig. 8D).

The hepatocytes of diabetic untreated animals had irregular shapes with numerous large fatty infiltrates in their cytoplasm (Fig. 8B), in contrast to the normal control (Fig. 8A), diabetic experimental animals treated with lactic acid fermented Amla beverage (NFAB and FAB) (Fig. 8D, 8E) and diabetic standard animals (Fig. 8C). The size and



DI- diabetes induced
PDI- postdiabetic induction
PTI- posttreatment initiation
Glibenclamide: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage

Fig. 6. Body mass of normal control, diabetic untreated, diabetic standard and diabetic experimental (with NFAB and FAB) at basal level (before treatment) and during 42 days of drug treatment (n=6) each column represents mean ±SE.

Table 3
Effect of non-fermented and lactic acid fermented Amla beverage on HbAlc, C-peptide and GLP-1 in diabetic rats (mean ±SD, n= 6).

Treatment	HbAlc (ng/ml)	C-peptide (ng/ml)	GLP-1 (pmol/l)
Control	17.95 ± 0.82	3.43 ± 0.41	24.08 ± 1.79
Diabetic	22.26 ± 1.04*	2.58 ± 0.28*	16.75 ±2.16*
Diabetic+Std	18.10 ± 0.93*	3.13 ± 0.17#	45.23 ± 2.40#
Diabetic+FAB	18.13 ± 0.94#	3.44 ± 0.19#	23.67 ± 1.17#
Diabetic +NFAB	19.61 ± 0.49#	3.32 ± 0.21#	26.02 ± 2.38#

Values are mean ± SD (n=6).
* Indicate significant difference p≤0.05 compared with control group.
# Indicate significant difference p≤0.05 compared with ethanol group.
Liv52: std. drug, NFAB-non-fermented Amla beverage, FAB-fermented Amla beverage.

number of fatty globules in hepatocytes of diabetic rats with NFAB and FAB were reduced, suggesting a possible hepatoprotective effect of the fermented beverage. This is consistent with previous findings by (Nayak et al., 2011) on the hypoglycemic and hepatoprotective properties of fermented noni fruit juice in diabetic rats. The authors suggested that fermented juice might be an excellent alternative to the hypoglycemic drug glibenclamide, as diabetic rats treated with fermented juice showed a reduction in hepatocyte fatty degeneration. The presence of triterpenes and saponins in fermented noni juice might be responsible for these properties.

The hypoglycemic effects of fermented Amla beverage may be closely related to its antioxidant activity, flavonoid content, and other bioactive compounds produced by functional LAB and their metabolites. These bioactive compounds may reduce glucagon levels and enhance glucose utilization, decreasing blood glucose. In contrast, glibenclamide acts by stimulating insulin secretion and inhibiting glucagon release. Both the Amla beverage and glibenclamide stimulate the remaining pancreatic cells, increasing serum insulin levels and decreasing blood glucose. The presence of rutin, a flavonoid composed of rutinose and quercetin, in fermented Amla beverage is thought to enhance insulin secretion by acting as a secretagogue similar to sucrose.

4. Conclusion

The present study suggests that the consumption of fermented Amla beverage may have a protective effect against chronic alcohol-induced toxicity by inhibiting lipid peroxidation and increasing levels of both enzymatic and non-enzymatic antioxidants in the liver. This effect may be attributed to the beverage's antioxidant properties, which help counteract ethanol-induced free radicals. LAB also been proven to alleviate ALD by improving the function of the intestinal barrier, regulating oxidative stress, inhibiting liver fat accumulation, protecting the gastric mucosa, and improving the nutrients supplement. The hypoglycemic effects of fermented Amla beverage may be closely related to its antioxidant activity, flavonoid content, and other bioactive compounds produced by functional LAB and their metabolites. These bioactive compounds may reduce glucagon levels and enhance glucose utilization, decreasing blood glucose. However, the molecular mechanism by which LAB protects is not fully understood; therefore, it is necessary to further study how fermented Amla beverage alleviates alcohol-induced liver damage and diabetes at the molecular and gene levels. All these functions provide a new target for the treatment of diabetes and liver diseases. However, there are few clinical research results, and a large number of experiments are needed to verify the effect of LAB on the prevention and remission of such diseases. Detailed study of the changes in gut microbiota caused by alcohol, and the analysis of which bacteria can effectively promote the development of these chronic disorders will lead to the exploration of targeted treatment. In addition, new probiotic strains and biological treatments that can regulate gut microbiota and prevent the development of alcohol-induced liver damage and diabetes should be identified. Large and long-term clinical experiments are needed to provide theoretical and technical support for the development

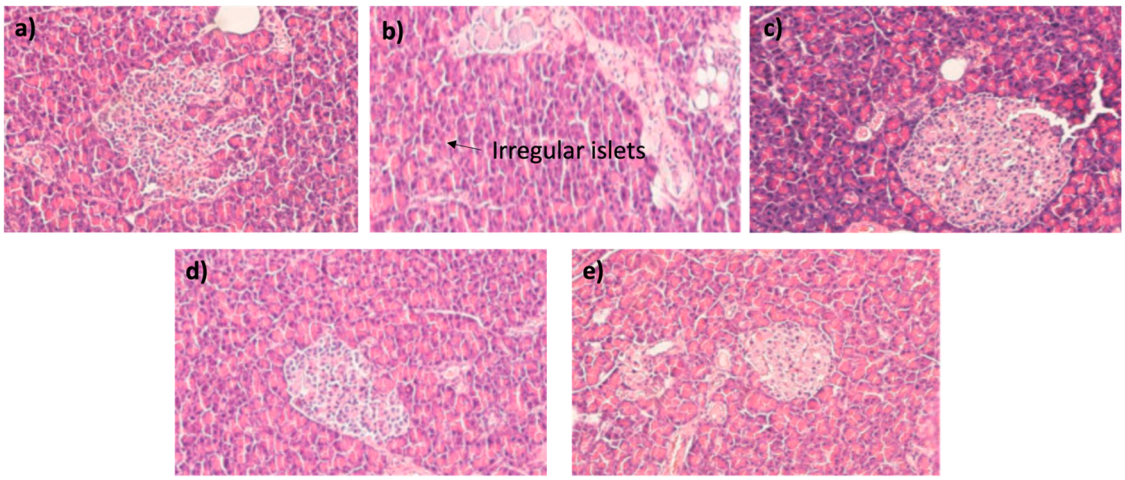


Fig. 7. Representative photomicrographs of the pancreas in control and Amla beverage-treated experimental diabetic rats (H&E stain) (a) Normal control rat pancreas, (b) Untreated diabetic rat pancreas, (c) Diabetic + Glibenclamide, (d) Diabetic + NFAB and (e) Diabetic +FAB.

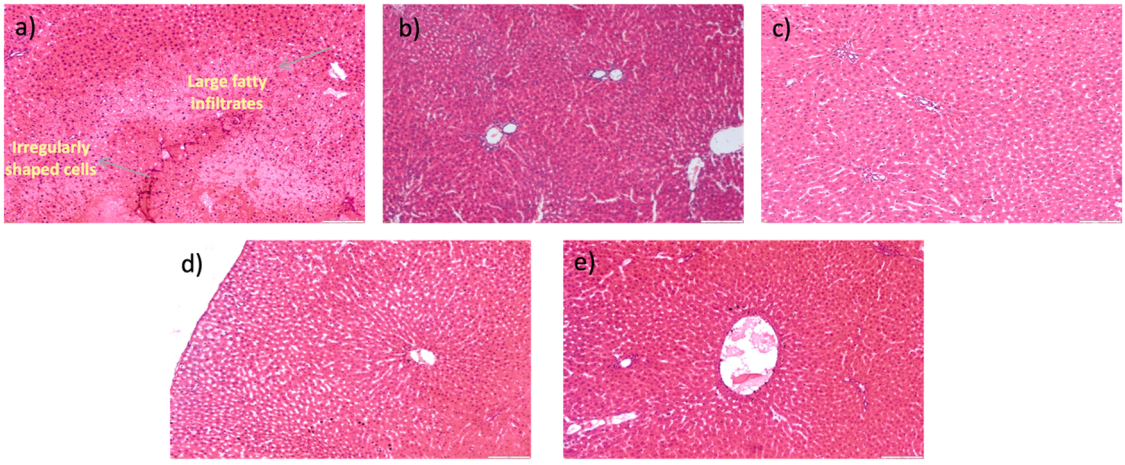


Fig. 8. Representative photomicrographs of the liver in control and Amla beverage-treated experimental diabetic rats (H&E stain) (a) Normal control rat liver, (b) Untreated diabetic rat liver, (c) Diabetic + Glibenclamide, (d) Diabetic + NFAB and (e) Diabetic +FAB.

of these new biological treatments with the ability to protect against such lifestyle disorders.

Ethical Statement

Wistar rats were procured from ISF College of Pharmacy, Moga, Punjab, India [Reg.No. No. 816/PO/a/04/CPCSEA].

The study was approved by the Ethics Committee of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India (IAEC approval number: *GADVASU/2020/IAEC/56/14)*.

All the experiments were conducted in accordance with the animal care guidelines of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2023.100155.

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