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A blend of Sphaeranthus indicus flower head and Terminalia chebula fruit extracts reduces fatty liver and improves liver function in non-alcoholic, overweight adults

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ABSTRACT

Background: Excessive accumulation of fat in the liver is a common clinical presentation in non-alcoholic fatty liver disease (NAFLD). The increasing prevalence of NAFLD is a growing health problem worldwide. The major risk factors of NAFLD include obesity, hyperlipidemia, and insulin resistance.

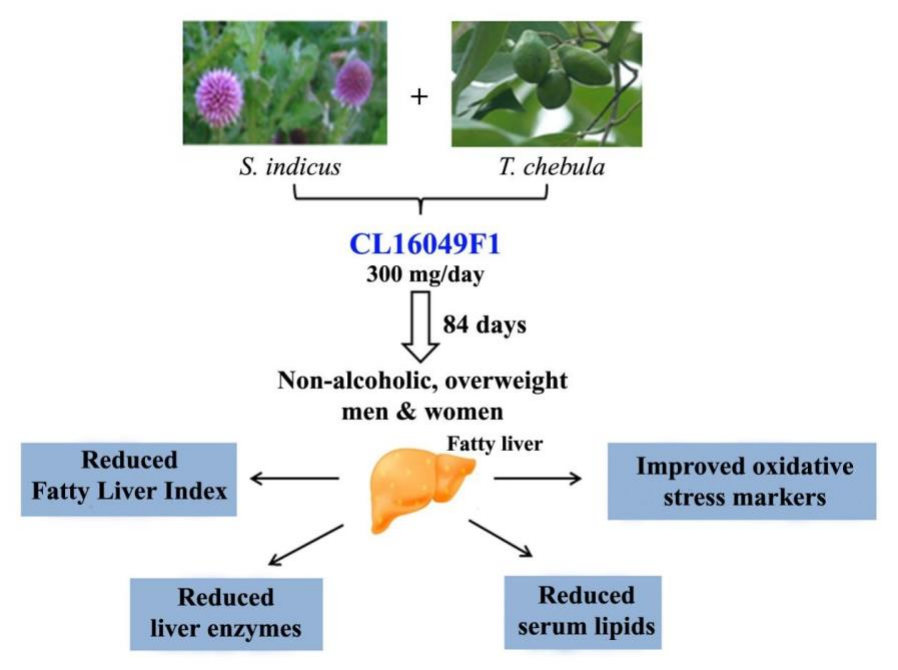
Objective: The aim of the present randomized, double-blind, placebo-controlled clinical trial was to assess the efficacy and tolerability of an herbal composition CL16049F1 in reducing fatty liver conditions and improving liver function in non-alcoholic, overweight individuals. CL16049F1 is a patented© blend of *Sphaeranthus indicus* flower head and *Terminalia chebula* fruit extracts.

Materials and Methods: Ninety male and female subjects (age: 25-60 yrs., BMI: 23-29 kg/m^2) with elevated Fatty Liver Index (FLI) between 31 and 59 were randomized into three groups (n=30). The participants received a daily dose of 300 mg CL16049F1, 320 mg Silymarin, or a placebo over a period of 84 consecutive days. FLI was evaluated as the primary efficacy measure. The secondary efficacy measures included the liver enzymes, lipid profile, and oxidative stress markers in serum, A 36-Item Short-Form Health Survey (SF-36), and Gastrointestinal symptoms (GIS) score were also evaluated. The clinical biochemistry, hematology, urine, and vital signs were evaluated as safety measures. Adverse events were also monitored.

Results: Eighty-eight subjects completed the study. Post-trial, the FLI score of CL16049F1-supplemented subjects was reduced by 13.81% ($p<0.05$) and 16.08% ($p<0.05$), while the Silymarin supplemented group exhibited reductions of 7.50% ($p<0.05$) and 7.27% ($p<0.05$) as compared to baseline and placebo, respectively. CL16049F1 supplementation significantly improved the liver enzymes, lipid profile, and oxidative markers in serum. The changes in the secondary efficacy measures in these treatment groups are comparable. No major adverse events were observed.

Conclusion: CL16049F1 is a well-tolerated and effective herbal formulation to reduce fatty liver and improve liver function in non-alcoholic, overweight subjects.

Keywords: Non-alcoholic fatty liver disease, Fatty liver index, CL16049F1, hepatoprotective botanical formulation.



INTRODUCTION

Fatty liver condition in non-alcoholic fatty liver disease (NAFLD) is an emerging public health issue, a major cause of liver dysfunction, liver-related morbidity, and mortality worldwide [1-2]. NAFLD refers to a progressive, broad spectrum of pathological liver conditions that includes fatty liver (steatosis), fibrosis, cirrhosis, and associated complications in non-alcoholic subjects [3]. The Dionysos Nutrition and Liver Study and the Dallas Heart Study estimated that 25% of the Italians and 30% of the Americans were suffering from NAFLD [4-5]. In the general population, NAFLD is prevalent among India's geographical regions, ranging from 9% to 53% [6]. A meta-analysis in 2019 suggested the global estimate of NAFLD prevalence was 25.2% [7]. NAFLD is prevalent up to 70% in obese; in contrast, it is estimated to be around

35% in non-obese subjects [8].

Measurement of the fatty liver index (FLI) has been considered a simple, reliable, and non-invasive method to monitor the progression of fatty liver in subjects with NAFLD. FLI is calculated using body mass index (BMI), waist circumference (WC), and levels of γ-glutamyl transferase (GGT) and triglycerides in serum. FLI can estimate fatty liver severity and hepatic fat accumulation with high accuracy [9-10].

Currently, no drug is approved to treat fatty liver conditions or restore normal liver function in NAFLD [11]. Among the nonpharmacological approaches, Silymarin (*Silybum marianum)* seed extract has been shown clinically effective in alleviating liver dysfunction, including fatty liver symptoms in non-alcoholic subjects. Silymarin scavenges free radicals and helps modulate the enzymes associated with liver toxicity, fibrosis, and cirrhosis [12]. Additionally, silymarin is a safe and efficacious botanical ingredient with a long traditional history of human consumption.

CL16049F1 is a patented© herbal combination containing extracts of *Terminalia chebula* fruit and *Sphaeranthus indicus* flower head to mitigate fatty liver conditions and improve liver function in non-alcoholic, overweight individuals. Earlier studies suggested that *Terminalia chebula* was hepatoprotective [13] and that *Sphaeranthus indicus* improved liver enzymes, lipid profiles, and glycemic indices in various preclinical animal models [14].

T. chebula fruit contains a variety of phytochemical constituents, including tannins, phenolic acids, and flavonoids [15]. Terminalia phenolics are considered the bioactive constituents of *T. chebula* fruit. Chebulinic acid (CA), a phenolic compound in *T. chebula* fruit, has a wide variety of bioactivities such as antioxidant, anticancer, and antihypertensive properties [15]. In a recent study, CA has demonstrated hepatoprotective activity in cellular and *in vivo* models [16].

Earlier, a methanolic extract of *Sphaeranthus indicus* enhanced the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) and diminished the quantity of lipid peroxides against acetaminophen-induced hepatotoxicity, suggesting a reduction of oxidative stress and displaying its hepatoprotective potential [17].

The present study demonstrates that the daily supplementation of CL16049F1 over a period of eighty-four consecutive days results in a significant reduction in the fatty liver index (FLI) of non-alcoholic, overweight participants. In parallel, CL16049F1 supplementation also improved the participants' liver function, lipid profile, gastrointestinal symptoms, and quality of life at the end of the trial. None of the participants reported any major adverse events following supplementation of CL16049F1; their vital signs, hematological, and biochemical parameters were not significantly different from the placebo. In summary, the herbal blend CL16049F1 reduced fatty liver and improved liver function in non-alcoholic, overweight subjects.

MATERIALS AND METHODS

Study Materials: CL16049F1 is a patented© composition containing a 95% blend of aqueous extracts of *Terminalia chebula* fruit and *Sphaeranthus indicus* flower heads at a 2:1 ratio. The extracts of *Terminalia chebula* fruit and *Sphaeranthus indicus* flower heads were blended into a homogeneous mixture with Maltodextrin and Silica as excipients. CL16049F1 was standardized to contain at least 1.5% gallic acid and 0.2% chlorogenic acids, which was produced in a cGMP-certified manufacturing facility

at Chemiloids Life Sciences Pvt. Ltd, Aswaraopet, Telangana, India.

Silymarin was procured from Panjin Jiangyuan Bio-Product Co. Ltd., Panjin City, China. According to the certificate of analysis provided by the manufacturer, the combined Silybin A and Silybin B content in the Silymarin sample was 58.13%. The daily dose of 320 mg Silymarin delivered 186 mg of Silybins.

Plant raw materials and extraction procedures: The plant raw materials *Terminalia chebula* fruit and *Sphaeranthus indicus* flower heads were procured from the approved vendors from Shimoga district, Karnataka, and Kalahandi district, Orissa, respectively, in India, and they were identified by a qualified herbalist and taxonomist. Those were compared with the authentic raw materials (RDM) and their voucher specimens (*S. indicus*: CL 6391, and *T. chebula*: CL 6274 are preserved in the Taxonomy Division of Chemiloids Life Sciences R&D Center, Ashwaraopet, India. *Terminalia chebula* fruit and *Sphaeranthus indicus* flower heads were processed separately. The dried raw materials were pulverized to coarse powders and extracted with water at 70-80°C under continuous percolation. The extracts were filtered and concentrated under vacuum at 60-70°C to obtain individual extracts as brown color thick paste and brown color dry powder, respectively.

HPLC Analysis: Analysis of CL16049F1 was carried out using Waters high performance liquid chromatographic system equipped with a thermostat-controlled column oven compartment, autosampler, photodiode array detector, and Empower 3 software (Waters Corporation, Milford, MA). The sample preparation includes the extraction of the sample using water-methanol (80:20), followed by filtration through a 0.22 µm PVDF filter. The sample solution was analyzed using Waters column, X Bridge C18 3.5 µm (100 x 4.6 mm).

A gradient elution system consists of solvent A [10 mM ammonium acetate in water; pH 4.0] and solvent B [Acetonitrile: Methanol (50:50)] as a mobile phase at a flow rate of 0.8 mL/min. The elution started at sample injection with a mixture of 97% A and 3% B as initial eluent, and a linear gradient was used to reach 82% A and 18% B in 20 minutes; then linear gradient was used to reach 80% A and 20% B in 5 minutes, and finally maintained an isocratic run at 80% A and 20% B for 5 minutes. The column oven compartment was maintained at 40°C.

Subject Recruitment and Randomization: The present clinical study protocol was approved by the institutional ethics committees of two independent sites in Andhra Pradesh, India, at Aditya Multi-Speciality Hospital, Guntur (IRB no. ECR/1347/Inst/AP/2020) and KIMS Super Speciality Hospital, Nellore (IRB no. ECR/453/Inst/AP/2013/RR-19). The approved protocol was registered in the Clinical Trial Registry of India (CTRI/2020/05/025322). The trial was conducted following the ethical principles of the Declaration of Helsinki and was compliant with the guidelines of Good Clinical Practice.

Participants visiting the outpatient departments of the clinics were selected for screening. After voluntarily signing informed consent, male and female participants were screened for enrollment in the study based on inclusion and exclusion criteria (Table 1). Briefly, the criteria for eligibility were that the participants between 25 and 60 years, BMI 23-29 $kg/m^{2,}$ and had a fatty liver

index between 31 and 59; their fatty liver condition was confirmed by abdominal ultrasound scan (BC5 Doppler System, Konica Minolta Healthcare India Pvt. Ltd., Mumbai, India). The individuals with HIV, autoimmune liver diseases, diabetes, or under medication for any chronic diseases, including hyperlipidemia, were not recruited for the study.

Table 1: Inclusion/ Exclusion Criteria

Criteria	Description
Inclusion	Ambulatory, male and female subjects 25–60 years of age
	Body mass Index (BMI) between 23.0 and 29.0 kg/m^2
	Fatty Liver Index (FLI) between 31 and 59, with no pre-existing medical conditions
	Fasting blood glucose levels <125 mg/dL
	Clinical lab observations were within normal range
	Not taking medicines or supplements for liver health benefits
	Non-alcoholic and non-smokers
	Agreed to maintain diet tracker
Exclusion	Hepatic abnormalities confirmed by ultrasound scan
	Gastrointestinal diseases such as crohn's disease or gastrointestinal surgery
	Viral Hepatitis or autoimmune liver diseases
	History of underlying biliary diseases such as jaundice, gallstones, hematological, renal or thyroid dysfunction or psychiatric disorder
	Pregnant, breast feeding or planning to become pregnant during the study period
	Subjects participated in any other clinical trials within 60 days before the screening
	HIV positive

Ninety enrolled participants were randomized into three groups, Placebo, CL16049F1, and Silymarin. Randomization code was generated by SAS 9.4 by block randomization using SAS procedure PROC PLAN. Each group contained thirty subjects. The subjects were advised to maintain their regular dietary habits and instructed to take one capsule of 300 mg CL16049F1, 320 mg Silymarin, or a matched placebo after dinner over a period of 84 consecutive days. The study consisted of a screening/randomization (visit 1), baseline (visit 2), the

first follow-up on day 28 (visit 3), the second follow-up on day 56 (visit 4), and the final visit on day 84 (visit 5). Subject enrollment, allocation, follow-up, and analysis are summarized in figure 1. Study capsules, compliance cards, a list of instructions, and dates of follow-up evaluations were provided to all participants at the baseline visit. A study dietitian shared the daily menu with each subject and provided counseling to assist participants in maintaining the standard diet during the study.

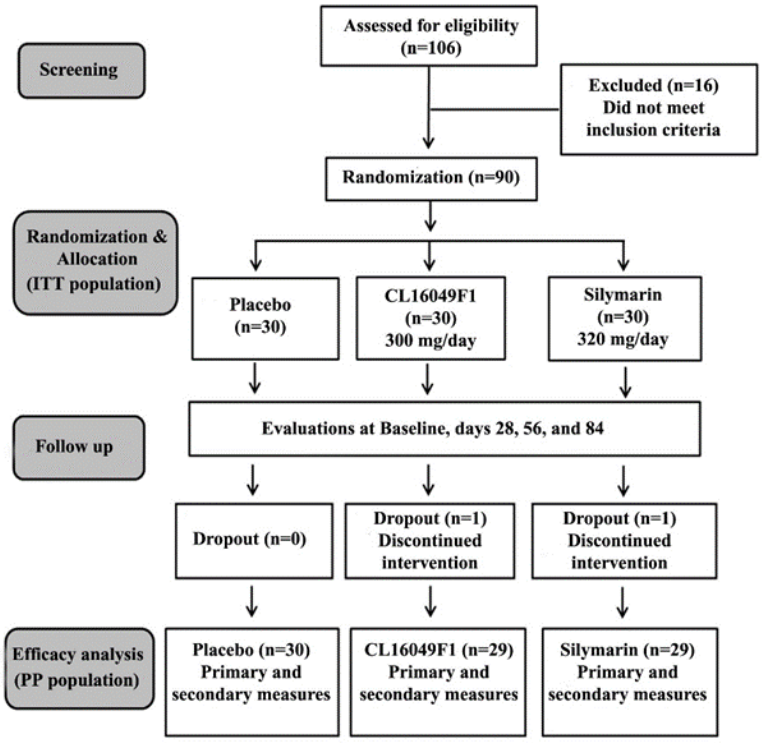


Figure 1: A Consort flow diagram presents participant enrollment and the subsequent steps of the trial.

Participants' body weights and waist circumference were measured using standard procedures at baseline and subsequent follow-up visits. The height of the participants recorded at the screening visit was considered for body mass index (BMI) calculations at all visits during the study.

The subjects registered their activities, including adverse events, in the daily diary and compliance card. The Compliance of the investigational products (IPs) administration was verified by checking the unused capsules returned to the study coordinator and the records in their daily diary and compliance card during the follow-up visits. The study coordinators were in regular touch with the study participants.

Assessment of the liver function tests (LFT), including ALT (Alanine transaminase), AST (Aspartate transaminase), GGT (γ-glutamyl transferase), ALP (Alkaline phosphatase), serum albumin globulin ratio (A/G ratio), and lipid profile was performed at baseline, follow-up visits and end of the study.

Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) of the participants was measured at baseline and end of the study using the formula: [fasting serum insulin (μU/mL) × fasting glucose (nmol/L)]/22.5 [18].

For safety evaluation, routine hematology, clinical

biochemistry, and urine analysis were performed at baseline and end of the study. In addition, important vital signs such as blood pressure (systolic/diastolic), pulse rate, respiratory rate, and body temperature were also monitored.

Fatty Liver Index (FLI): The FLI was calculated utilizing the waist circumference, body mass index (BMI), serum triglyceride, and γ-glutamyl-transferase (GGT) of the participants using the following formula [9]:

$$FLI = [e^{0.953}\times\log_e(TG) + 0.139\times BMI + 0.718\times\log_e(GGT) + 0.053\times WC - 15.745)] / [1+e^{0.953}\times \log_e(TG) + 0.139\times BMI + 0.718\times\log_e(GGT) + 0.053\times WC - 15.745] \times 100.$$

Serum triglyceride (TG) was measured in mmol/l, GGT in U/l, and waist circumference (WC) in cm.

Clinical Biochemistry, Hematology, and Urinalysis: In clinical biochemistry, serum fasting glucose, creatinine, uric acid, blood urea nitrogen, the LFT parameters, high-density lipoproteins (HDL), low-density lipoprotein (LDL), triglycerides (TG), total cholesterol (TC), and very-low-density lipoprotein (VLDL), sodium, potassium, albumin, and globulin were measured. Routine hematological assessments, including counts of red blood cells, platelet, total and differential leukocytes, hemoglobin, and ESR were conducted. Clinical biochemistry and hematological parameters were assessed using Rx-Daytona+ Automated Analyzer (Randox Laboratories, Kearneysville, WV), and an automated 6-part differential hematology analyzer (XN-450, Sysmex India Pvt Ltd, Mumbai, Maharastra, India), respectively.

In routine urine analysis, color, specific gravity, pH, glucose, protein measurements, and RBC counts were included. Urine analysis was carried out using DIRUI H10 Urine Test Strip (Dirui Industrial Co., Ltd. Chang Chun, China) and by microscopy (Vision 2000, Labomed Inc., Los Angeles, CA).

Serum Biomarkers: Oxidative stress markers viz., superoxide dismutase [SOD; Kinesis Dx, CA, USA], malondialdehyde [MDA; Krishgen Biosystems, Mumbai, India], thiobarbituric acid reactive substances [TBARS; R&D Systems, MN, USA], reduced glutathione [GSH; Biovision, CA, USA], and a kidney function marker Cystatin C [Brno, Czech Republic] were assessed in the serum samples following the manufacturer's protocol. The assay sensitivities of SOD, MDA, TBARS, GSH, and Cystatin C kits were 4 ng/mL, 0.14 nmol/mL, 0.024 µmol, 80 pmol/mL and 0.25 ng/mL, respectively.

Short Form-36 health survey (SF-36): SF-36 is a tool used for assessing health-related quality of life. SF-36 score consists of generic questions© that measure physical and mental health status [19]. The questionnaire comprises eight domains: (A) physical functioning, (B) physical role functioning, (C) bodily pain, (D) general health perceptions, (E) vitality, (F) social functioning, (G) emotional role functioning, and (H) mental health. The scores for the SF-36 scales range from 0 to 100, with a higher score indicating a better health status.

Gastrointestinal symptoms (GIS) questionnaire: GIS questionnaire is an evaluation for identifying gastrointestinal symptoms and is used in a standardized form to ascertain dyspeptic symptoms in patients with functional dyspepsia [20]. These symptoms are assessed individually based on a 5-point Likert scale from 0,

indicating no complaints, to 4, indicating very severe discomfort.

Sample size calculation and statistical Analysis: The sample size was calculated considering approximately 26 subjects per treatment group to provide 90% power to detect a treatment effect in the primary efficacy variable at a two-sided significance level of 5% (α = 0.05). A mean difference of -22.0 and a deviation size of approximately 23.8 was considered for sample size calculation based on an earlier clinical study of *Cynara cardunculus* extract supplementation in subjects with metabolic syndrome [21]. Considering an assumption of 10 to 15% dropout (~ 4 subjects per group), approximately 30 subjects per group were randomized to obtain a power of 90% of meeting the primary objective.

All data are expressed as mean ± SD. The efficacy parameters were tested for significance using paired t-tests for intra-group comparisons and ANCOVA for inter group comparisons. Tukey's multiple comparison test was used for the pairwise treatment comparisons in ANCOVA for multiplicity adjustment. One-way ANOVA was used to evaluate baseline characteristics, vital signs, and laboratory parameters with the statistical significance level at $p<0.05$. Descriptive statistics were conducted on demographics and baseline characteristics, clinical laboratory assessments, and vital signs. The statistical tool used for data analysis was SAS 9.4.

RESULTS:

HPLC analysis of CL16049F1: A typical HPLC chromatographic profile of CL16049F1 at 240 nm shows three peaks at 2.082-, 5.787-, and 23.715 min, representing gallic acid, 5-caffeoylquinic acid, and 3, 4-dicaffeoylquinic acid, respectively (Figure 2). The identification of these peaks was confirmed using respective reference standards.

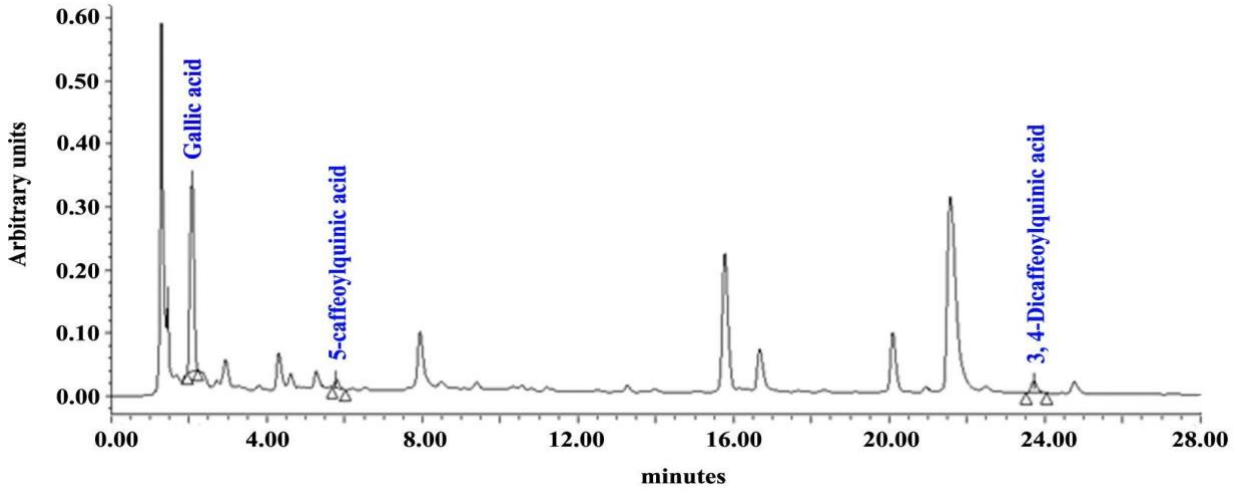


Figure 2: The chromatographic profile of CL16049F1 at 240 nm. Three peaks at 2.082, 5.787, and 23.715 min correspond to Gallic acid, 5-caffeoylquinic acid, and 3, 4-Dicaffeoylquinic acid, respectively.

Demographic and baseline characteristics: The demographic variables and baseline characteristics are summarized in table 2. Participants were randomly distributed into placebo and treatment groups. Overall, the active group receiving CL16049F1 (300 mg/day, n = 30), Silymarin (320 mg/day, n=30), or placebo (n = 30) were not statistically different at baseline with respect to any demographic characteristic (Table 2).

Table 2: Baseline demographic characteristics of the participants in placebo, CL16049F1, and silymarin supplemented groups

Characteristics	Placebo (n=30)	CL16049F1 (n=30)	Silymarin (n=30)
Men (%)	16 (53.3)	16 (53.3)	14 (46.7)
Women (%)	14 (46.7)	14 (46.7)	16 (53.3)
Age (yrs)	36.6 ± 7.5	36.9 ± 9.2	40.2 ± 10.1
Body Weight (kg)	73.7 ± 8.1	74.6 ± 7.6	74.7 ± 7.6
BMI (kg/m²)	26.5 ± 1.5	27.1 ± 1.6	27.1 ± 1.6
Waist circumference (cm)	91.12 ± 8.52	91.67 ± 8.68	94.30 ± 7.55
Fatty liver index (FLI)	44.83 ± 8.61	47.38 ± 9.12	48.71 ± 7.84

Fatty Liver Index (FLI): In the current study, CL16049F1 supplementation significantly reduced the FLI as early as 56 days, while in the Silymarin group, the active comparator, the improvement was observed at the end of the study (84 days) as compared to the baseline. Changes from baseline FLI in these three groups are summarized in table 3. CL16049F1 supplementation over a period of 84 days exhibited a 13.80% reduction in FLI from baseline ($p<0.0001$). Whereas, in the placebo group, FLI was significantly ($p=0.0284$) increased by 7.67% from baseline. It is interesting to note that the CL16049F1 group significantly ($p=0.0123$) reduced the mean FLI at 56 days of supplementation from baseline, while the reduction in FLI in the Silymarin group was not statistically significant ($p=0.5631$). At the end of 84 days of supplementation, a significant reduction of 16.07% in FLI ($p<0.0001$) was observed in the CL16049F1 group as compared to the placebo.

Table 3: Reduction in the fatty liver index (FLI) in the Placebo, CL16049F1, and Silymarin groups.

Evaluations	Placebo (n=30)	CL16049F1 (n=29)	Silymarin (n=29)
Baseline	44.83 ± 8.61	47.00 ± 9.02	48.39 ± 7.77
Day 28	48.53 ± 8.92*	45.49 ± 10.34$	47.05 ± 9.32
Day 56	46.22 ± 9.02	43.25 ± 10.33*$	46.81 ± 6.91
Day 84	48.27 ± 8.12*	40.51 ± 9.67*$	44.76 ± 6.87*$

*and $ indicate significances ($p<0.05$) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

Metabolic parameters: Analysis of secondary outcomes evidenced that CL16049F1 intake significantly reduced the level of liver enzymes, including ALT, AST, ALP, and GGT (Table 4). Improvement in liver function on day 56 of supplementation was observed with a 17.30% reduction in serum GGT in the CL16049F1 group ($p<0.0007$) from baseline. Eighty-four days supplementation of CL16049F1 significantly reduced AST (14.26%; $p=0.0104$), ALP (8.91%; $p=0.0003$), A/G ratio (12.67%; $p<0.0001$, and HOMA-IR (16.66%; $p=0.0018$), compared with baseline (Table 4). Also, the changes in these parameters in the CL16049F1 group are significant as compared to the placebo. As expected, on day 84, the Silymarin-supplemented group also showed significant reductions in GGT (9.62%; $p=0.0420$), A/G ratio (8.10%; $p=0.0154$), HOMA-IR (8.64%; $p=0.0101$) from baseline and ALT (21.91%; $p=0.0052$) from placebo (Table 4). At the end of the study, the changes in serum Cystatin C levels in the placebo and CL16049F1 groups were not significant, but the Silymarin group showed a significant increase in Cystatin C levels from baseline (Table 4).

Table 4: Changes in serum metabolic parameters in the Placebo, CL16049F1, and Silymarin groups

Parameters	Evaluations	Placebo (n=30)	CL16049F1 (n=29)	Silymarin (n=29)
GGT (U/L)	Baseline	27.29 ± 8.79	27.05 ± 9.87	27.13 ± 10.46
	Day 28	26.87 ± 8.23	26.71 ± 12.80	27.16 ± 11.36
	Day 56	27.39 ± 10.27	22.37 ± 7.08*$	23.82 ± 7.58*
	Day 84	32.82 ± 8.21*	21.73 ± 6.99*$	24.52 ± 5.73*$
ALT/SGPT (IU/L)	Baseline	15.40 ± 7.90	17.79 ± 8.78	16.07 ± 7.19
	Day 28	18.87 ± 6.92	18.00 ± 7.80	15.97 ± 6.17
	Day 56	18.63 ± 6.90	16.76 ± 8.29	17.21 ± 5.25
	Day 84	19.03 ± 7.05*	15.24 ± 5.15$	14.86 ± 4.62$
AST/SGOT (IU/L)	Baseline	22.53 ± 5.70	25.66 ± 8.26	25.59 ± 8.38
	Day 28	30.27 ± 7.57*	25.97 ± 4.99$	26.69 ± 5.81
	Day 56	31.93 ± 10.48*	24.21 ± 5.09$	26.59 ± 5.96$
	Day 84	25.70 ± 7.56	22.00 ± 5.63*$	24.38 ± 4.77
ALP (U/L)	Baseline	92.57 ± 15.44	90.10 ± 12.98	96.34 ± 16.31
	Day 28	96.77 ± 20.33	92.69 ± 19.16	98.14 ± 30.15
	Day 56	92.97 ± 19.32	82.14 ± 34.90	84.55 ± 24.64*
	Day 84	92.90 ± 14.64	82.07 ± 16.99*$	89.79 ± 16.57
Albumin/globulin ratio (A/G ratio)	Baseline	1.38 ± 0.31	1.42 ± 0.35	1.48 ± 0.41
	Day 28	1.35 ± 0.28	1.39 ± 0.36	1.45 ± 0.43
	Day 56	1.35 ± 0.21	1.33 ± 0.35*	1.42 ± 0.44
	Day 84	1.40±0.26	1.24 ± 0.32*$	1.36 ± 0.43*
HOMA-IR	Baseline	2.20 ± 0.73	2.34 ± 1.04	2.20 ± 0.93
	Day 84	2.09 ± 0.51	1.95 ± 0.48*	2.01 ± 0.47*
Cystatin C (mg/L)	Baseline	0.83 ± 0.09	0.88 ± 0.10	0.83 ± 0.13
	Day 84	0.88 ± 0.16	0.90 ± 0.13	0.91 ± 0.19*

*and $ indicate significances ($p<0.05$) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

Serum lipid profile: Eighty-four days of CL16049F1 supplementation significantly reduced serum LDL (16.24%; p<0.0001) and total cholesterol levels (9.80%; p=0.0031) as compared to the baseline (Table 5). The CL16049F1 group also exhibited significant reductions in triglyceride (15.16%; p=0.0492) and cholesterol (10.22%, p=0.0125) levels as compared to the placebo. Interestingly, on day 56, the reductions in serum LDL and total cholesterol levels were also significant as compared to placebo and baseline (Table 5). Post-trial, the Silymarin group also exhibited reductions in the serum LDL (9.84%; p=0.0390) and cholesterol (6.14%; p=0.0396) levels as compared to the baseline (Table 5).

Table 5: Changes in serum fat metabolism markers in the Placebo, CL16049F1, and Silymarin groups

Parameters	Evaluations	Placebo (n=30)	CL16049F1 (n=29)	Silymarin (n=29)
Triglycerides (mg/dL)	Baseline	145.23 ± 52.74	142.07 ± 46.96	128.17 ± 38.02
	Day 28	170.57 ± 53.55*	141.69 ± 46.00$	123.41 ± 35.87$
	Day 56	155.47 ± 42.30*	140.03 ± 36.78	136.38 ± 37.18
	Day 84	155.27 ± 63.93	131.72 ± 33.28$	124.31 ± 32.46$
LDL (mg/dL)	Baseline	107.53 ± 21.49	112.10 ± 30.37	113.41 ± 27.42
	Day 28	110.63 ± 16.78	108.10 ± 16.60	108.83 ± 21.77
	Day 56	110.73 ± 23.47	99.97 ± 15.79*$	103.93 ± 16.00*
	Day 84	108.53 ± 27.92	93.90 ± 9.99*$	102.24 ± 22.86*
HDL (mg/dL)	Baseline	40.63 ± 4.21	40.97 ± 7.56	40.72 ± 5.61
	Day 28	39.53 ± 3.68	41.14 ± 5.30	42.21 ± 5.39$
	Day 56	41.97 ± 4.66	42.59 ± 9.08	45.17 ± 10.90*
	Day 84	41.87 ± 3.18	43.45 ± 4.13*	41.62 ± 5.21
VLDL (mg/dL)	Baseline	28.67 ± 11.10	28.41 ± 9.39	25.63 ± 7.60
	Day 28	33.77 ± 11.00*	28.34 ± 9.20$	24.68 ± 7.17$
	Day 56	30.39 ± 8.12	28.01 ± 7.36	27.28 ± 7.44
	Day 84	30.50 ± 13.20	26.34 ± 6.66	24.86 ± 6.49
Total cholesterol (mg/dL)	Baseline	176.83 ± 24.08	181.48 ± 33.30	179.77 ± 30.20
	Day 28	183.93 ± 22.33	177.58 ± 19.92	175.72 ± 26.95
	Day 56	183.95 ± 24.36	170.56 ± 15.83*$	176.38 ±17.94
	Day 84	182.33 ± 36.84	163.69 ± 12.66*$	168.72 ± 26.24*

*and $ indicate significances (p<0.05) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

Oxidative stress markers: The effect of CL16049F1 supplementation on oxidative stress markers in the serum samples is presented in table 6. Post-trial, CL16049F1 significantly reduced MDA (26.50%, vs. baseline p<0.0001; 22.64%; vs. placebo p= 0.0213) and TBARS (6.61%, vs. baseline p=0.0002; 8.13%, vs. placebo p=0.0141) levels in the serum samples. Also, CL16049F1 supplementation increased serum SOD (33.76%, p=0.0087 vs. placebo; 40.64%, p= 0.0001 vs. baseline) and GSH (44.31% p=0.0112 vs. placebo; 36.87%, p= 0.0004 vs. baseline) levels at the end of the study (Table 6). Eighty-four days supplementation of Silymarin also significantly increased the serum SOD (20.13%; p=0.0404) and GSH levels (37.98%; p=0.0006) as compared to the baseline. Silymarin supplementation also exhibited significant reductions in TBARS levels as compared to both placebo and baseline (Table 6).

Table 6: Changes in Oxidative stress markers in the serum samples of CL16049F1, Placebo, and Silymarin groups

Parameters	Evaluations	Placebo (n=28)	CL16049F1 (n=28)	Silymarin (n=28)
SOD (ng/ml)	Baseline	35.93 ± 14.05	33.95 ± 9.41	34.56 ± 8.27
	Day 84	35.69 ± 11.69	47.74 ± 21.71*$	41.52 ± 16.40*
MDA (nmol/ml)	Baseline	8.04 ± 4.09	7.81 ± 3.33	7.77 ± 3.26
	Day 84	7.42 ± 3.74	5.74 ± 1.82*$	6.91 ± 2.52
TBARS (µM/L)	Baseline	2.49 ± 0.28	2.42 ± 0.32	2.46 ± 0.34
	Day 84	2.46 ± 0.28	2.26 ± 0.24*$	2.29 ± 0.26*$
GSH (µM/L)	Baseline	5.79 ± 2.76	6.21 ± 2.99	5.87 ± 2.78
	Day 84	5.89 ± 2.98	8.50 ± 4.07*$	8.10 ± 3.85*$

*and $ indicate significances (p<0.05) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

SF-36 Score: Changes in SF-36 scores for the Placebo, CL16049F1 and Silymarin groups are summarized in table 7. Eighty-four days supplementation of CL16049F1 exhibited significant improvements in the Physical functioning (15.20%; p=0.0003), physical role functioning (11.57%; p=0.0144), emotional role functioning (13.22%; p=0.0027), vitality (16.70%; p<0.0001), social role functioning (7.72%; p=0.0282) and general health perceptions (7.86%; p<0.0001) as compared to baseline (Table 7). Also, improvement in the physical functioning score (20.10%, p=0.0002) of CL16049F1 group is significant at the end of 84 days of supplementation as compared to the placebo. The Silymarin supplemented group exhibited significant improvements in physical functioning (8.89%, vs. baseline p=0.0025 and 18.48%, vs. placebo p=0.0008); emotional role functioning (28.33%, vs. baseline p<0.0001; and 7.65%, vs. placebo p=0.0380) and vitality (22.04%, vs. baseline p<0.0001 and 12.39%, vs. placebo p= 0.0072) at the end of the study (Table 7).

Table 7: Changes in SF-36 scores in Placebo, CL16049F1 and Silymarin groups

SF-36 Domains	Evaluations	Placebo (n=30)	CL16049F1 (n=29)	Silymarin (n=29)
Physical functioning	Baseline	68.33 ± 20.01	66.90 ± 18.00	69.83 ± 17.19
	Day 28	68.67 ± 21.53	71.03 ± 13.85	73.10 ± 10.13
	Day 56	69.67 ± 15.42	73.45 ± 9.17*	74.31 ± 8.21*
	Day 84	64.17 ± 18.39	77.07 ± 9.40*$	76.03 ± 9.20*$
Physical role functioning	Baseline	85.00 ± 25.93	81.90 ± 28.27	87.93 ± 19.62
	Day 28	88.33 ± 25.20	89.66 ± 20.61*	89.66 ± 18.32
	Day 56	94.17 ± 15.65*	91.38 ± 19.22*	90.52 ± 18.19
	Day 84	85.00 ± 24.21	91.38 ± 21.42*	93.97 ± 17.24*
Emotional role functioning	Baseline	80.00 ± 35.67	78.16 ± 35.94	68.97 ± 45.37
	Day 28	93.33 ± 16.14*	89.66 ± 22.01*	93.10 ± 22.50*
	Day 56	88.89 ± 20.22*	90.80 ± 21.63*	88.51 ± 25.63*
	Day 84	82.22 ± 29.99	88.51 ± 22.32*	88.51 ± 27.13*$
Vitality	Baseline	66.67 ± 22.72	69.14 ± 18.37	65.69 ± 22.07
	Day 28	69.50 ± 18.91	72.24 ± 15.15*	68.62 ± 17.57
	Day 56	71.33 ± 16.18*	76.21 ± 13.41*	70.86 ± 16.48*
	Day 84	71.33 ± 17.07	80.69 ± 11.00*$	80.17 ± 11.91*$
Mental health	Baseline	78.40 ± 22.32	74.34 ± 25.45	73.10 ± 27.06
	Day 28	78.67 ± 21.25	75.03 ± 23.34	77.10 ± 22.04*
	Day 56	78.80 ± 21.91	75.45 ± 22.19	77.66 ± 22.00*$
	Day 84	76.13 ± 25.85	75.03 ± 24.11	75.59 ± 23.53*$
Social role functioning	Baseline	78.75 ± 24.16	78.02 ± 24.01	78.45 ± 22.88
	Day 28	78.75 ± 24.16	78.45 ± 22.63	78.45 ± 21.88
	Day 56	80.42 ± 23.83	78.02 ± 24.24	79.31 ± 23.69
	Day 84	79.17 ± 24.64	84.05 ± 22.88*	81.90 ± 22.06
General health perceptions	Baseline	69.17 ± 17.57	70.17±15.95	68.45 ± 15.82
	Day 28	70.67 ± 15.58	73.62±10.77*	70.52 ± 14.16
	Day 56	69.83 ± 16.99	73.45±11.58*	71.38 ± 13.62*
	Day 84	72.83 ± 13.50*	75.69±13.54*	72.76 ± 12.22*
Bodily pain	Baseline	79.42 ± 14.89	73.62±16.21	80.00 ± 13.21
	Day 28	81.25 ± 14.32	74.66±15.14	78.02 ± 15.12
	Day 56	82.42 ± 13.40	78.36±15.67	79.31 ± 15.31
	Day 84	86.42 ± 12.28*	79.57±15.95	83.53 ± 15.71

*and $ indicate significances ($p<0.05$) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

Gastrointestinal symptoms (GIS) Score: Table 8 summarizes the changes in total and each symptom score of GIS in the participants of the study. As compared to baseline and placebo, CL16049F1 supplementation significantly reduced the total GIS score by 43.18% (p=0.0078) and 43.02% (p=0.0278), respectively, at the end of the trial. In individual symptoms score analysis, the CL16049F1-supplemented group reduced epigastric pain (80.37%; p=0.0046 vs. baseline; 82.92%, p=0.0092 vs. placebo), and general intestinal symptoms (46.10%, p=0.0306 vs. baseline; 52.26% p=0.0321 vs. placebo) (Table 8). Similarly, post-trial, Silymarin supplementation also reduced the total GIS scores (36.33%, p= 0.0174 vs. baseline; and 50.96 %, p=0.0462 vs. placebo) and general intestinal symptoms (42.89%, p=0.0304 vs. baseline; 58.31%; p=0.0320 vs. placebo) (Table 8).

Table 8: Changes in gastrointestinal scores in Placebo, CL16049F1and Silymarin supplemented groups

GIS Domains	Evaluations	Placebo (n=30)	CL16049F1 (n=29)	Silymarin (n=29)
Abdominal pain	Baseline	1.40±1.16	1.69±1.83	1.07±1.03
	28 days	1.93±2.29	1.59±1.24	1.52±0.95
	56 days	1.60±2.37	1.34±1.04	1.24±0.58
	84 days	1.53±2.66	1.31±2.07	1.00±0.65
Epigastric pain	Baseline	1.00±1.36	1.07±1.44	0.90±0.77
	28 days	1.07±1.20	0.90±0.94	0.97±1.05
	56 days	0.77±1.65	0.52±0.69*	0.72±1.07
	84 days	1.23±2.75	0.21±0.49*$	0.59±1.05
General intestinal symptoms	Baseline	4.10±5.81	4.10±5.47	3.38±8.62
	28 days	4.40±5.59	3.93±4.31	2.79±2.40
	56 days	4.00±5.72	3.03±3.51	2.83±2.22
	84 days	4.63±7.99	2.21±2.23*$	1.93±1.85*$
Dysphagia	Baseline	0.50±1.17	0.45±1.21	0.24±1.12
	28 days	0.50±1.20	0.45±1.24	0.14±0.35
	56 days	0.53±1.14	0.34±1.17	0.21±0.49
	84 days	0.50±1.28	0.34±1.23	0.10±0.31
Stools	Baseline	3.47±3.18	3.55±3.07	2.76±2.60
	28 days	2.83±2.41	3.62±3.37	2.41±1.62
	56 days	2.90±4.10	2.59±3.02	2.38±1.37
	84 days	2.93±4.08	2.10±2.04*	1.69±0.85*
Total score	Baseline	10.47±11.58	10.86±11.97	8.34±12.54
	28 days	10.73±10.84	10.48±9.91	7.83±4.94
	56 days	9.80±11.98	7.83±8.67*	7.38±4.35
	84 days	10.83±16.01	6.17±7.22*$	5.31±3.51*$

*and $ indicate significances (p<0.05) in intragroup comparison (vs. baseline) using paired t-test and in intergroup comparison (vs. placebo) using ANCOVA model, respectively.

Table 9: Incidences of adverse events

Symptoms	Severity	Placebo (%)	CL16049F1 (%)	Silymarin (%)
Fatigue	Mild	2 (7)	2 (7)	1 (3)
Headache	Mild	1 (3)	0 (0)	1 (3)
Nausea	Mild	1 (3)	0 (0)	1 (3)
Bloating	Mild	0 (0)	1 (3)	0 (0)
Total number of incidences		4	3	3

Adverse events and dropouts: During 84 days of supplementation, no serious adverse events were reported. However, the minor adverse events reported by the participants are summarized in table 9.

Two subjects dropped out of the study; one participant from each CL16049F1 and Silymarin group did not continue due to personal reasons. The remaining participants adhered well to the study protocol, instructions, and consumption of investigational products (IP). The mean ± SD of the IP compliances were 90.19 ± 5.85% in placebo, 90.36 ± 6.17% in CL16049F1, and 89.63 ± 6.28% in Silymarin groups. The efficacy analyses were performed on the data from the subjects who completed the study, viz., placebo (n=30), CL16049F1 (n=29), and Silymarin (n=29).

DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver dysfunctions, representing approximately 25% of global prevalence (7). Subjects suffering from NAFLD have increased risks of hepatic or non-hepatic malignancies, cardiovascular complications, compromised immune competence, [22] and increased mortality [23]. Recent studies have shown that overweight and obese individuals are associated with a greater risk of NAFLD than non-obese individuals [24].

Earlier, in HepG2 human liver cells, a 2:1 combination of Sphaeranthus *indicus* flower head and *Terminalia chebula* fruit aqueous extracts (CL16049F1) exhibited synergistic antioxidant potential through scavenging ROS production and NADPH oxidase inhibition (Unpublished observation). Antioxidants have been demonstrated to have excellent hepatoprotective potential against chemicals or dietary factors-induced toxicity [25-26]. Therefore, the potent antioxidant potential of CL16049F1 raised the possibility that this synergistic combination may have significant efficacy in reducing hepatotoxicity and fatty liver conditions in non-alcoholic subjects. Therefore, the present study aimed to evaluate the efficacy of CL16049F1 in reducing the fatty liver condition and assess its tolerability in non-alcoholic, overweight subjects with elevated FLI.

The present 84-day clinical study demonstrates that CL16049F1 supplementation significantly reduced the FLI and improved overall liver function compared to placebo and baseline. FLI is a simple, non-invasive, and validated algorithm to assess fatty liver condition in humans [9, 10]. Generally, NAFLD subjects have elevated levels of serum TC, TG, LDL, and VLDL and reduced levels of HDL [27]. In this context, assessment of these blood lipid metabolites is important to evaluate the efficacy of CL16049F1. Interestingly, our results revealed that the serum levels of TC, TG, LDL, and VLDL were significantly reduced, while HDL was increased following CL16049F1 supplementation. These observations indicate that CL16049F1 could normalize the lipid metabolism,

including fat mobilization and ameliorates hepatic injury, and prevents the progression of NAFLD. Furthermore, elevated levels of liver enzymes, including ALT, GGT, ALP, and AST in serum, are indicators of hepatotoxicity. These enzymes are secreted into the circulation due to the hepatocytes' membrane damage and are considered surrogate markers of hepatotoxicity in NAFLD [28]. It is noteworthy to mention that in the present study, CL16049F1-supplemented participants exhibited reduced serum levels of these liver enzymes, thus suggesting an improved liver function in the herbal-supplemented subjects.

Another interesting observation in the present study was that the CL16049F1-supplemented participants showed a significant reduction in HOMA-IR, a widely used biomarker for the estimation of insulin resistance [18]. Insulin resistance is closely associated with fat accumulation in the liver, liver dysfunction, and the progression of the fatty liver condition to fibrosis [29-30].

Sphaeranthus indicus flower head extracts and *Terminalia chebula* fruit contain a significant amount of phenolic and flavonoid compounds, including ellagic acid, gallic acid, chebulic ellagitannins, and quinic acid [31-32]. These phytochemicals exhibit potent antioxidant efficacy and are proven to normalize the liver enzyme levels, thus, providing significant hepatoprotection [32-33]. Therefore, we believe that the effect of CL16049F1 supplementation in normalizing liver enzymes and fat mobilization is due to its antioxidant potential [34].

Amelioration of oxidative stress could be regarded as an anti-NAFLD viable mechanism. Due to the accumulation of triglycerides and free fatty acids in NAFLD, excessive ROS are generated in the liver. The free radical homeostasis in the body is mainly maintained by the antioxidant balance of SOD and GSH-Px [35]. In NAFLD, the activity SOD is significantly decreased [36]. In the present study, 84 days of supplementation of CL16049F1 significantly increased the levels of circulating SOD and GSH and significantly reduced lipid peroxidation in the participants. The presence of elevated lipid peroxidation and its end product MDA are reported in both alcoholic and non-alcoholic fatty liver diseases [37]. The oxidative stress marker MDA is reported to stimulate hepatic stellate cells to produce collagen, resulting in fibrosis [36]. Together, our observations demonstrate a potential role of CL16049F1 in providing a strong antioxidant defense that helps to maintain the normal physiological condition of the liver in the volunteers. It is of note that following the classification and regulation of functional foods proposed by the Functional Food Center, Dallas, TX, the pharmacological actions of the active phytochemicals and the clinical benefits of CL16049F1 observed in the present study offer a potential possibility of considering this botanical formulation as a functional food ingredient [38].

Earlier toxicological studies in preclinical models have concluded that the extracts of *T. chebula* and *S. indicus* are safe [39-40]. Also, *T. chebula* fruit has been reported to reduce lead- and aluminum-induced genotoxicity [41]. Earlier, a clinical study in psoriasis patients demonstrated good tolerability of *S. indicus* extract when consumed over a long time period [40]. Importantly, an age-old history of traditional usage of *Terminalia chebula* fruit and *Sphaeranthus indicus* flower heads is indicative of the safety of CL16049F1. Furthermore, no adverse events were reported.

CONCLUSION

In conclusion, the findings of the present proof-of-concept study established the efficacy of CL16049F1 supplementation in reducing fatty liver index (FLI) and oxidative stress level, as well as improving lipid profile in non-alcoholic subjects with fatty liver. These indicate that

CL16049F1 supplementation helps improve liver function in the study participants. Also, this botanical formulation is well tolerated.

List of Abbreviations: ALP: Alkaline phosphatase, ALT: Alanine transaminase, AST: Aspartate transaminase. CAT: Catalase, FLI: Fatty liver index, GGT: γ-glutamyl transferase, GSH-px: Glutathione peroxidase, HDL: High-density lipoprotein, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance, LDL: Low-density lipoprotein, NAFLD: Non-alcoholic fatty liver disease, ROS: Reactive oxygen species, SOD: Superoxide dismutase, TG: Triglyceride

Authors' Contributions: VKPR, NTG designed the trial; VKPR, SK, SKB NTG conducted the study; SKB captured the data; SK, VKPR analyzed the data; SKB drafted the manuscript; NTG reviewed and finalized the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest in conducting the research and publishing the data.

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