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Ciplukan (*Physalis Angulata* Linn.) Extract Potential on High-Fat Diet-Induced Non-alcoholic Fatty Liver Disease (NAFLD) for Liver Anti-Fibrotic Drug Development

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ABSTRACT

An increasing proportion of the global population have or are at risk of developing non-alcoholic fatty liver disease (NAFLD). Fibrosis is the most important predictor of prognosis in NAFLD patients. Ciplukan (*Physalis angulata* Linn.) has been reported to have antifibrotic potency in CCl4-induced liver fibrotic rats, so this study was designed to evaluate the antifibrotic effect of ciplukan extract on liver function, inflammation, and cholesterol levels. The liver fibrosis model was established using 20% margarine injected subcutaneously 8 times twice a week for 4 weeks in 35 male and 35 female Wistar rats. The rats were then treated orally with ciplukan extract (CPL) in 70% alcohol, starting the 6th week of treatment with 2 different doses, namely 13.5 mg (CPL-1) and 27 mg (CPL-2) every day for 4 weeks. The histopathological changes in the liver were analyzed by Haematoxylin Eosin (HE) staining. Serum IL-6 and TGF-β1 levels were determined by ELISA. ALT and cholesterol levels were measured using a diagnostic kit. Single and multiple doses of ciplukan extract with or without standard therapy (Vitamin E) reduced the fibrotic scores to 1.30±0.95 (p=0.001), TGF-β1 levels to 24.20±2.02 ng/mL (p = 0.000), IL-6 levels to 1.68±0.52 pg/mL (p=0.156), ALT levels to 104.57±2.02 U/mL (p=0.001), and cholesterol levels to 81, 07±2.02 mg/dL (p=0.000). In conclusion, the ciplukan herb ethanol extract possesses potent liver antifibrotic activity, thus it is a potential new liver antifibrotic drug.

Keywords: *Physalis angulata* Linn (Ciplukan), liver antifibrotic, NAFLD, ALT, Cholesterol, IL-6

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease diagnosed with secondary causes of steatosis, such as excessive alcohol consumption, long-term use of steatogenic substances, and monogenic liver disorders. NAFLD

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is characterized by morphological and biochemical changes typical of alcoholic steatohepatitis but may occur in individuals consuming less than 20 g alcohol per day for women, and less than 30 g alcohol per day for men, depending on the excess (more than 5% of dry organ mass) accumulation of triglycerides in the liver (Chalasani *et al*., 2018). Currently, NAFLD is the most common chronic liver disease with an estimated global prevalence of 25% (Younossi *et al*., 2016). Liver fibrosis results in abnormal proliferation and accumulation of tough fibrous connective tissue (scar tissue) in the liver. Although scar tissue formation is a normal physiological response to injury, this healing process is disrupted in fibrosis. The normal process of wound healing involves collagen deposition, however, chronic activation of this healing mechanism leads to liver pathology (Ahmad & Ahmad, 2012).

Animal model experiments have suggested an important in vivo role for TGF-β in the pathogenesis of fibrotic conditions, as TGF-β induction and activation were consistently observed in experimental models of tissue fibrosis (Biernacka *et al*., 2011). TGF-β, together with PDGF, is the most potent inducer of liver fibrosis (Dewidar *et al*., 2015). The TGF-β superfamily consists of 33 members, of which TGF-β1 plays an important role in liver fibrogenesis (Dropmann *et al*., 2016). The diagnosis of NAFLD should be considered in any patient with a mild elevation of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) (rarely above 300 IU/l) after ruling out other causes of liver disease. In most cases, the ALT level is higher than the AST. Given the risk of progression, non-invasive methods of evaluating fibrosis are essential. Ultrasonography combined with elastography is useful for NAFLD diagnosis, with a sensitivity of 87% and specificity of 91% (EASD, 2016). Liver biopsy and histopathological evaluation should also be performed to differentiate simple NAFLD from NASH or fibrosis, as well as to predict the disease course (Jeznach-Steinhagen *et al*., 2019).

Since liver fibrosis is reversible at an early stage, effective treatment is of clinical importance and therapeutic strategies are necessary to prevent the progression of liver fibrosis to cirrhosis and liver cancer (El-Shawi *et al*., 2022). Currently, physical exercise, a weight loss diet, or bariatric surgery can effectively relieve or treat NAFLD but there are no approved drugs to treat NAFLD specifically, and potential drugs for NAFLD have side effects that may lead to drug safety problems (Ren *et al*., 2021).

Several plant extracts and natural products have been recommended for the treatment of various liver diseases (El-Shawi *et al*., 2022). Indeed, various natural products and phytochemicals found in food and used as dietary extracts can prevent or slow the development of liver fibrosis in various animal models (Brancaccio *et al*., 2018). Ciplukan (*Physalis angulata* Linn.) is a well-known native Indonesian medicinal plant (Dewi *et al*., 2019). Rohmawaty *et al*. (2021) reported that an ethyl acetate fraction of ciplukan had an antifibrotic effect on CCl_4-induced rat liver fibrosis by lowering ALT levels and significantly improving liver fibrosis. These effects may be caused by several bioactive substances found in ciplukan plants including flavonoids, polyphenols, alkaloids, saponins, vitamin C, stearic acid, palmitic acid, and physalins (Fan *et al*., 2017; Hasyim *et al*., 2022). Therefore, this study was designed to evaluate the antifibrotic potential of ciplukan extract in the NAFLD rat model induced by a high-fat diet.

MATERIALS AND METHODS

Plant Sample Preparation

Ciplukan herbs include all components of the ciplukan plant (*Physalis angulata* Linn.) except the roots. The ciplukan plants were collected from various locations in West Java, Indonesia between December 2019 and February 2022 and confirmed as ciplukan by the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. The plants were extracted by a cold maceration process at room temperature with 70% alcohol for 3 x 24 hours. The filtrate was subjected to rotary vacuum evaporation and the dry extract was obtained by freeze-drying (Rohmawaty *et al*., 2021).

Experimental Animal Model

This study received ethical approval from the Research Ethics Committee of the Faculty of Medicine, Padjadjaran University, and Dr. Hasan Sadikin Bandung (ref. no: 530/UN6.KEP/EC/2022.) Experimental animals were male and female Wistar rats aged 3 months with a body weight of 150-200 g. Experimental rats were obtained from the Animal Laboratory Section of PT. Biofarma in Parongpong, West Java, Indonesia, and housed in cages in the Animal Laboratory of the Physiology Section of the Faculty of Medicine, Universitas Padjadjaran.

High Fat Diet Administration

Liver fibrosis was induced by a high-fat diet containing trans fatty acids, namely 20% margarine (Blueband, Indonesia) for 4 weeks. Fibrosis was confirmed by histopathological examination of the rat liver.

Treatments

Ciplukan extract (CPL) was orally administered to rats every day for 4 weeks. The extract was administered at week 5 from the first day of induction. To determine the dose in rats, the human dose (750 mg and 1500 mg) was multiplied by the Laurence-Bacharach coefficient for rats of 0.018, so that the dose of extract in rats was 13.5 mg (CPL-1) and 27 mg (CPL-1). Standard therapy for liver fibrosis is vitamin E (Vit E) in humans at a dose of 900 mg/day (Hickman and MacDonald, 2007). To determine the dose of Vit E in rats, the dose in humans was multiplied by the Laurence-Bacharach coefficient for rats, which was 0.018, so that the dose in rats was 14.4 mg/rat/day.

The experimental rats were randomly divided into seven groups (n=5): group (K-): negative control, which received only 20% margarine; group (K+): positive control, received 20% margarine + Vitamin E; group (P1): received 20% margarine + 13.5 mg single dose (CPL-1); group (P2): received margarine 20% + double dose of 27 mg (CPL-2); group (P3): received margarine 20% + Vitamin E + a single dose of 13.5 mg (CPL-1); group (P4): received margarine 20% + Vitamin E + a double dose 27 mg (CPL-2); and group (N=normal): no treatment.

After 28 days of treatment, the rats were anesthetized using Ketamine HCL, and blood samples were taken before they were sacrificed to harvest the livers. The livers were removed as soon as possible and weighed, then washed using physiological NaCl solution and formalin-fixed for H&E staining.

Histopathological Assessment

The rat liver was placed in 10% Neutral Buffered Formalin (NBF) for 24 h. The tissue was then divided into 1 x 1 x 1 cm squares and put into cassettes before dehydration in serial alcohol dilutions (70%, 80%, 90%, and 96%). The tissue samples were embedded and blocked before 4-5 mm sections were cut using a microtome and mounted on glass slides (Rahmi *et al*., 2015). Liver histopathology was assessed using a fibrosis score based on a scoring system adapted from AASLD (American Association for the Study of Liver Diseases) (Veteläinen *et al*., 2006) (Table I).

Table I. Histopathology score of hepatic lesions

Histological criteria	Severity	Description	Score
Steatosis	Absent	<10%	0
	Mild	10-30%	1
	Marked	31-60%	2
	Severe	>60%	3
Inflammation	None	Scattered	0
	Moderate	Foci	1
	Marked	Diffuse	2
	Severe	0%	3
Necrosis	Absent	0%	0
	Mild	<10%	1
	Marked	10-50%	2
	Severe	>50%	3
Fibrosis	Absent		0
	Mild		1
	Marked		2
	Severe		3

TGF-β1 and IL-6 ELISAs

TGF-β1 and IL-6 levels in rat blood samples were measured by commercially available ELISA kits (E-EL-0162 and E-EL-M0044, Elabscience, USA) according to the manufacturer's protocol. The absorbance was measured at a wavelength of 450 nm.

Alanine transaminase (ALT) Measurements

The ALT levels in rat blood samples were measured using a commercially available kit (Glory Diagnostic, LOT 16887, Linear Chemical S, SLU., Spain) according to the kit protocol.

Cholesterol Measurements

The cholesterol levels in rat blood samples were determined using a Glory Diagnostic kit (LOT16777, Linear Chemical S, SLU., Spain) according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics for Windows, version 22, IBM Corp., Armonk, New York). The data were analyzed using One-way ANOVA. The Mann-Whitney U-test was used to determine the significance of differences for the non-parametric test and Duncan's T3 for the parametric test.

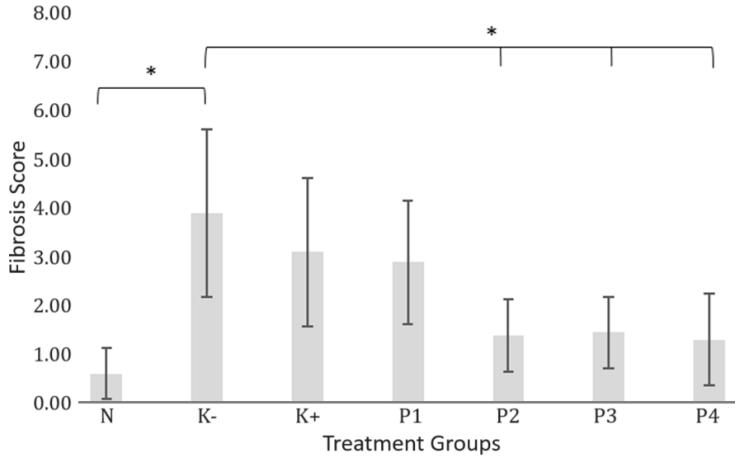

Figure 1. Comparison of the fibrous score between the treatment groups of high-fat-diet-induced rats.
Note: N = Normal Group (No Treatment); K- = Negative Control (Margarine 20%); K+ = Positive Control (Margarine 20%+ Vit E); P1 = Margarine 20%+ Ciplukan Extract Dose 1; P2 = Margarine 20% + Ciplukan Extract Dose 2; P3 = Margarine 20% + Ciplukan Extract Dose 1 + Vit E; P4 = Margarine 20% + Ciplukan Extract Dose 2 + Vit E
*Data presented as mean±SD (n=5). The experiment was performed in triplicate. * $p < 0.05$.

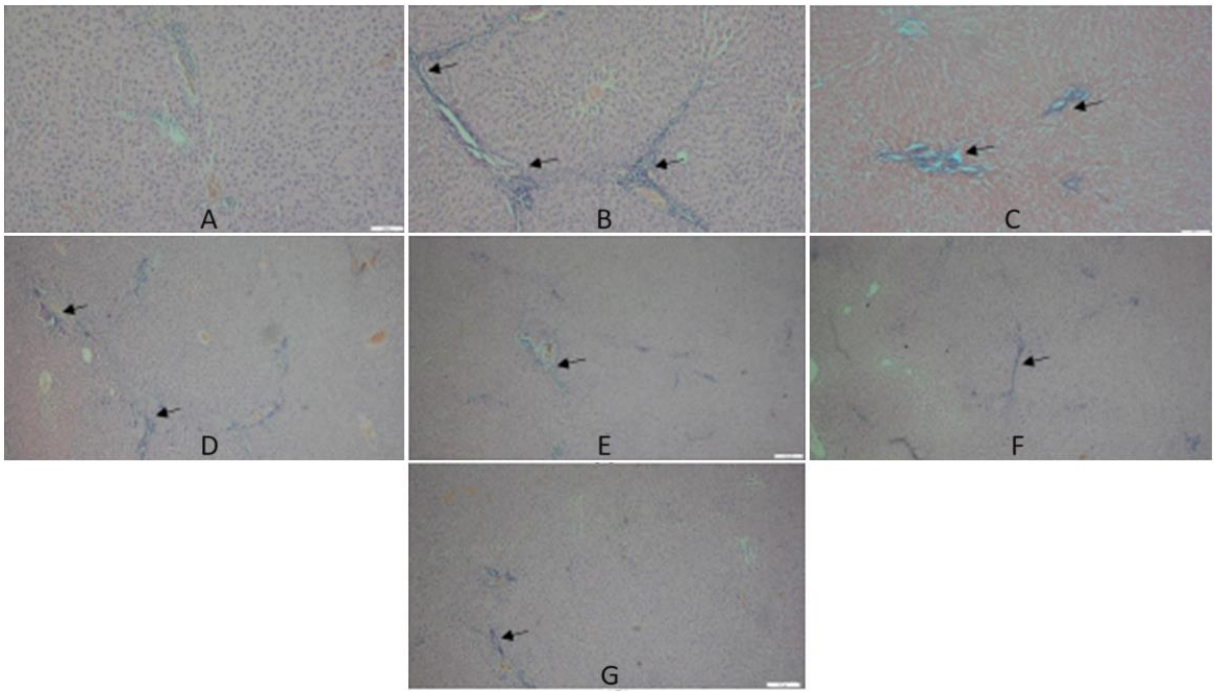

Figure 2. Progression of fibrosis according to the AASLD scoring system in photomicrographs of liver biopsy specimens. (A) Normal Group, 100x (No Treatment); (B) Negative Control (Margarine 20%), 100x; (C) Positive Control, (Margarine 20%+ Vit E); (D) NAFLD + CPL-1, 100x; (E) NAFLD + CPL-2, 100x; (F) NAFLD + Vit E +CPL-1, 100x; (G) NAFLD + Vit E +CPL-2, 100x.

RESULTS AND DISCUSSION

NAFLD is a broad-spectrum disease ranging from hepatic steatosis (non-alcoholic fatty liver – NAFL) to inflammation, often with fibrosis (non-alcoholic steatohepatitis – NASH). Among the known risk factors for NAFLD development, inadequate dietary habits are recognized as pivotal. Excessive lipid intake, in particular, is associated with fat accumulation in the liver. Moreover, a high-fat diet induces fat accumulation in the liver (steatosis), even without changes in body weight (Sales *et al*., 2018), as well as insulin resistance, inflammation, and oxidative stress (Yustisia *et al*., 2022). Margarine is a source of lipids that are known to increase the risk of fatty liver disease (Saber & Noshahry, 2021). In this study, a high-fat diet of 20% margarine induced NAFLD as confirmed by the increased liver fibrosis scores and TGF-β1, IL-6, ALT, and cholesterol levels. This is in line with Longhi (2019) who stated that the ingestion of margarine containing the highest trans fatty acids (TFA) in rats showed the strongest inflammatory effect and increased plasma triglyceride and total cholesterol levels.

Effect of Ciplukan Extract on Rat Liver Fibrosis

The histopathological investigation of liver tissues of the high-fat diet-induced rats showed fibrous septa of connective tissue flowing together, penetrating the parenchyma, and progressive fibrous septa formation resulting in nodule formation. The negative control group exhibited progressive deterioration with a severe degree of fibrinolysis. There was progressive destruction of fibrous septa with a mild degree of fibrinolysis in the CPL-1 and CPL-2 treated groups with or without vitamin E.

The high-fat diet induction increased the fibrosis score (Figure 1 and 2) (Table II), characterized by thick fibrous septa (arrows, blue color) (Figure 2B). The ciplukan extract treatment groups demonstrated significantly lower scores than standard therapy (Vitamin E), especially at multiple doses with or without Vitamin E (p-values of 0.001 and 0.002, respectively). Thin fibrous septa were also observed in the ciplukan extract treatment groups (Figures 2D, 2E, 2F, and 2G).

Effect of Ciplukan Extract on IL-6 and TGF-β1 Levels in Rat Liver Fibrosis

Excessive lipid accumulation increases oxidative stress and induces an inflammatory response, the two main causes of NAFLD pathogenesis (Xia *et al*., 2019). The complex interaction of inflammatory stress and lipid accumulation aided by mediators such as pro-inflammatory interleukins and TGF-β1 forms the basis for NAFLD development. The persistent increase in cytokines shifts the disease from an acute to a chronic state (Ahmed *et al*., 2021).

In the early stages of NAFLD, trans-differentiated hepatic stellate cells (HSCs) play an important role in TGF-β1 production, thereby accelerating disease progression and activating HSCs that increase the production of extracellular matrix (ECM) fibroblasts. Excessive ECM production causes tissue parenchymal damage and deterioration of vascular structures, thereby disrupting the liver structure.

TGF-β1 is mainly involved in myofibroblast activation leading to injury in liver cirrhosis. It acts through the ALK-5 receptor and triggers the SMAD signaling pathway, phosphorylating SMAD 2/3 protein and forming a trimeric complex with SMAD 4 protein that enters the nucleus and initiates gene expression for fibrotic liver disease. It also stimulates other pathways such as JNK, MAPK, PI3K/Akt, Rho, and Ras which are involved in cirrhosis and chronic liver injury in NAFLD which leads to hepatocellular carcinoma (HCC) (Ahmed *et al*., 2021; Abo-Zaid *et al*., 2020).

The present study showed that high-fat diet induction increased TGF-1 levels in the negative controls (Figure 3, Table II). TGF-β1 levels significantly (p=0.000) decreased after treatment with single and multiple doses of ciplukan herb ethanol extract, with and without a combination of standard therapy (Vit E). The ciplukan extract suppressed TGF-β1 levels in NAFLD rats. Ciplukan contains the bioactive compound physalin D, which can reduce liver fibrosis by blocking the TGF-β/SMAD and YAP (Yes-associated Protein) signaling pathways (Xiang *et al*., 2020). Furthermore, decreased TGF-β1 may correlate with inhibition of NF-B activation via TAK1 inactivation, phosphorylation of the IKK complex, and Iκβ degradation. Physalin D also effectively reduces NF-κB receptor activation (Ding *et al*., 2020). In line with the TGF-β1 levels, the high-fat diet induction increased IL-6 levels (p = 0.897), which could be reduced by the ciplukan extract at both doses and combined with vitamin E as the standard therapy for liver fibrosis (Figure 3).

Table II. Comparison of the Fibrosis Score Between the Treatment Groups of High Fat Diet-induced Rats

Variable	N	Fibrosis Score			p-value
		Mean±SD	Median	Range (min-max)	
Normal	10	0.60±0.52	1	0-1	0.000*
NAFLD	10	3.90±1.73	3	1-6	
NAFLD	10	3.90±1.73	3	1-6	0.387
NAFLD + Standard Therapy Vit E	10	3.10±1.73	3	1-6	
NAFLD	10	3.90±1.73	3	1-6	0.107
NAFLD + CPL-1	9	2.89±1.27	3	2-6	
NAFLD	10	3.90±1.73	3	1-6	0.002*
NAFLD + CPL-2	8	1.38±0.74	1.5	0-2	
NAFLD	10	3.90±1.73	3	1-6	0.001*
NAFLD + Standard Therapy+ CPL-1	9	1.44±0.73	2	0-2	
NAFLD	10	3.90±1.73	3	1-6	0.001*
NAFLD + Standard Therapy + CPL-2	10	1.30±0.95	1	0-3	

Notes: The Mann-Whitney test was used for the analysis as the data were not normally distributed or homogenous. * $p < 0.05$.

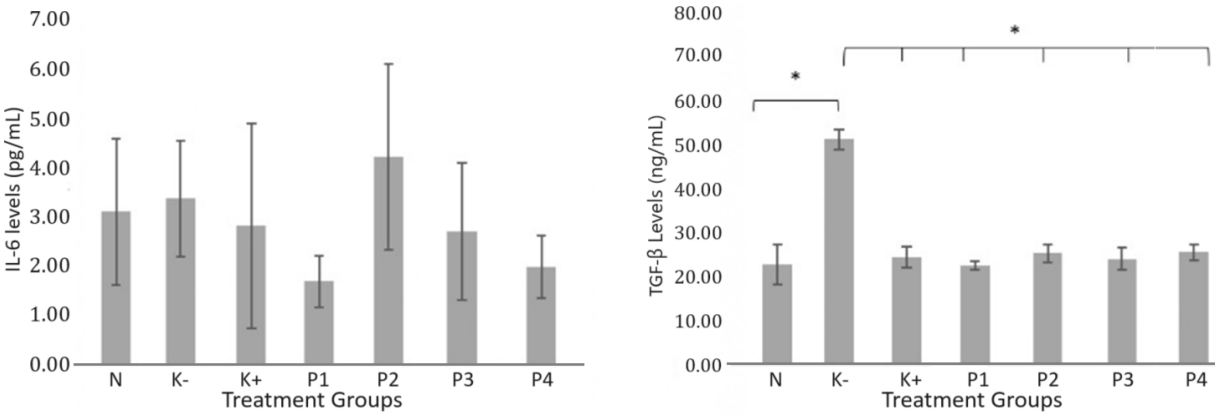

Figure 3. The effect of ciplukan ethanol extract on TGF-β1 and IL-6 levels in the NAFLD Rat Model.
Note: N = Normal Group (No Treatment); K- = Negative Control (Margarine 20%); K+ = Positive Control (Margarine 20%+ Vit E); P1 = Margarine 20%+ Ciplukan Extract Dose 1; P2 = Margarine 20% + Ciplukan Extract Dose 2; P3 = Margarine 20% + Ciplukan Extract Dose 1 + Vit E; P4 = Margarine 20% + Ciplukan Extract Dose 2 + Vit E
* Data presented as mean±SD (n=5). The experiment was performed in triplicate. * $p < 0.05$.

Several pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are critically involved in inflammation, fibrosis, and cancer development associated with hepatocyte apoptosis and steatosis (Del Campo *et al*., 2018). IL-6 mainly contributes to the activation of HSCs which produce ECM in the liver. TNF-α and IL-1β contribute to fibrosis by upregulating TIMP-1 levels, and IL-6 contributes to fibrosis through STAT3 activation (Chen and O'Shea, 2008; Ouyang *et al*., 2008). IL-6 plays an active role in liver fibrosis, mediating the differentiation of Th17 cells that interact with TGF-β.

The ciplukan extract could reduce IL-6 levels in NAFLD rats induced by a high-fat diet except for CPL-2 without standard vitamin E therapy. Yamaguchi *et al*. (2015) suggested blockade of IL-6 signaling may ameliorate hepatic steatosis by modulating insulin resistance in mice and higher levels of IL-6 induced by a high-fat diet (Yamaguchi *et al*., 2015). Physalin B from ciplukan herbs has been shown to protect against liver injury without causing toxicity (Zhang *et al*., 2021).

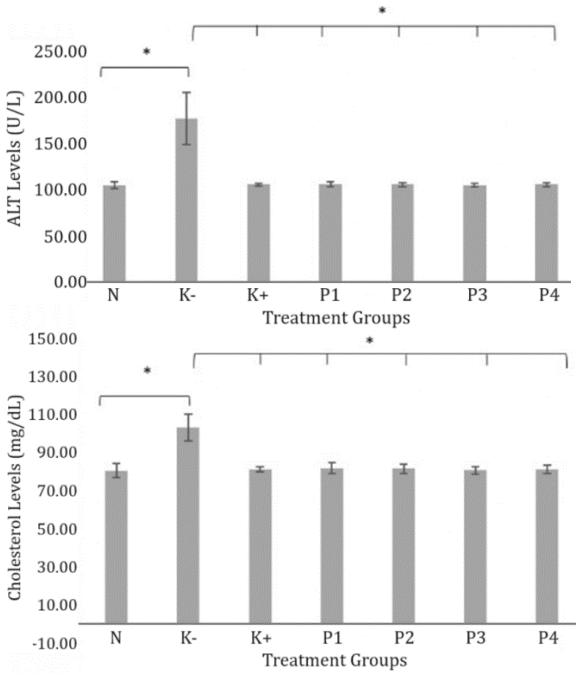

Figure 4. Effects of ciplukan ethanol extracts on ALT and cholesterol levels in the NAFLD rat model. Note: N = Normal Group (No Treatment); K- = Negative Control (Margarine 20%); K+ = Positive Control (Margarine 20%+ Vit E); P1 = Margarine 20%+ Ciplukan Extract Dose 1; P2 = Margarine 20% + Ciplukan Extract Dose 2; P3 = Margarine 20% + Ciplukan Extract Dose 1 + Vit E; P4 = Margarine 20% + Ciplukan Extract Dose 2 + Vit E *Data presented as mean±SD (n=5). The experiment was performed in triplicate. * $p < 0.05$.

Effect of Ciplukan Extract on ALT and Cholesterol Levels in Rat Liver Fibrosis

ALT is an abundant enzyme in the hepatocyte cytosol. ALT levels increase significantly after apoptosis and hepatocyte injury, so ALT levels are commonly used to assess liver function. Higher ALT levels are closely associated with a higher risk of NAFLD, especially with NASH (Ma *et al*., 2020; Rosada *et al*., 2022). In the present study, ALT levels were significantly increased compared to the normal group (p= 0.002) in the NAFLD rat model. As shown in Figure 4, the administration of both single and multiple doses of ciplukan extract significantly reduced ALT to levels similar to the standard therapy group. However, treatment with both doses of ciplukan extract combined with standard therapy reduced ALT levels more than the use of ciplukan extract alone. In line with Xia *et al*. (2019), ALT and IL-6 levels increased in the NAFLD rat model and decreased significantly after treatment with green tea polyphenols improved liver function and attenuated the HFD-induced inflammatory response.

The cholesterol levels in the liver fibrosis rat model were also higher than in normal rats (Figure 4; p=0.000). All treatment groups significantly reduced cholesterol levels, with the most effective treatment being both doses of the ciplukan extract combined with standard therapy (vitamin E) (p= 0.002 and p=0.001).

A high-cholesterol diet and increased cholesterol influx to the hepatocytes increase de novo lipogenesis and hepatic steatosis (Lonardo *et al*., 2021). Unesterified free cholesterol promotes pro-inflammatory and pro-fibrotic pathways that facilitate the progression of NAFLD to NASH and/or cirrhosis (Lonardo *et al*., 2021). Unesterified free cholesterol is toxic to hepatocytes by promoting liver inflammation and subsequent fibrosis (Ioannou *et al*., 2015). Furthermore, the antioxidant actions of ciplukan herbs may be beneficial against NAFLD/NASH. The present study showed that ciplukan extract can suppress cholesterol levels in NAFLD rats in line with Ramadhan (2012) who showed that ciplukan herb medicine also protects the liver from oxidative stress and reduces the amount of fatty liver that develops due to a high-fat diet.

CONCLUSION

The ethanol extract of ciplukan herb demonstrated antifibrotic activity in the liver of a NAFLD rat model, as evidenced by decreased liver fibrosis scores, TGF-β1, IL-6, ALT, and cholesterol levels. Thus, ciplukan herb is a promising candidate antifibrotic drug for liver fibrosis and can also be used together with vitamin E.

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