Research Protocol

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Ayurveda Section

Hepatoprotective Effect of *Centella asiatica* Extract Against High-fat Diet-induced NAFLD in Wistar Rats: A Research Protocol

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ABSTRACT

Introduction: Non-Alcoholic Fatty Liver Disease (NAFLD) is a rapidly increasing global health problem characterised by excessive fat accumulation in hepatocytes, leading to oxidative stress, inflammation, and hepatocellular injury. The growing prevalence of NAFLD is strongly associated with unhealthy dietary habits, sedentary lifestyles, obesity, and metabolic disorders. If left untreated, NAFLD may progress to advanced liver diseases such as Non-Alcoholic Steatohepatitis (NASH), fibrosis, and cirrhosis.

Need of the study: The NAFLD affects a significant proportion of the global population, and currently available pharmacological options remain limited. Plant-based therapeutic agents with antioxidant and anti-inflammatory properties have gained attention as potential alternatives. *Centella asiatica* has demonstrated antioxidant, anti-inflammatory, and hepatoprotective activities in experimental studies, suggesting its possible role in the management of NAFLD.

Aim: To evaluate and compare the hepatoprotective efficacy of the hydroalcoholic extract of *Centella asiatica* with vitamin C, vitamin E, caffeine, and metformin in High-fat Diet (HFD) -induced NAFLD in male Wistar rats.

Materials and Methods: The present preclinical experimental study will be conducted at the Central Preclinical Research Facility, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research (DMIHER), Salod, Wardha, Maharashtra, India, from December 2025 to February 2026. Seven groups of male Wistar rats (n=6 per group), aged 10-12 weeks and weighing 150-200 g, will be included. Group I will receive a standard pellet diet, while Groups-II to VII will receive a HFD alone or in combination with *Centella asiatica* extract, vitamin E, vitamin C, metformin, or caffeine, respectively. Hepatoprotective activity will be assessed using Liver Function Tests (LFT), lipid profile analysis, estimation of oxidative stress markers such as Superoxide Dismutase (SOD) and Glutathione (GSH), and histopathological examination of liver tissue. Statistical analysis will be performed using statistical software Statistical Package for Social Sciences (SPSS) version 17 and the differences between multiple groups will be analysed by One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test, with a p-value <0.05 considered statistically significant.

Keywords: Antioxidant, Fatty liver, Lipid metabolism, Non alcoholic fatty liver disease, Oxidative stress, Superoxide dismutase, Steatosis

INTRODUCTION

The NAFLD is a growing global health concern, affecting 32.4% of adults worldwide [1]. NAFLD is characterised by excessive fat accumulation in liver cells, leading to inflammation, oxidative stress, and hepatocyte damage [2]. NAFLD can develop into more serious liver diseases such as NASH, fibrosis, cirrhosis, and hepatocellular carcinoma if treatment is not received [3]. NAFLD has a complicated and multifaceted aetiology that includes genetic susceptibility, insulin resistance, and dyslipidaemia [4]. Obesity, type 2 diabetes, metabolic syndrome, and dyslipidaemia are all closely associated with NAFLD. Diets high in fructose and saturated fats, along with physical inactivity, also contribute to its development. The diagnosis of NAFLD relies on imaging techniques, such as ultrasound and Magnetic Resonance Imaging (MRI), as well as liver biopsy. Lifestyle modifications, including weight loss, dietary adjustments, and increased physical activity, remain the primary strategies for managing NAFLD. Pharmacological interventions, such as vitamin E and metformin, may be employed in selected cases. Overall, NAFLD is a complex, multi-factorial condition that requires a comprehensive approach to diagnosis and management [5]. Currently, few effective treatments are available for NAFLD, and the mainstay of care is still lifestyle changes including diet and exercise [6]. However, the search for effective pharmacological interventions has led to a renewed interest in traditional herbal medicines, which have been used for centuries to treat various liver disorders [7].

Centella asiatica, also known as *mandukaparni* in Ayurveda, is a perennial herb belonging to the family Apiaceae. It has *tikta rasa* (bitter taste with astringent after taste), *laghu guna* (light property), *sheeta virya* (cooling potency), and *medhya prabhava* (cognitive-enhancing effect) [8]. It is a traditional herbal medicine that has been used in Ayurvedic and traditional Chinese medicine for centuries to treat various ailments, including liver disorders [9]. The herb has been reported to possess hepatoprotective effects, including anti-inflammatory, antioxidant, and anti-fibrotic activities [10,11]. Bioactive constituents reported in *Centella asiatica* include: triterpenoids (asiatic acid, madecassic acid, and madecassoside); flavonoids (quercetin and kaempferol); alkaloids (brahmine); saponins (centelloside, brahmoside, asiaticoside); and volatile compounds including sesquiterpenes [12]. These bioactive compounds underpin the wide range of medicinal properties attributed to *Centella asiatica*, including hepatoprotective, anti-inflammatory, anti-proliferative, antioxidant, anti-ulcer, anti-steatotic, anti-cancer effects as well as it has neuroprotective and cognitive-enhancing functions [13]. Asiatic acid, madecassic acid, and asiaticoside, have been shown to modulate various molecular mechanisms involved in the pathogenesis of NAFLD [14].

Centella asiatica has been reported to possess hepatoprotective effects in fatty liver (excessive fat accumulation in liver cells). The hepatoprotective effect of *Centella asiatica* in fatty liver is to reduce lipid accumulation [15], inhibit inflammatory pathways [16], improve insulin sensitivity and protect against hepatocyte damage [17].

Molecular mechanisms: The hepatoprotective effects of *Centella asiatica* in fatty liver are mediated by several molecular mechanisms, including: Activation of Adenosine Monophosphate-Activated Protein Kinase (AMPK), Inhibition of Nuclear Factor-Kappa B (NF-κB), Activation of peroxisome Proliferator-Activated Receptor-alpha (PPAR-α) and inhibition of oxidative stress and inflammation [11].

REVIEW OF LITERATURE

The NAFLD encompasses a wide range of diseases, including simple steatosis, NASH, liver fibrosis, cirrhosis, and hepatocellular cancer [2]. With the rising prevalence rates of metabolic disorders, including diabetes, obesity, dyslipidaemia and metabolic syndrome, NAFLD incidence is growing year by year [3]. *Centella asiatica* is a traditional herbal medicine which has hepatoprotective effects, including antioxidant, anti-inflammatory and anti-fibrotic activities [9,10]. Several studies have reported the hepatoprotective and antioxidant effects of *Centella asiatica*.

Ha SK et al., created four groups of eight rats each. For six weeks, rats were given a HFD and two different doses of the HM (herbal mixture containing *C. asiatica)* (50 and 200 mg/kg body weight). The present study showed that an herbal mixture containing *C. asiatica* decreased liver weight, visceral fat, serum triglyceride, total cholesterol, and Low-Density Lipoprotein (LDL) levels in the rats. While increasing High-Density Lipoprotein (HDL), and histological analysis confirmed reduced hepatic lipid accumulation in a dose-dependent manner [15].

Akrom et al., study used 21 male Wistar rats. The rats were split into seven groups at random: Group 1 was the normal control group, which received standard food; Group 2 was the HFD control group, which received induced HFD; Group 3 was the positive control group, which received induced HFD and simvastatin; Group 4 was the placebo control group, which received induced HFD and a placebo; Group 5-7 was the treatment group, which received CTCE (Chewable Tablet of *Centella asiatica* Extract) at doses of 100, 200, and 300 mg/kg Body Weight (BW), respectively. The CTCE was administered for one week only in last week of intervention, while the HFD induction lasted five weeks. The study results showed reduced serum triglyceride levels and Serum Glutamic Pyruvic Transaminase (SGPT) activity in Wistar rats fed a HFD [18].

In Vishnumukkala T et al., study, 30 male albino Wistar rats were given oral *Centella asiatica* extract (100, 200, and 300 mg/kg/day) for 70 days after nephrotoxicity was produced using AlCl3 and D-galactose. The study concluded that *Centella asiatica* reduces oxidative stress markers, increasing SOD and catalase, and decreasing MDA levels, along with attenuating histopathological changes such as necrosis and fibrosis [19].

Similarly, Choi MJ et al., found that *Centella asiatica* leaf extract protected against Dimethylnitrosamine (DMN) induced liver injury by decreasing liver weight, reducing inflammation, lowering Malondialdehyde (MDA) levels, and enhancing antioxidant enzymes including SOD, Glutathione (GSH) , and catalase, also the assessment of the liver histology showed reduced mass periportal ± bridging necrosis, intralobular degeneration, and regional necrosis, fibrosis of liver tissues [20].

Despite these findings, evidence on the hydroalcoholic extract of *Centella asiatica* specifically in HFD-induced NAFLD in Wistar rats remains limited. Key aspects such as standardised dosing, long-term administration, and mechanistic pathways are yet to be fully explored.

The present study is aimed to investigate the hepatoprotective and antioxidant potential of *Centella asiatica* hydroalcoholic extract against HFD induced NAFLD in male Wistar rats.

Primary objectives:

- To evaluate the hepatoprotective effects of *Centella asiatica* hydroalcoholic extract against HFD-induced NAFLD in male Wistar rats.
- To evaluate the hepatoprotective effects of *Centella asiatica* hydroalcoholic extract with standard therapeutic agents such as metformin, caffeine, vitamin C, and vitamin E.
- To examine the histopathological changes in liver tissue of rats treated with *Centella asiatica* hydroalcoholic extract.

Secondary objectives:

- To compare the hepatoprotective efficacy of *Centella asiatica* hydroalcoholic extract with standard therapeutic agents such as metformin, caffeine, vitamin C, and vitamin E.

Null Hypothesis: The hydroalcoholic extract of *Centella asiatica* has no significant effect on the reduction of NAFLD in male Wistar rats.

Alternative Hypothesis: The hydroalcoholic extract of *Centella asiatica* significantly reduces NAFLD in male Wistar rats.

MATERIALS AND METHODS

The present preclinical experimental animal study will be carried out at the Central Preclinical Research Facility, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research (DMIHER)'s registered animal house in Salod, Wardha, Maharashtra, India from December 2025-February 2026. The ethical approval is obtained from the Institutional Animal Ethics Committee (IAEC) (DMIHER/IAEC/2024-25/50). Vitamin E, Vitamin C, Metformin and Caffeine will be procured from local pharmaceuticals for the present study. *Centella asiatica* extract will be prepared by Soxhlet extraction method [21]. HFD will be procured from a certified pharmaceutical supplier. Total 42 male Wistar rats will be taken for study and will be divided into seven groups of six rats each by simple random sampling technique.

Inclusion and Exclusion criteria: Healthy male rats of age 10-12 weeks weighing 150-200 gm will be included in the present study. Diseased rats, under or overweight rats and rats aged less than 10 weeks will be excluded.

Study Procedure

Whole plants of *Centella asiatica* will be obtained from the herbal garden of Mahatma Gandhi Ayurvedic College and will be authenticated by the Department of *Dravyaguna*. The plant material will be thoroughly rinsed with water to remove dirt and other contaminants. The cleaned material will be shade-dried at room temperature for approximately one week, after which it will be coarsely powdered using a mechanical grinder. The powdered drug will be stored in an airtight container until further use.

The extraction will be carried out using a hydroalcoholic solvent mixture prepared in a 70:30 ratio of ethanol to distilled water. A measured quantity of the dried plant powder will be placed in a cellulose thimble and extracted using a Soxhlet apparatus. The system will be operated for approximately 6-8 hours at a controlled temperature range of 40-60°C, ensuring continuous circulation of the solvent until it becomes clear. After completion of the extraction process, the extract will be filtered through Whatman No.1 filter paper and concentrated under reduced pressure using a rotary evaporator maintained at 40-45°C. The partially concentrated extract will be further dried on a water bath at low temperature to obtain a thick, semisolid residue. The dried extract will be weighed to calculate the extraction yield and stored in amber-coloured airtight containers at 4°C until further experimental use [21].

The high-fat diet will be prepared as an emulsion containing corn oil, saccharose, milk powder, cholesterol, sodium deoxycholate, Tween-80, propylene glycol, vitamin mixture, cooking salt, mineral mixture, and distilled water, providing a high-energy formulation [Table/Fig-1]. Animals in the HFD group will be fed this diet daily for 90 days, while those in the Trial Group (TG) will receive the same HFD supplemented with hydroalcoholic extract of *Centella asiatica*.

Male Wistar rats aged 10-12 weeks, weighing between 150-200 g, will be selected for the experiment. A total of 42 rats will be randomly

S. No.	Component	High fat emulsion (gm)
1.	Corn oil	400
2.	Saccharose	150
3.	Total milk powder	80
4.	Cholesterol	100
5.	Sodium deoxycholate	10
6.	Tween 80	36.4
7.	Propylene glycol	31.1
8.	Vitamin mixture	2.5
9.	Cooking salt	10
10.	Mineral mixture	1.5
11.	Distilled water	300
12.	Total energy	4342 Kcal

[Table/Fig-1]: High-Fat Diet (HFD) composition.

divided into seven groups, with six animals in each group, using a computer-generated random sequence. Each group will be housed in a separate enclosure under standard animal house conditions with a 12-hour light and dark cycle. The animals will be provided with a regular pellet diet and free access to water throughout the present study period. All animal experimental procedures will be conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The animals will be acclimatised for one week prior to the commencement of the experiment, and only healthy, age-matched rats will be included in the current study. Group allocation will be performed using a computer-generated random sequence, and investigators involved in sample analysis will be blinded to the treatment groups. All procedures, including animal handling, dosing, and sample collection will be carried out by trained personnel using minimally stressful techniques. Animals will be monitored daily for behavioural and clinical signs, and humane endpoints will be strictly followed. At the end of the present study, euthanasia will be performed using CPCSEA-approved methods, ensuring minimal pain and distress [22].

The experimental groups will be as follows: [Table/Fig-2]

- **Group 1:** Sham group, which will receive only distilled water
- **Group 2:** HFD control group
- **Group 3:** HFD + *Centella asiatica* hydroalcoholic extract
- **Group 4:** HFD + Vitamin E
- **Group 5:** HFD + Vitamin C
- **Group 6:** HFD + Metformin
- **Group 7:** HFD + Caffeine

On the first day, prior to administration of the test drugs, blood samples will be collected from all 42 animals through orbital puncture for the assessment of Liver Function Tests (LFTs), lipid profile, SOD, and GSH levels. After 90 days of administration of the final dose of the respective treatments, the animals will be allowed an overnight rest period. Blood samples will then be collected again through orbital puncture for reassessment of LFTs, lipid profile, SOD, and GSH.

Subsequently, three animals from each group will be sacrificed using a light dose of ether anaesthesia. The liver tissues will be carefully excised and preserved in 10% formalin for histopathological examination.

Primary outcomes:

- Histopathological evaluation of liver tissue to assess structural changes, including the degree of hepatic steatosis, inflammation, and architectural alterations, following 90 days of treatment.
- Improvement in liver morphology as evidenced by reduced fatty changes and hepatocellular damage in treated groups compared to the HFD control group.

Secondary outcomes:

- Biochemical assessment of liver function, including LFTs such as serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP), measured on Day 1 and Day 90.
- Evaluation of lipid profile parameters, including total cholesterol, triglycerides, LDL, and HDL levels, assessed at baseline (Day 1) and at the end of the present study (Day 90).
- Assessment of antioxidant status, including estimation of GSH and SOD levels, conducted at baseline (Day 1) and after 90 days of treatment.

STATISTICAL ANALYSIS

All analyses will be performed using statistical software SPSS version 17. All data management will be done in an excel sheet. All the values will be expressed as mean±standard deviation. Differences between multiple groups will be analysed by one-way ANOVA followed by Tukey's post-hoc test for parametric data or by Kruskal-Wallis test followed by Dunn's multiple comparison for non-parametric data. Repeated measures such as body weight over time will be analysed using repeated measures ANOVA. A p-value <0.05 will be considered statistically significant.

Data Management

The data management plan will ensure rigorous procedures for data entry, coding, security, and storage to maintain integrity throughout the trial. Standardised data entry protocols will be implemented to minimise errors, potentially including double data entry for critical information. Variables will be planned to be coded according to established guidelines to facilitate analysis. Security measures will include restricted electronic access and encryption of sensitive data, with physical documents planned to be stored securely. Data will be centralised on secure servers with regular backups planned to prevent loss. Range checks during data entry will be conducted to promptly identify outliers or errors. Detailed data management procedures will be outlined in the protocol documentation for transparency and adherence to guidelines.

Groups	Group name	Age	Group code	Sample size	Intervention/drug	Route of drug administration	Duration
1	Sham group	10-12 weeks	SG	6	Standard pellet diet	Oral	90 days
2	HFD Group	10-12 weeks	HFD	6	HFD 20 gm/day	Oral	90 days
3	Trial Group (TG) with hydroalcoholic leaf extract of *Centella asiatica*	10-12 weeks	TG	6	HFD 20 g/day *Centella asiatica* hydroalcoholic extract 60 mg/day	Oral	90 days
4	Comparison Group 1	10-12 weeks	CG 1	6	HFD 20 g/day + Vitamin E 20 mg/day	Oral	90 days
5	Comparison Group 2	10-12 weeks	CG 2	6	HFD 20 g/day + Vitamin C 50 mg/day	Oral	90 days
6	Comparison Group 3	10-12 weeks	CG 3	6	HFD 20 g/day+ Metformin 50 mg/day	Oral	90 days
7	Comparison Group 4	10-12 weeks	CG 4	6	HFD 20 g/day + Caffeine 6 mg/day	Oral	90 days

[Table/Fig-2]: Experimental grouping and posology.
SG: Sham group; HFD: High-Fat Diet; TG: Trial group; CG: Comparison group

Declaration of Interests

Principal investigators overseeing the entire trial and at each study site will transparently disclose any financial or other competing interests that could potentially influence the study's integrity or interpretation of results.

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