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FULL ARTICLE

Curcuma longa and *Trigonella foenum graecum*-enriched nutrient mixture from germinated *Macrotyloma uniflorum* and *Vigna radiate* ameliorate nonalcoholic fatty liver diseases in rats

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Abstract

The prevalence of nonalcoholic fatty liver is increasing due to modern lifestyle. Germinated and dehulled *Macrotyloma uniflorum* and *Vigna radiate* were shown to have enhanced nutrients. *Curcuma longa* and *Trigonella foenum graecum* were proven hepatoprotective.The supplementation of the nutrient herbal mixture to the MCD diet-induced steatosis shows reduced hepatic fat accumulation and lipid profile, and liver injury markers in serum also reserved in normal. Increased serum albumin in the treatment group indicates that the liver function is enhanced than that of steatosis. The supplementation of the herbal mixture has preserved the hepatic antioxidant. Zymographic analysis of matrix metalloproteinase, western blot determination of α-SMA, and histological evolution (H&E, Sirius red) depicted reduced fibrosis and reveled management of hepatic stellate cells in quiescent form. The present study concludes that the herbal mixture has reduced hepatocyte fat accumulation in steatotic animals, and curtailed the oxidative stress, further it prevents the progression of steatohepatitis.

Practical applications

Fatty liver diseases can be treated by modulating the diet composition such as consuming food rich in the nutrient herbal mixture. In this study, the nutrient mixture was made with dynamic food processing techniques such as germination, dehulling, and milling to augment the nutritional contents. Besides, *Macrotyloma uniflorum*, *Vigna radiate*, *Curcuma longa*, and *Trigonella foenum graecum* were used to improve the medicinal value and antioxidant. This formulation could target the various stages of NAFLD. This study revealed that the nutrient herbal mixture reduces the steatosis of the liver and curtailed the progression of steatohepatitis from hepatic steatosis. Since the edible foodstuff was used to make the nutrient mixture, it has excellent clinical application.

KEYWORDS

food processing, lipotoxicity, nutritional therapy

Subramaniyam Nithyananthan, Dangudubiyyam Sushmaa and Ibansiewdor Myrthong are contributed equally to this study.

1 | INTRODUCTION

The liver is the glandular regenerative organ that carries out many of the metabolic functions. Liver diseases account for roughly two million deaths per year. It comprises of hepatitis B virus, hepatitis C virus infections, alcoholic liver diseases, nonalcoholic fatty liver diseases, and hepatocellular carcinoma (HCC) which are having a foremost impact on the global burden of liver diseases (Asrani, Devarbhavi, Eaton, & Kamath, 2019; Wang, Fan, et al., 2014). Among the liver diseases, fatty liver is common due to both unhealthy and unbalanced diets, which leads to the accumulation of fat in parenchymal liver cells. It is mostly due to the augmented de novo synthesis of fatty acids in hepatocytes or impaired apolipoprotein secretion or β-oxidation in hepatocytes (Kneeman, Misdraji, & Corey, 2012). The prevalence of nonalcoholic fatty liver diseases (NAFLD) is quickly increasing worldwide and it is now the most common liver malady in the world. It is an intricate continuum of liver diseases stretching from benign asymptomatic hepatic steatosis to its more aggressive necroinflammatory nonalcoholic steatohepatitis (NASH) (Hansen et al., 2017). NAFLD represents a spectrum ranging from simple steatosis to hepatic inflammation, hepatocyte ballooning, presence of Mallory-Denk bodies, and fibrosis, which is referred to as NASH in case of proven alcohol abstinence. The distinction between steatosis and steatohepatitis that steatosis is less likely to evolve to severe liver diseases, whereas NASH leads to cirrhosis and HCC (Bettermann, Hohensee, & Haybaeck, 2014). Intracellular deposition of lipid leads to oxidative stress in hepatocytes and also induces cytokine-mediated injury (Parola & Robino, 2001). An elevated level of cytokines and macrophages in systemic circulation remodels the extracellular matrix and leads to a more lethal condition like inflammation, fibrosis, and HCC (Decaris et al., 2017; Schulz et al., 2015).

Plants like *Camellia sinensis*, *Cissus quadrangularis*, *Curcuma longa*, *Nelumbo nucifera*, and *Zingiber officinale* are the preventive remedies for steatosis and steatohepatitis (Jadeja, Devkar, & Nammi, 2014). In the Indian subcontinent, a wide array of cereals, grains, and herbs are used as conventional food that has great nutritional value (Perumpail et al., 2018). The horse gram and green grams have a good reservoir of essential amino acids, protein, fiber, and carbohydrate content. Food processing such as germination, dehulling, and milling increases the nutritional value of these grains as well as reduces the anti-nutritional factors (Oghbaei & Prakash, 2017; Prasad & Singh, 2015). Fenugreek has widely used a legume in India as a spice and taste enhancer, boost up the immunity and has anticancer potential (Wani & Kumar, 2018). Curcumin is having a spectrum of beneficial effects like antioxidants, anti-inflammation, hyperglycemia, hypertension, and obesity (Hewlings & Kalman, 2017; Sahebkar, Serban, Ursoniu, & Banach, 2015).

The effective therapeutic strategy for NASH is changing the lifestyle and having effective preventive strategies. Since diagnosis of fatty liver can be effectively achieved at NASH stage where majority of hepatocytes were damaged than hepatic steatosis condition. So the effective prevention strategy is essential to avoid the development of steatosis and further into NASH which is also in association with malnutrition. The nutrient mixtures were made with the germinated horse gram and green gram whose medicinal values were improved with fenugreek and turmeric to target various stages of NASH progression.. In the first phase of our study, we found that newly made herbal nutritional mixture has rich nutrition factors with reduced anti-nutritional factors and also prevented CCl_4-induced liver cirrhosis (Nithyananthan et al., 2020). In the current investigation, we intended to evaluate the therapeutic application of nutrient mixture against NAFLD and its preventive effect on the progression of steatohepatitis from hepatic steatosis.

2 | MATERIALS AND METHODS

Vigna radiata (green gram), *Macrotyloma uniflorum* (horse gram), *Trigonella foenum graecum* (fenugreek), and *Curcuma longa* (turmeric) were purchased from the local market of Puducherry, India. Methionine and choline-deficient (MCD) diet were procured from (Catalogue no: 960439) MP Biomedicals, USA. All other chemicals used were of analytical grade and procured from HiMedia Pvt. Limited, India.

2.1 | Mixture preparation

The nutrient herbal mixture was prepared as explained in Nithyananthan et al. (2020). In crisp, the germinated seeds were manually dehulled, dried under the shade at room temperature (RT) further milled into fine powders (Oghbaei & Prakash, 2017). *Trigonella foenum graecum* (100grams) was roasted under mild heat, cooled, and powdered. The nutrient mixture was prepared by suspending powders of *Vigna radiate* (0.5%), *Macrotyloma uniflorum* (0.5%), *Trigonella foenum graecum* (0.025%), and *Curcuma longa* (0.025%) in distilled water and allowed to boil over a hot plate for 5 min with constant stirring. After cooling to RT, the nutrient mixture filtrate was used for further study.

2.2 | Animal studies

Male, Wistar albino rats (n = 48, weighing between 200 and 250 grams) from Biogen animal facility, Bangalore, India, were housed in a polypropylene cage and were maintained at a temperature of 23°C ± 1°C and relative humidity of 60 ± 10% with 12 hr light and dark cycle in Central Animal House Facility, Pondicherry University, India. All the animal experiments were conducted as per the instructions and guidelines by the Institutional Animal Ethics Committee, Pondicherry University (PU/CAHF/22nd IAEC/2018/01, Dated 03.09.2018). The animals were allowed free access to drinking water and feed ad libitum. After a week of quarantine period and acclimatization, the animals were randomly allocated into eight groups (n = 6). Volume and concentration of the herbal nutrient mixture were arrived through previous research articles (Nithyananthan et al., 2020; Senadheera, Ekanayake, & Wanigatunge, 2014). Figure 1: Represent experimental details.

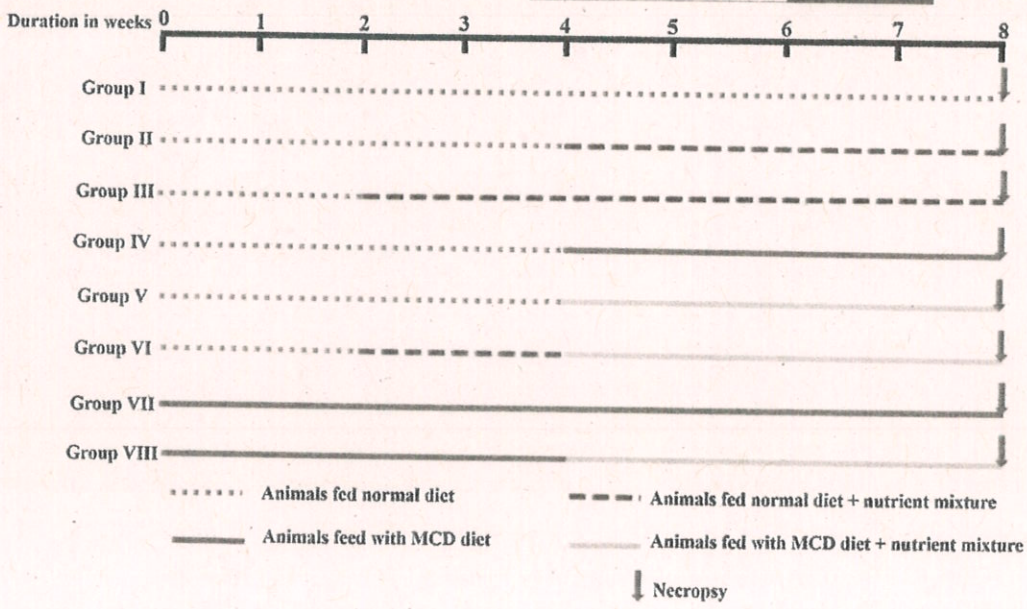

FIGURE 1 The representation of the experimental design

Group I: Control animals fed with normal rodent diet *ad libitum* (Control) (n = 6).

Group II: Animals were administered with freshly prepared nutrient herbal mixture (8 ml/kg body weight, orally) on every morning between 9:30 and 10:30 a.m. for 4 weeks.

Group III: Animals were administrated with freshly prepared nutrient herbal mixture (Dose as in Group II) for 6 weeks

Group IV: Nonalcoholic hepatic steatosis (NAHS)-induced animals (fed with MCD for 4 weeks *ad libitum* (Barakat & Hamza, 2011).

Group V: NAHS-induced animals (as in group IV) co-administered with nutrient herbal mixture (as in group II) for 4 weeks

Group VI: Animals administered with nutrient herbal mixture (as in group II) for 2 weeks and MCD diet *ad libitum* as in group IV (NAHS) for 4 weeks, while continuing the nutrient herbal mixture for 4 weeks, (Total 6 weeks as in group III) (serving as the chemopreventive study).

Group VII: Nonalcoholic steatohepatitis (NASH)-induced animals (MCD diet as *ad libitum* for 8 weeks) (Saleh, Ahmed, & Amin, 2017).

Group VIII: NASH-induced animals but the last 4 weeks in addition to the MCD diet as *ad libitum* animals were administered with nutrient herbal mixture for 4 weeks (as in group I). This group asses the ability of the nutrient mixture to prevent the progression of NASH from nonalcoholic hepatic steatosis.

At the end of experimental period, blood samples were collected by retro-orbital punturing with isoflurane anesthesia. The animals were subsequently euthanized by administering thiopental-sodium (50mg/kg body Wt. i.p.). The liver was excised and washed with phosphate-buffered saline then the wet weight was noted. A portion of the median lobe of the liver was excised and fixed in 10% neutral buffered formalin. The rest of the liver tissue and separated sera were stored at −80°C until further analysis.

2.3 | Serum analysis

The commercial kits from the Agappe Diagnostics Pvt. Ltd. and Beacon Diagnostics Pvt. Ltd. were used to analyze serum albumin levels and serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) activities.

2.4 | Serum and liver lipid profiling

Liver tissue (200mg) was suspended in 3 ml of the chloroform–methanol mixture (2:1 ratio) and gently crushed using a glass rod to extract the lipids. The tubes were then allowed to stand for 24 hr at RT and then centrifuged at 10,000 rpm for 5 min. The upper organic phase containing the lipids was collected and used for assays (Folch, Lees, & Sloane Stanley, 1957). The serum and hepatic lipids such as triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were determined using the commercial kid from Beacon Diagnostics Pvt. Ltd.

2.5 | Hepatic antioxidant analysis

Hepatic antioxidants such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced glutathione (GSH), and oxidative stress marker such as lipid peroxidation was determined as previously described and the specific enzyme activity is used to represent the antioxidant enzyme activity. (Ellman, 1959; Marklund & Marklund, 1974; Ohkawa, Ohishi, & Yagi, 1979; Rotruck et al., 1973; Sinha, 1972).

2.6 | Zymography

Liver samples were homogenized in RIPA (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) containing protease inhibition cocktail. About 8% nonreducing SDS-PAGE having 0.2% gelatin was used to resolve 50 μg of protein samples. The gel was washed with water and incubated for 4 hr in 2.5% triton X100 to remove SDS and re-nature protein. Incubation of this gel with developing buffer (50 mM Tris buffer pH 7.4, 10 mM CaCl2, 0.15 M NaCl, and 0.02% NaN3) for 48 hr at 37°C accelerates the matrix metalloproteinase (MMP) activity. Subsequently, the gel was stained with Coomassie brilliant blue for 30 min and de-stained. Image J analysis of the gel (Densitometry) revealed the degree of gelatin degradation by MMP-2 and MMP-9 (Domitrović, Jakovac, Tomac, & Šain, 2009).

2.7 | Western blot

Liver protein (50 μg) was resolved through 12% reducing SDS-PAGE, transferred on to a PVDF membrane subsequently blocked with 5% skimmed milk powder in TBST (Tris-buffered saline, 0.01% Tween 20) for 2 hr at room temperature and washed with TBST (3 × 10 min, each). The membrane was incubated with primary antibody (1:1,000 dilution) of β-actin (Santa Cruz Biotechnologies, SC-47778) and α-smooth muscle actin (α-SMA) (Santa Cruz Biotechnologies, SC-130617) for overnight at 4°C and washed with TBST. The membrane was further incubated with HRP-conjugated secondary antibody (Sigma Aldrich, A-9044, 1:18,000 dilution) for 2 hr at RT again washed with TBST. The membrane was developed by enhanced chemiluminescence reaction and exposed to an X-ray film. Image J software (NIH, Bethesda, USA) was used to quantitate the band's intensity (Subastri et al., 2018).

2.8 | Histological evolution

The neutral buffered 10% formalin-fixed liver samples were embedded in paraffin block and sectioned with a microtome (LeicaTM, Germany). The tissue sections were stained with hematoxylin and eosin (H&E), and Sirius red (0.1%) in saturated picric acid and observed under bright-field microscopy. The images were photographed using Olympus U-RFLT50 equipped with an imaging system. The liver sections were scored according to Ishak scoring system by accounting the properties such as ductal reaction (0–3), portal and lobular inflammation (0-none, 1-mild, 2-moderate, 3-severe), presence of oval cells, steatosis (0-none, 1 < 30%, 2 for 30%–60%, 3-more than 60%), and ballooning degeneration. The Sirius red-stained liver sections were staged by the Ishak method for liver cirrhosis where 0-no fibrosis, 1-few portal fibrosis with or without septa, 2-moderate portal fibrosis with or without bridges, 3-moderate fibrosis with few bridges, 4-liver fibrosis with multiple bridges, 5-fibrosis with occasional nodulation, and 6-cirrhosis (Goodman, 2007; Traber et al., 2013).

2.9 | Statistical analysis

All data were expressed as the mean ± *SEM*. Statistical analysis was performed with one-way ANOVA followed by the Tukey's multiple test using GraphPad Prism software. $p < .001$, $p < .01$, and $p < .05$ were considered as statistically significant.

3 | RESULTS

3.1 | Changes in body and liver weight and liver index

The percentage body weight change of the animals feed with the nutrient mixture and the MCD diet is monitored every week and displayed in Figure 2. As observed by other investigators, an increase in the duration of MCD diet supplementation (Groups IV and VII), further reduced ($p < .001$) its body weight (Figure 2) than that of control animals (Group I). Nutrient mixture co- and pre-supplementation to NAHS rats (Groups V, VI, and VIII) increased the body weight ($p < .001$) when compared with their respective MCD diet feed rats (Groups V and VI vs. Group IV and Group VII vs. Group VIII).

Table 1 represents the absolute final body weight and liver index of the different experimental animals. MCD diet feed animals (Groups IV and VII) showed a significant reduction ($p < .001$) in final body weight and liver index when compared with healthy control animals (Group I). Nutrient mixture co- and pre-supplementation to NAHS rats (Groups V, VI, and VIII) increased the body weight ($p < .001$), and liver index than that of respective MCD diet feed rats (Groups V and VI vs. Group IV and Group VII vs. Group VIII). We did not observe a statically significant difference in 4 weeks (Group II) nutrient mixture supplied rats when compared with control animals (Group I), moreover, 6 weeks (Group III) supplementation showed $p < .05$ level increase in final body weight without affecting the liver index. Figure 3 displays the gross morphology of the control and the nutrient mixture supplied healthy and NAFLD.

3.2 | Level of liver injury markers in serum

Table 2 represents the levels of serum liver function markers such as albumin, SGOT, SGPT, and ALP. Induction of NASH by MCD diet (Group VII) displayed decreased albumin ($p < .01$) than control rats (Group I). Supplementation of the nutrient mixture to NAHS rats (Group VIII) demonstrated a significant increase ($p < .05$) in albumin than that of NASH animals (Group VII); however, no significant deviation was observed when compared with control (Group I). We did not witness any statistical difference in the serum albumin level of other experimental animals when compared with respective control groups. NAHS developed rats (Group IV) and NASH rats (Group VII) depicted an elevated level of SGOT ($p < .001$), SGPT ($p < .001$), and

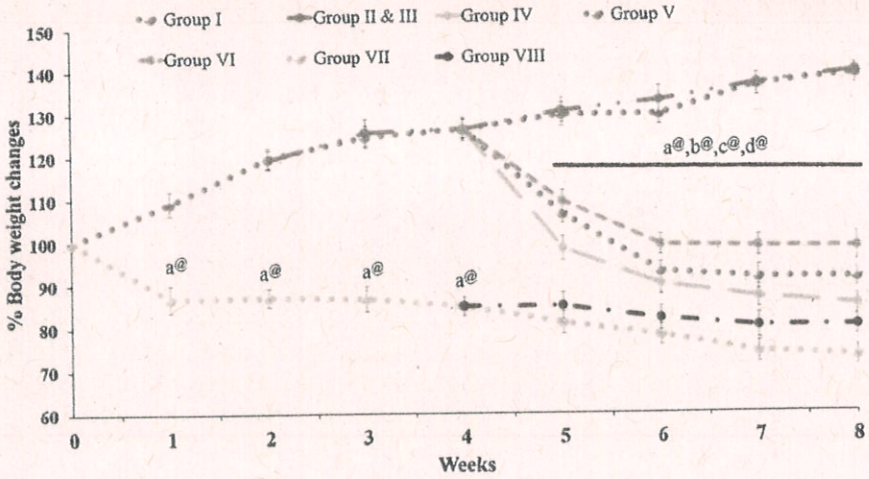

FIGURE 2 Percent body weight changes in different experimental groups. The representation of the protective role of the nutrient mixture on the % body weight change with MCD diet-induced steatosis and NASH. All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V) and "d" versus NASH rats (Group VII). The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly

TABLE 1 Shows the absolute body weight and liver index at the end of the experiment

Groups	Body weight (g)	Liver index
I	334.75 ± 6.48	34.90 ± 0.09
II	338.08 ± 14.26	32.84 ± 0.21
III	350.67 ± 8.9 a^{*}	35.35 ± 0.34
IV	220.95 ± 3.72 $a^{\&}$	24.22 ± 0.45 $a^{\&}$
V	244.08 ± 7.64 $a^{\&}$, b^{*}	28.42 ± 0.49 $a^{\&}$, b^{*}
VI	250.13 ± 5.32 $a^{\&}$, b^{*},d^{*}	31.08 ± 0.01 a^{*}, b^{*},$c^{\&}$
VII	203.72 ± 9.18 $a^{@}$	21.83 ± 0.17 $a^{@}$
VIII	216.66 ± 4.48 $a^{@}$	27.38 ± 0.88 $a^{\&}$, $d^{@}$

Notes: All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly.

ALP ($p < .001$) than control animals (Group I). Nutrient mixture co-supplemented (Group IV), pre-supplemented (Group VI), and steatosis (Group VIII) rats displayed reduced ($p < .001$) serum SGOT, SGPT, and ALP activities than control animals (Group I), and respective pair-fed control animals (Groups V and VI vs. Group IV and Group VII vs. Group VIII). Interestingly the nutrient mixture pre-supplemented animals (Group VI) showed reduced activities of SGOT ($p < .001$), SGPT ($p < .001$), and ALP ($p < .001$) when compared with nutrient mixture co-supplemented (Group V) animals. These findings show that pre-supplementation has a better effect than that of co-supplementation. Administration of nutrient mixture to normal rats (Groups II and III) has displayed nil statistical difference in hepatocyte damage markers than control animals (Group I).

3.3 | Serum lipid profile

Table 3 displays the serum lipid profile such as TG, TC, LDL-C, and HDL-C of the nutrient mixture supplied healthy and NAFLD rats. MCD diet-induced NAHS (Group IV) has reduced ($p < .001$) TG and HDL-C levels and elevated ($p < .001$) TC and HDL-C than control animals (Group I). Co-administration (Group V) and pre-administration (Group VI) of nutrient mixture displayed increased TG ($p < .001$) and HDL-C ($p < .05$) and decreased ($p < .001$) TC and HDL-C than NAHS rats (Group IV). Interestingly, nutrient mixture pre-supplementation (Group VI) has provided significantly reduced TG ($p < .05$) and increased HDL-C ($p < .01$) than rats co-supplied with the nutrient mixture (Group V). The nutrient mixture supplied NASH rats (Group VII) were securitized to have increased TG ($p < .01$), TC ($p < .01$), HDL-C ($p < .01$), and LDL-C ($p < .001$) than control animals (Group I). Nutrient mixture administration to NASH animals (Group VIII) has a reduction in the levels of TG, TC, and HDL-C ($p < .05$) than Group VII (NASH) rats, however, group VIII animals compared with group I has $p < .001$ level elevation is noticed in above said lipid profile. Nutrient mixture alone supplementation to healthy rats (Groups II and III) displayed an insignificant change in serum lipid profile from control rats (Group I).

3.4 | Hepatic lipid content

Table 4 displays the altered lipid content in the NAHS liver and the reduction of fat loading in steatosis by the nutrient mixture. The hepatic NAHS animals (Group IV) and NASH animals (Group VII) displayed markedly elevated ($p < .001$) TG, TC, HDL-C, and LDL-C than healthy control animals (Group I). The nutrient mixture co-administration (Group V) and pre-supplemented (Group IV) NAHS rats displayed a reduction

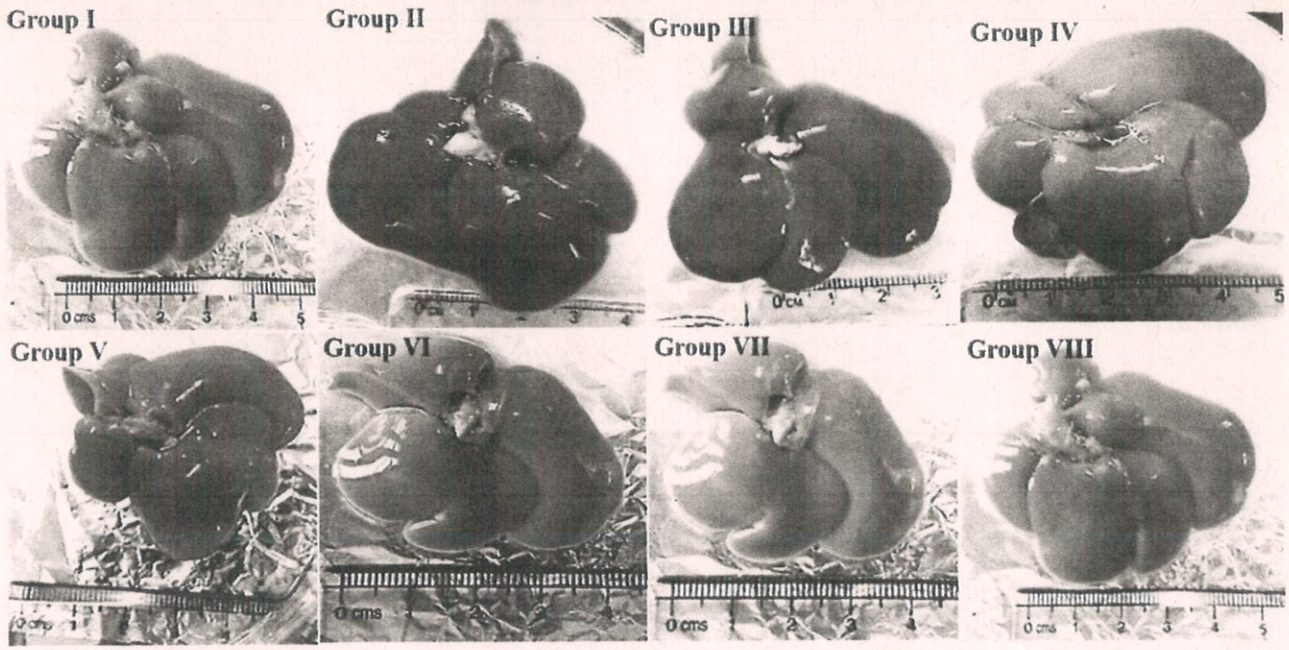

FIGURE 3 Gross morphology of nutrient mixture supplied healthy, steatotic and steatohepatitis liver

TABLE 2 The representation of the serum albumin, SGOT, SGPT and ALP in nutrient mixture supplied healthy and NAFLD rats

Group	Albumin (g/dl)	SGOT	SGPT	ALP
I	3.02 ± 0.14	41.18 ± 3.25	13.61 ± 1.7	95.4 ± 8
II	2.94 ± 0.10	46.28 ± 3.28	13.18 ± 3.7	98.9 ± 5.3
III	2.89 ± 0.12	47.03 ± 6	12.36 ± 3.5	87.53 ± 4.2
IV	3.14 ± 0.02	71 ± 1 $a^{\&}$	33.38 ± 2.3 $a^{\&}$	158.4 ± 3.1 $a^{\&}$
V	3.05 ± 0.07	53.8 ± 0.61 $b^{\&}$	16.32 ± 1.2 $b^{\&}$	113.97 ± 4.9 $b^{\&}$
VI	3.09 ± 0.04	51.41 ± 0.9 $b^{\&}$, c^{*}	14.14 ± 1.32 $b^{\&}$, c^{*}	107.94 ± 2.3 $b^{\&}$, c^{*}
VII	2.44 ± 0.03 $a^{@}$	84.49 ± 1.49 $a^{@}$	42.99 ± 3.40 $a^{\&}$	137.01 ± 5.72 b^{*}
VIII	2.78 ± 0.19 $d^{@}$	54.62 ± 4.55 $d^{@}$	29.59 ± 2.92 $d^{@}$	108.09 ± 4.8 $d^{@}$

Notes: All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly.

TABLE 3 Depicts the changes in the serum TG, TC, HDL-C, and LDL-C in nutrient herbal mixture supplied healthy and NAFLD rats

Group	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)
I	55.79 ± 5.3	43.63 ± 0.22	32.9 ± 3.2	11.07 ± 0.85
II	52 ± 4.7	45.25 ± 1.3	38.4 ± 3.5	10.22 ± 0.97
III	62.38 ± 6	42.82 ± 1.8	33.22 ± 2.03	9.16 ± 4
IV	28.8 ± 6.9 $a^{\&}$	38.09 ± 1.3 a^{*}	23.97 ± 0.97 $a^{\&}$	14.35 ± 0.49 $a^{\&}$
V	44.6 ± 2.1 a^{*}, $b^{\&}$	47.03 ± 1.5 $b^{\&}$	27.09 ± 0.43 $a^{@}$, b^{*}	12.04 ± 1.8 a^{*}, $b^{\&}$
VI	49.18 ± 2.3 c^{*}	45.67 ± 2.3	29.77 ± 1.08 $c^{@}$	11.86 ± 1.4 $b^{\&}$
VII	26.24 ± 7 $a^{\&}$	31.86 ± 1.09 $a^{\&}$	20.47 ± 1.47 $a^{\&}$	24.72 ± 0.4 $a^{\&}$
VIII	46.19 ± 0.8 $d^{\&}$	46.02 ± 0.4 $d^{\&}$	29.52 ± 0.43 $d^{\&}$	19.41 ± 0.4 $a^{\&}$, $d^{\&}$

Notes: All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly.

in TG ($p < .001$), TC ($p < .01$), HDL-C ($p < .001$), and LDL-C ($p < .001$) when compared with Group IV (NAHS) rats. Group VI (Nutrient mixture pre-supplied steatotic rats) demonstrated significantly ($p < .05$) reduced hepatic lipid accumulation than nutrient mixture co-supplied rats (Group V). The nutrient mixture administration to NASH animals (Group VIII) reduced levels of TG, TC, and HDL-C ($p < .05$) when compared with Group VII (NASH rats). Nutrient mixture supplementation to healthy rats (Groups II and III) does not alter the hepatic lipid content in a significant fashion from healthy control (Group I).

3.5 | Hepatic antioxidant status

Table 5 represents the hepatic antioxidant alterations in different experimental animals. Hepatic steatosis (Group IV) and NASH (Group VII) rats have $p < .001$ significant reduced hepatic enzymatic antioxidants such as CAT, SOD and GPx, and nonenzymatic antioxidants such as GSH than healthy animal (Group I). Supplementation of nutrient mixture before (Group VI) and during (Group V) MCD diet-induced steatotic animals has improvement in hepatic antioxidant such as CAT ($p < .05$), SOD ($p < .05$), GPx ($p < .01$), and GSH ($p < .01$) than steatotic rats (Group IV). The preconditioning of the liver with the nutrient mixture and subsequent steatosis (Group VI) showed convalescent antioxidant content (CAT and SOD, $p < .01$ and GPx and GSH, $p < .05$) than nutrient mixture co-supplied NAHS rats (Group V) depicting the hepatoprotective effect of nutrient mixture. Even though, the nutrient mixture supplied NASH animals (Group VIII) has reduced antioxidant ($p < .001$) than control animals (Group I) is statistically improved ($p < .05$ and $p < .01$) than NASH rats (Group VII), depicting the hepatoprotective effect of nutrient mixture. Administration of nutrient mixture alone to healthy rats (Groups II and III) does not alter hepatic antioxidants at a statistically significant level.

3.6 | Change in lipid peroxidation in the liver of different experimental animals

The rate of lipid peroxidation in control and experimental animals was determined in terms of thiobarbituric acid reactive substance (TBARS) and is represented in Table 5. The development of steatosis (Group IV) has manifested 15.7-fold ($p < .001$) enhanced TBARS level than control (Group I). But nutrient mixture co-administration (Group IV) has

TABLE 4 Hepatic lipid profiling of nutrient mixture supplied healthy and NAFLD rats

Group	Triglycerides (mg/g of liver)	Total cholesterol (mg/g of liver)	HDL-cholesterol (mg/g of liver)	LDL-cholesterol (mg/g of liver)
I	233.7 ± 10	57 ± 20	2.36 ± 0.14	1.32 ± 0.3
II	177.52 ± 16.7	83 ± 10	2.33 ± 0.20	2.8 ± 0.26
III	169.65 ± 12.45	75 ± 16	2.60 ± 1.33	2.38 ± 0.76
IV	342.7 ± 16.9 $a^{\&}$	739 ± 8 $a^{\&}$	8.65 ± 0.13 $a^{\&}$	17.5 ± 1.8 $a^{\&}$
V	242.63 ± 8.55 $b^{\&}$	563 ± 19 $a^{\&}$, $b^{@}$	6.53 ± 0.04 $a^{\&}$, $b^{\&}$	13.3 ± 0.5 $a^{\&}$, $b^{\&}$
VI	240.53 ± 6.64	545.79 ± 20 c^{*}	5.63 ± 0.07 c^{*}	7.96 ± 2.27 $c^{\&}$
VII	385.39 ± 17.58 $a^{\&}$	749.88 ± 14 $a^{\&}$	7.65 ± 0.09 $a^{\&}$	16.02 ± 1.96 $a^{\&}$
VIII	304.49 ± 1.50 d^{*}	621.84 ± 12.04 $d^{@}$	4.91 ± 0.29 $d^{\&}$	17.60 ± 1.85

Notes: All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly.

TABLE 5 The represent the antioxidants status and oxidative stress in the nutrient herbal mixture supplied healthy and NAFLD rat's liver

Group	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	GSH (mg/g of tissue)	Lipid peroxidation (nM TBARS/mg of protein)
I	42.72 ± 3.4	62.59 ± 6.4	7.41 ± 0.7	170.25 ± 13.61	34.5 ± 3.7
II	44.91 ± 3.6	66.45 ± 2.7	7.37 ± 0.09	163.98 ± 7.2	25.67 ± 2.7
III	38.28 ± 4.3	67.45 ± 3.2	8.19 ± 0.39	201.84 ± 5.6	25.02 ± 7.3
IV	14.18 ± 1.8 $a^{\&}$	27.11 ± 0.5 $a^{\&}$	4.97 ± 0.40 $a^{\&}$	107.37 ± 2.22 $a^{\&}$	540 ± 90 $a^{\&}$
V	26.34 ± 0.74 $a^{\&}$, b^{*}	45.56 ± 2.9 $a^{\&}$, b^{*}	6.76 ± 0.19 $b^{\&}$	154.63 ± 8.9 a^{*}, $b^{\&}$	286.3 ± 100 $a^{\&}$, $b^{\&}$
VI	35.45 ± 2.51 $c^{@}$	52.19 ± 4.1 $c^{@}$	7.32 ± 0.32 c^{*}	160.84 ± 2.1 c^{*}	135.67 ± 50 a^{*}, b^{*}, $c^{@}$
VII	8.42 ± 2.12 $a^{\&}$	10.43 ± 1.45 $a^{\&}$	4.24 ± 0.223 $a^{\&}$	105.712 ± 1.26 $a^{\&}$	1,077.11 ± 86.09 $a^{\&}$
VIII	21.57 ± 1.9 $a^{\&}$, d^{*}	26.41 ± 1.07 $a^{\&}$, d^{*}	6.43 ± 0.19 $d^{@}$	118.05 ± 3.56 $a^{\&}$, d^{*}	764.55 ± 72.75 $a^{\&}$, $d^{@}$

Notes: All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly.

1.9-fold lesser ($p < .001$) TBARS than steatotic rats (Group III). Whereas nutrient mixture treated health liver revealed a 1.34-fold decline in lipid peroxidation, indicating the antioxidant nature of the nutrient mixture. The pre-administration of the nutrient mixture to animals and following fatty liver induction (Group VI) exhibited $p < .05$ diminished TBARS level than steatotic rats (Group IV) and nutrient mixture co-supplemented with steatosis (Group VI). NASH rats (Group VII) have exhibited 26.4-fold ($p < .001$) intensified TBARS level than control (Group I), but nutrient mixture administration to NASH rats (Group VIII) has portrait 22-fold ($p < .001$) level of TBARS than control which is 1.2-fold lesser ($p < .001$) than NASH rats (Group VII).

3.7 | Histological alterations of the liver in various experimental animals

Figure 4A is the histopathological analysis of H&E stained liver sections of control, nutrient mixture supplied healthy, and steatotic animals (100×). Figure 4B describes the steatotic area calculated by Image J software and Figure 4C outlines the Ishak scoring. Figure 5 shows the 400× magnified H&E stained liver sections of the nutrient mixture supplied healthy and NAFLD rats. The control and nutrient mixture feed healthy rats (Groups I, II, and III) confirmed typical architecture of the liver but the development of steatosis (Group IV) revealed prominent macrovesicular steatotic changes in zone 3 areas. Image J quantitation found a higher % steatosis area ($p < .001$) than control (Group I) and gained the Ishak steatosis score of 3 ($p < .001$) than control. The steatotic liver (Group IV) also displayed increased hepatocyte ballooning degeneration, immune cell infiltration, lobular portal inflammation (Ishak score of 3), and portal fibrosis accompanied by ductal reactions. Both pre-and co-administration of the nutrient mixture (Groups V and VI) have reduced the macrovesicular changes and depicted numerous microvesicular ($p < .001$) than steatosis rats (Group IV). Nutrient mixture pretreated steatotic rats (Group VI) displayed $p < .001$ significantly reduced % area of steatosis than

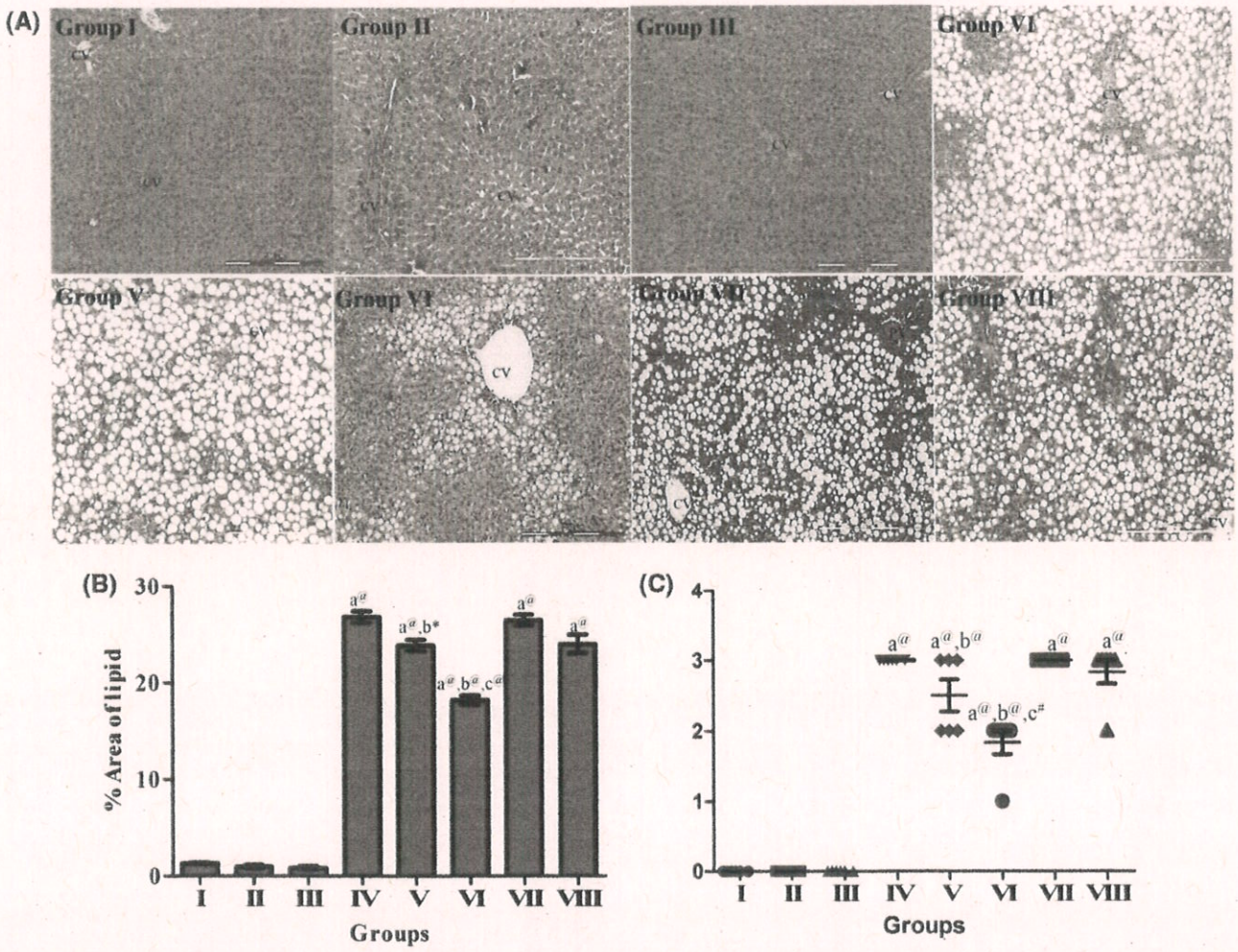

FIGURE 4 The representation of the histology (100×) of the nutrient mixture supplied healthy and NAFLD liver. (A) H&E stained liver sections of the nutrient mixture supplied healthy and steatosis liver sections. (B) The image J quantified % area of steatosis. (C) Represents the steatosis score for NAFLD. All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly. CV—central vein and PV—portal vein

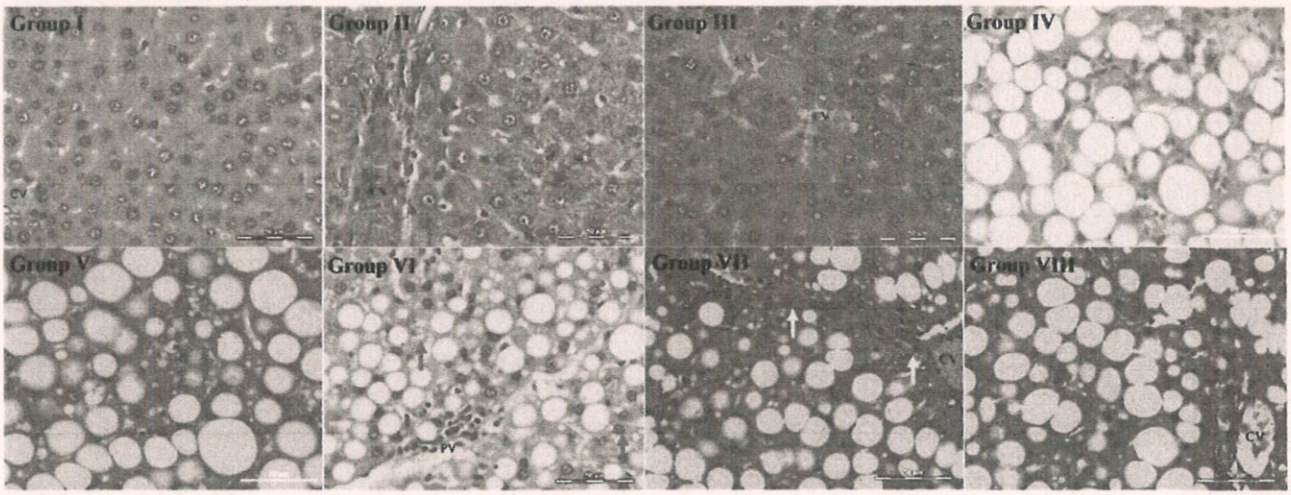

FIGURE 5 Shows the histology (400×) of the nutrient mixture supplied healthy and NAFLD liver. Red arrow indicates the existence of microvesicular changes and yellow arrows represent the fibrosis

nutrient mixture co-supplemented steatotic rats (Group V). The development of NASH (Group VII) presented prominent macrovesicular steatotic changes and higher % steatosis area ($p < .001$) than control (Group I), meanwhile gained the Ishak score of 3 ($p < .001$) than control and the nutrient supplementation (Group VIII) showed significant no change. The NASH animals (Group VII) also displayed increased hepatocyte ballooning degeneration, immune cell infiltration, lobular portal inflammation (Ishak score of 4), and portal fibrosis accompanied by ductal reactions.

Figure 6A depicts Sirius red staining of the liver sections and Figure 6B&C is the further Image J quantitation and fibrosis scoring of Sirius red positive area. Control and nutrient mixture supplied healthy liver (Groups I, II, and III) exhibited intense Sirius red positivity to the endothelial lining of central and portal veins, and displayed negligible area of fibrosis and its scoring. The steatosis rats displayed a few extend portal fibrosis, which was insignificant, but the onset of NASH has developed hepatic fibrosis. The NASH rats (Group VII) displayed portal to central and portal to portal numerous extend fibrosis, which was $p < .001$ high in % area of fibrosis and gained Ishak fibrosis score of 4 compared with control (Group I). The nutrient mixture treatment to NASH (Group VIII) unveiled extended portal fibrosis without septa (Ishak fibrosis score of 3) and noted that $p < .001$ reduced Sirius red positivity than NASH rats (Group VII).

3.8 | Activity of matrix metalloproteinase

Figure 7 is the zymography analysis of MMP-2 and MMP-9 activity. Control and nutrient mixture-treated healthy rats displayed the basal level activity of both MMP-2 and 9. Induction of NAHS (Group IV) has improved MMP-2 and MMP-9 activity without any significant changes. NASH liver (Group VII) has increased MMP-2 and MMP-9 activity and attains a significant difference of $p < .001$ than control rats (Group I). Nutrient mixture administration to NASH rats (Group VIII) documented $p < .001$ reductions in MMP-2 and MMP-9 levels than NASH rats (Group VII), indicating the rescue from hepatic fibrosis (Group VIII).

3.9 | α-Smooth muscle actin expression

Figure 8A represents the western blot determination of activated stellate cell marker, α-SMA in the NASH condition and Figure 8B is the Image J densitometry quantitation of α-SMA. Western blot analysis of α-SMA has displayed $p < .001$ intense expressions in NASH rats (Group VII) than control (Group I). The nutrient mixture administration to NASH rats (Group VIII) evidenced increased α-SMA level ($p < .001$) than control (Group I) meanwhile, its expression was noticed to be lesser ($p < .001$) than NASH rats (Group VII).

4 | DISCUSSION

The liver, the major metabolic organ, plays a vital role in the living organism, especially in the metabolism of absorbed nutrients and the biotransformation of xenobiotics. NAFLD is the excessive hepatic fat accumulation (steatosis in > 5% of hepatocytes) and is associated with insulin resistance (IR). Reduced lipid export from the liver to peripheral organs and an increased rate of lipid import to the liver leads to lipotoxicity of the liver (Machado & Diehl, 2016). The incidence of NAFLD is about 20%-30% in western countries and 5%-18% in Asian countries and tends to be 10% higher in overweight individuals than the lean individual (Benedict & Zhang, 2017; Sayiner, Koenig, Henry, & Younossi, 2016). NASH is an underlying cause of cirrhosis, and the bridging of the septa is a marker of fibrosis, and its progression leads to liver cirrhosis (Brunt, 2001). The progression of NAFLD to NASH has been expounded by the two-hit hypothesis. The first hit is IR causing lipolysis, thereby increasing free fatty acid (FFA) in the serum. The first hit makes the liver more vulnerable to the second hit mediated by the increased oxidative stress, inflammatory mediators,

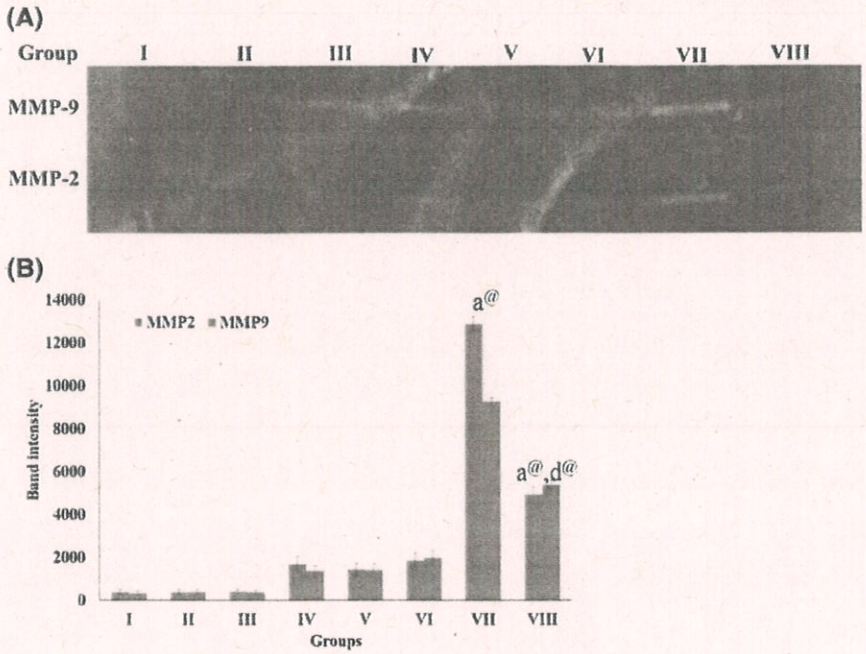

FIGURE 7 (A) Representation of the MMP zymogram pattern and (B) The densitometry analysis of MMP-2 and MMP-9 activity by Image J. All data were presented with mean ± *SEM*. "a" versus Control rats (Group I), "b" versus MCD diet-induced steatotic rats (Group IV), "c" versus the nutrient mixture co-supplemented steatotic rats (Group V), and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly

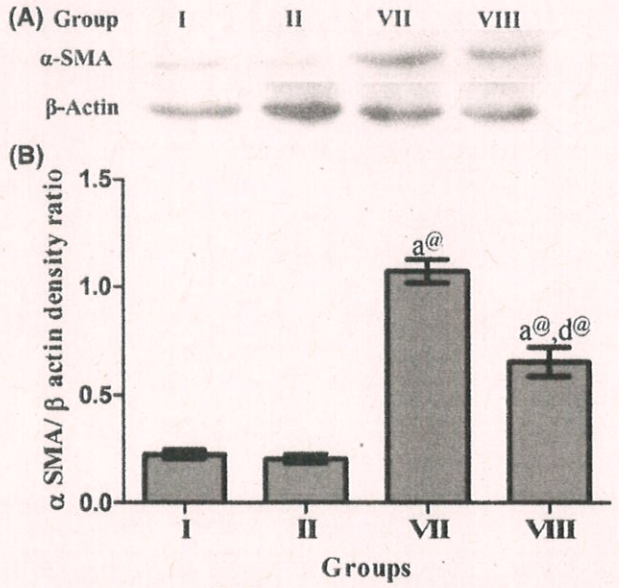

FIGURE 8 (A) The western blot analysis of the hepatic stellate cell marker such as α smooth muscle actin (α-SMA) and the (B) represents the corresponding Image J densitometry analysis. [Since there was no significant difference noticed in the fibrotic markers such as MMP, Sirius red staining positivity in the steatotic liver, the same is omitted for this study and only considered NASH groups for α-SMA determination]. All data were presented with mean ± *SEM*. "a" versus Control rats (Group I) and "d" versus NASH (Group VII) rats. The symbol "&, @, *" represent the statistical significance levels at $p < .001$, $p < .01$, and $p < .05$ accordingly

legumes have also shown increased protein digestibility due to the activation of hydrolytic enzymes such as protease, α amylase, β-amylase, and glucanase. It is also a rich source of carbohydrate (22%–45%) (Benítez et al., 2013). The prepared medicinal value-enriched nutritional mixture has increased antioxidants, nutritional value, reduced anti-nutritional value, and also has reduced CCl_4-induced liver cirrhosis (Nithyananthan et al., 2020). The reduced anti-nutritional value of this nutrient mixture could be due to the effective food processing procedure like germination and dehulling (Coulibaly, Kouakou, & Chen, 2011; Oghbaei & Prakash, 2017). The administered nutrient mixture to the steatotic animals (8 ml/kg body weight) is equivalent to the normal human consumption volume. During the 4 weeks of the study, the rats in group IV (MCD) showed significant weight loss, which is the characteristic of NAFLD induced by the MCD diet (Schattenberg & Galle, 2010). Supplementation of the nutrient mixture during the steatosis development and NASH has reduced loss in body weight and managed liver index close to control, and the pre-supplementation of the nutrient mixture has protected animals significantly than the diseased animals. In our study, there was no significant decrease in the serum albumin levels in steatosis and nutrient mixture supplied steatosis animals when compared to the control, indicating the preliminary compensated condition. In steatohepatitis rats, the levels of serum albumin are found to decrease significantly and nutrient mixture supplementation to NASH rats has improved the serum albumin level (Hadizadeh, Faghihimani, & Adibi, 2017). The active hepatocyte is enriched with the ALT and AST to carry out the normal metabolic function. The onset of direct

hepatocyte injury releases such markers into the bloodstream and indicates the abnormality of the liver and is noticed to be elevated in rats fed with the MCD diet (Hadizadeh et al., 2017). The co- and pre-administration of the nutrient mixture to steatosis rats resulted in the decrease of all of these markers, indicating the role of the nutrient mixture as a hepatoprotective agent by reducing the damage done to the hepatocytes by other metabolic factors (Cunningham et al., 2018; Kargulewicz et al., 2014).

There was a marked decrease in the levels of serum TG, TC, HDL-C, and an increase in LDL-C in NAFLD when compared to healthy control animals (Huang, Tung, Hsia, Wu, & Yen, 2017; Wang, Li, et al., 2014). Supplementation of the nutrient mixture to NAFLD rats led to an increase in serum TG, TC, HDL-C, and a decrease in serum LDL-C levels, indicating the nutrient mixture's anti-steatotic effects, which has been established in earlier studies (Nithyananthan et al., 2020). Studies have revealed that *V. radiata* regulates the growth of gut microbiota and decreases the absorption of toxic substances through the intestine and also reduces the risk of development of hypercholesterolemia and cardiovascular disease (Kruawan, 2012). Other studies involving the supplementation of *V. radiata* powder and extracts to hyperlipidemic rabbits and normal rats have shown a decrease in serum cholesterol levels. This was accounted as follows: The polysterols in the seed extract prevents the synthesis of cholesterol and its absorption (Bei, Guo, Wu, & Cao, 2012; Tang, Dong, Ren, Li, & He, 2014). Supplementation of extracts of *M. uniflorum* to high-fat diet-fed rats significantly decreased serum TG, TC, LDL, VLDL, SGOT, and SGPT levels and elevated HDL levels in previous studies (Kumar, Prashanthi, Avasarala, & Banji, 2013). *T. foenum graecum* was noticed to possess anti-hyperlipidemic ability by modulating the lipogenic enzyme activity in alloxan-induced diabetes rats and maintains glucose homeostasis (Raju, Gupta, Rao, Yadava, & Baquer, 2001). One or many of these mechanisms by the components of the nutrient mixture may involve in the reduction of steatosis.

In the case of hepatic lipid content, the results were as follows—MCD fed rats had increased hepatic levels of TC, TG, HDL, and LDL levels and supplementation of the nutrient mixture has resulted in a significant decrease in all of these parameters, demonstrated the anti-lipidemic ability of the nutrient mixture. Curcumin supplementation to MCD diet fed mice has decreased CYP2E1 & Prx1 expression, up-regulated of Prx6 thereby decreased intrahepatic FFA and TG level (Lee, Kang, Iqbal, & Kwon, 2015). Curcumin acts on the bile acid metabolism pathways intestinal gut microflora causing NAFLD (Rao, Sekhara, Satyanarayana, & Srinivasan, 2018). In a study involving high-fat diet-fed hamsters, the supplementation of curcumin was found to decrease the hepatic lipid accumulation by downregulating the hepatic fatty acid synthase enzyme and upregulation of β-oxidation of fatty acids (Jang et al., 2008). All of these studies demonstrate possible mechanisms by which the nutrient mixture might interact with the hepatic fatty acid metabolism pathways or the intestinal microbiota to ameliorate the diet-induced hepatic steatosis in these animals.

Significant increase in the anti-oxidant parameters such as CAT, SOD, GPx, GSH, and the decrease of TBARS levels in the nutrient mixture supplied NAFLD depicts the antioxidant nature of the nutrient mixture. All the substances in the nutrient mixture contain the well-established anti-oxidant capacity and medicinal property may function through one or many of the pathways as follows. The seeds of *V. radiata* are known for their radical scavenging activities against DPPH and also have a significant role in their action against lipid peroxidation of the membrane by their ferric reducing antioxidant power (Lee et al., 2011). A study on the supplementation of a green gram to rats has significantly increased the levels of antioxidant enzymes (Rajagopal, Pushpan, & Antony, 2017). The polyphenol in these pulses can scavenge free radicals and reactive oxygen species (Kawsar, Huq, Nahar, & Ozeki, 2008; Tang et al., 2014). In addition to this, sprouted seeds have significant increased levels of total polyphenols in pulses (Kim, Jeong, Gorinstein, & Chon, 2012). The antioxidant capacity of *T. foenum graecum* aqueous extract was studied in rats. The study showed that the high ferric reducing activity of extract by the polyphenols, flavonoids, and other water-soluble components had protected Fe^{2+}-ascorbate-induced lipid peroxidation (Dixit, Ghaskadbi, Mohan, & Devasagayam, 2005). Studies have also been performed to demonstrate the effect of *T. foenum graecum* to elevate the levels of other antioxidant enzymes such as SOD, GPx, and GST (Sushma & Devasena, 2010). Curcumin, the bioactive compound found in *Curcuma longa*, is known to possess anti-oxidant properties (Jiang et al., 2017). The decreased percentage area of lipid accumulation and the degree of fibrosis was evidenced (histological studies) in the nutrient mixture supplied steatotic rats. The nutrient mixture supplementation has reduced de novo lipogenesis, increased β-oxidation, and VLDL secretion. The expression of α-SMA is markedly increased in NASH rats and was significantly decreased in the nutrient mixture supplied NASH rats (Washington et al., 2000). The MMPs are zinc-dependent endopeptidases that participate in the extracellular matrix (ECM) remodeling. The activity of MMP-2 and MMP-9 has been noted to increase in the early-stage liver disease itself and the inflammatory period of NASH (D'Amico et al., 2010; Palladini et al., 2019). In the present study, nutrient mixture supplementation has reduced MMP-9 in NASH animals and these findings are consisting of reduced Sirius red positivity for fibrosis. These findings depict that the nutritional support provided by the nutrient mixture enriched with the medicinal value by curcumin and fenugreek could manage liver in a healthy status. The antioxidant support from this nutrient mixture manages oxidative stress created by the lipotoxicity and the hepatic stellate cells in an inactive stage thereby delays the steatohepatitis. The preventive studies with this nutrient mixture have displayed better protection than the treatment condition and consumption of this nutrient mixture in day to day life could prevent and protect the NAFLD.

5 | CONCLUSION

In the complete, the adjustment of diet could be an efficient therapy for the treatment of NASH. Combination of different herbals having

different biological roles and biological targets could work on various stages of NAFLD pathogenesis. The nutrient mixture made with various germinated cereals, fenugreek and turmeric displayed, nutritional value, antioxidant, and anti-inflammatory properties and could be the reason to protect the liver from NASH and maintains the hepatic stellate cells in the inactive stage. The nutrient mixture supplementation effectively prevented the progression of NASH from hepatic steatosis. The pre-supplementation of the nutrient mixture has better NAFLD preventive and ameliorative potential.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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