EVALUATING THE ACUTE TOXICITY AND HEPATOPROTECTIVE EFFECT OF PROTECFUL HARD CAPSULE ON PARACETAMOL-INDUCED ACUTE HEPATITIS IN MICE

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Drug-induced liver injury is characterized by liver damage due to pharmaceutical compounds, resulting in a wide range of liver disorders, from asymptomatic elevations in liver enzymes to full liver failure. It is a leading cause of acute liver failure in many countries. This study investigated the acute toxicity and evaluated hepatoprotective effects on paracetamol-induced acute hepatitis of PROTECFUL hard capsules in Swiss mice. The acute toxicity was assessed through oral administration, and a fifty percent lethal dose was determined using the Litchfield-Wilcoxon method. In the paracetamol-induced acute hepatitis model, mice were orally administered distilled water, silymarin at 140 mg/kg per day, or PROTECFUL hard capsules at 345.6 and 1036.8 mg/kg/day. All indexes, including biochemical indicators (AST, ALT), liver malondialdehyde, glutathione concentration, and microscopic liver images, were evaluated. Regarding acute toxicity, our findings showed that the maximum tolerated dose was 35.4 times higher than the expected human dose without symptoms of acute toxicity. PROTECFUL-treated groups at both doses tended to decrease liver weight and lower liver enzyme activity compared to the model group. The histopathological examination of the liver in the PROTECFUL-treated groups tended to reduce liver degeneration and inflammatory cell infiltration. In summary, these findings suggest that PROTECFUL shows no acute toxicity and tends to have a hepatoprotective effect against paracetamol-induced liver injury.

Keywords: PROTECFUL, hepatoprotective, acute toxicity, paracetamol-induced acute hepatitis, *Swiss* mice.

I. INTRODUCTION

The liver, an essential human organ, performs critical functions such as detoxification, metabolism, digestion, and synthesis of essential proteins.[1] However, the liver is vulnerable to various diseases, notably drug-induced liver injury, which is a significant cause of liver damage when drugs or their metabolites induce hepatotoxicity. Drug-induced liver injury can be caused by a broad range of medications, especially paracetamol. It can present as acute or chronic hepatitis, cholestasis, or a mixed pattern of injury, each with distinct mechanisms and challenging prognoses.[2] This disease accounts for many acute liver failure cases in developed countries. Symptoms can range from mild liver enzyme elevations to severe liver failure, necessitating thorough evaluation and prompt cessation of the offending drug.[3] Consequently, there is an increasing need for enhanced support for drug-induced liver injury, prompting researchers to develop more preventive and therapeutic medications.

PROTECFUL hard capsules contain milk

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thistle extract (*Cardus marianus* extract) containing 60% silymarin. They also include additional ingredients to improve liver health, such as choline bitartrate, dried artichoke extract, L-ornithine-L-aspartate, curcumin (95%), and B vitamins. Silymarin has potent antioxidant, anti-inflammatory, and antifibrotic effects. It is beneficial in treating various liver disorders such as chronic liver diseases, cirrhosis, and hepatocellular carcinoma, as well as mitigating alcohol-related damage and non-alcoholic fatty liver disease progression.[4] Meanwhile, artichoke extract exhibits choleretic, bile-enhancing, and liver-protective properties;[5] choline decreases hepatosteatosis and liver cell death;[6] L-ornithine L-aspartate increases ammonia removal by residual hepatocytes and skeletal muscle of patients with cirrhosis.[7] Curcumin can be helpful in the modulation of oxidative stress conditions and inflammation cascades.[8] While each ingredient has demonstrated hepatoprotective effects in traditional medicine and scientific studies, there still needs to be comprehensive knowledge and safety evidence regarding this mixture's combined effects and potential toxicities.[8] Therefore, the present study investigated the acute toxicity and evaluated hepatoprotective effects on paracetamol-induced acute hepatitis of PROTECFUL hard capsules in *Swiss* mice.

II. MATERIALS AND METHODS

1. Subjects

Ingredients

Each PROTECFUL hard capsule with 720mg mixture includes: 262.5mg Milk thistle extract (*Cardus marianus* extract standardized to 60% silymarin); 125mg choline bitartrate; 50mg dried artichoke extract (equivalent to 500mg of dry medicinal herbs, ratio 10:1); 50mg L-ornithine-L-aspartate; 15mg curcumin 95%; 2,5mg vitamin B1 (thiamine mononitrate); 3mg vitamin B2 (riboflavin); 5mg vitamin B5 (calcium D-pantothenate); 5mg vitamin B6 (pyridoxine hydrochloride). PROTECFUL hard capsules are manufactured by Tam Minh General Trading Company with production batch number 0223/ The manufacture date was November 7, 2023, and the expiry date is November 7, 2026. Research products achieve the manufacturer's standard.

The expected clinical dose is two tablets daily, equivalent to 1440mg daily. PROTECFUL was dissolved in distilled water before being administered to mice.

Animals

Healthy *Swiss albino* mice of both sexes weighing 25 - 30g were provided by the National Institute of Hygiene and Epidemiology. Experimental animals were kept under a temperature of 25 ± 1°C, appropriate air humidity, and lighting. The animals were acclimated to housing in the Department of Pharmacology, Hanoi Medical University laboratory for 7 days before and during the study period; they were fed with standard food and unlimited water intake.

2. Methods

Chemicals and Equipment

Silymarin (Legalon®) 70mg, 5,5-Dithiol-bis (2-nitrobenzoic acid) (Sigma Aldrich, Germany), acid thiobarbituric (Sigma Aldrich, Germany). Other chemicals used for histopathology and related studies that meet testing standards are provided by the Department of Pathology-E Hospital and the National Institute of Medical Materials.

Biochemical analyzer ERBA chem. (Germany) and commercial ERBA diagnostic kits used for quantifying enzymes and metabolites in blood: ALT (alanine aminotransferase), AST (aspartate aminotransferase), GGT (Gamma-

glutamyltransferase), total bilirubin, albumin.

Himac CT6e centrifuge from Himac (Japan), LX 220A analytical balance from Precisa (Switzerland), Micropipettes from Eppendorf (Germany), Specord210 ultraviolet spectrometer from AnalytikJena (Germany), HumanReader HS (German).

Acute toxicity

An acute toxicity study of the research product was conducted on mice orally, and the LD_{50} (fifty percent lethal dose) was determined according to the Litchfied-Wilcoxon method.[9]

Before this experiment, *Swiss albino* mice were fasted on the initial night and subsequently segregated into four distinct groups. These groups were administered PROTECFUL orally in escalating dosages to ascertain the minimal dose which caused 100% mortality, and the maximal dose, which resulted in no fatality (0% death). The condition of the experimental animals was monitored for 72 hours after PROTECFUL administration. Finally, we took care of the mice until the 14th day of this experiment.

Assess hepatoprotective effects of PROTECFUL hard capsule on paracetamol-induced acute hepatitis mice

Mice were given silymarin/PROTECFUL or distilled water continuously every morning for 10 days. On the 10th day, two hours after taking the reagent (mice were fasted for 16 - 18 hours before), proceed to cause liver cell damage by giving mice from groups 2 to 5 a single dose of paracetamol (400 mg/kg).[10]

A)

Group 1	Group 2	Group 3	Group 4	Group 5
Normal control	Model	Silymarin 140 mg/kg	PROTECFUL 345.6 mg/kg/day	PROTECFUL 1036.8 mg/kg/day
	Induce liver damage with a 400 mg/kg dose of paracetamol.			

B)

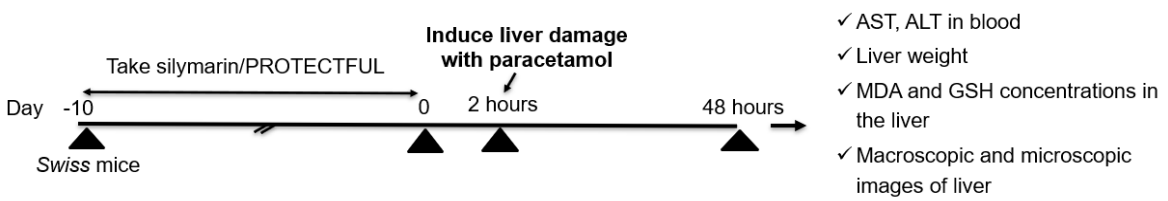

Figure 1. Experimental protocol of paracetamol-induced acute hepatitis study

After 48 hours of paracetamol-induced hepatitis in mice, blood samples from the carotid artery were taken to assess AST and ALT enzyme activity. The liver weight of each mouse was measured. In each group, liver histopathology was conducted on 30% of mice, involving microscopic and macroscopic imaging evaluation. This assessment aimed to evaluate hepatocellular damage, the degree of polymorphonuclear (PMN) infiltration, the extent of steatosis, lobular fibrosis, fibrosis stage, and the presence and site of bilirubinostasis.[11] We also quantified the concentration of malondialdehyde (MDA) using the thiobarbituric acid reaction[12] and measured the concentration of glutathione (GSH) based on the yellow color produced by 5,5-dithiol bis (2-nitrobenzoic acid).[13]

Statistical analysis

Statistical analysis utilized SigmaPlot 12.0 software. Results are presented as mean ± SD. Group differences were described using

one-way ANOVA followed by the Student-Newman-Keuls post-hoc test for pairwise comparisons. LD_{50} in the acute toxicity study was determined using the Litchfield-Wilcoxon method. Significance was defined as $p < 0.05$.

III. RESULTS

1. Acute toxicity

The study findings indicate no acute toxic effect or abnormal signs observed in mice administered from 10 capsules/kg (*equivalent to 7200 mg/kg*) to 17 capsules/kg (*equivalent to 12240 mg/kg*). The maximum tolerated dose of PROTECFUL hard capsules is estimated to be 35.4 times higher than the anticipated human dose.

Table 1. Acute toxicity study of PROTECFUL

Group	n	Dose (capsules/kg)	Dose (mg/kg)	The proportion of deaths (%)	Other abnormal signs
Group 1	10	10	7200	0	No
Group 2	10	12	8640	0	No
Group 3	10	14	10080	0	No
Group 4	10	17	12240	0	No

2. Assess hepatoprotective effects of PROTECFUL hard capsule on paracetamol-induced acute hepatitis mice

The results in Chart 1 showed that the liver weight of mice in the model group increased significantly compared to the normal control group ($p < 0.05$). The liver weight of mice in the PROTECFUL groups tended to decrease compared to the model group. However, the difference was not statistically significant ($p > 0.05$).

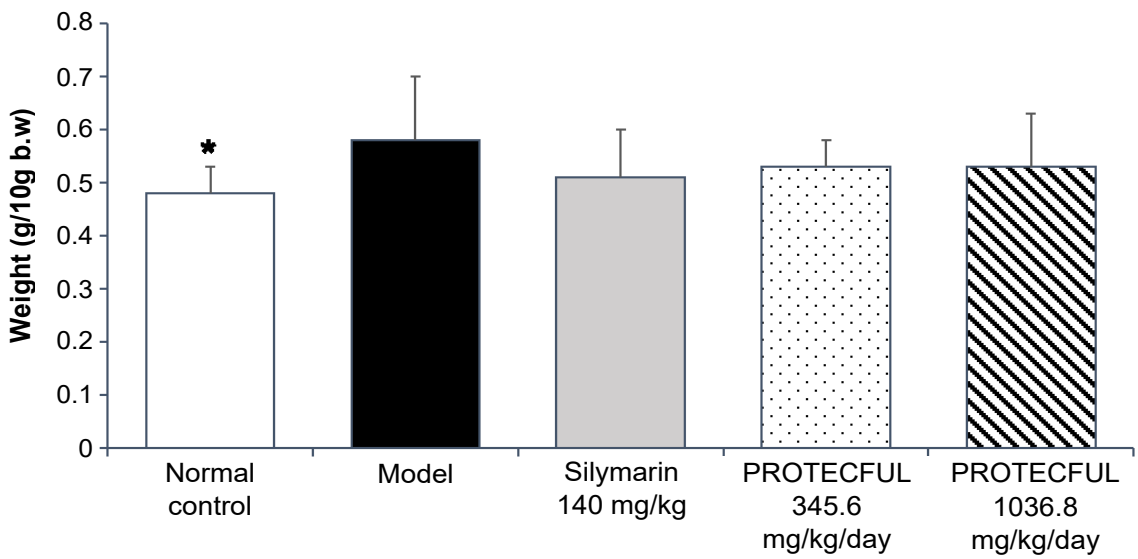

Chart 1. Effects of PROTECFUL on the liver weight of mice

**p < 0.05: compared to model group*

The study results indicated that AST activity followed a similar pattern to ALT, with significant increases in the model group compared to the normal control in Chart 2. Additionally, the PROTECFUL groups at both administered doses showed a tendency for AST activity to decrease when compared to the model group, yet these reductions were not statistically significant ($p > 0.05$).

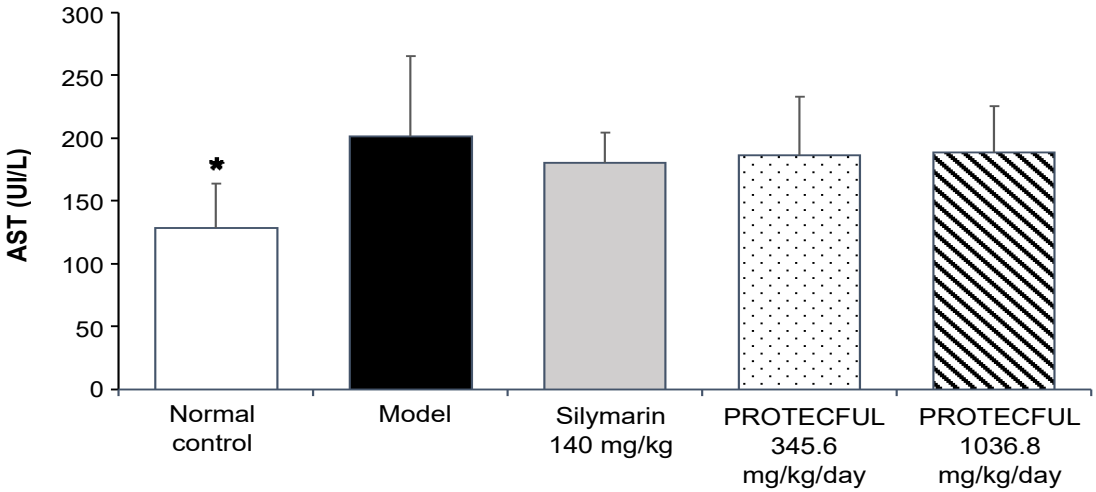

Chart 2. Effects of PROTECFUL on AST in the blood of mice

**p < 0.05: compared to model group*

In the study results presented in chart 3, the ALT activity in the model group showed a significant increase compared to the normal control group ($p < 0.001$), indicating liver damage or dysfunction. However, when treated with silymarin, the ALT levels significantly decreased ($p < 0.05$) compared to the model group. In contrast, the PROTECFUL groups, despite showing a trend toward reduced ALT levels, did not achieve statistically significant differences compared to the model group ($p > 0.05$).

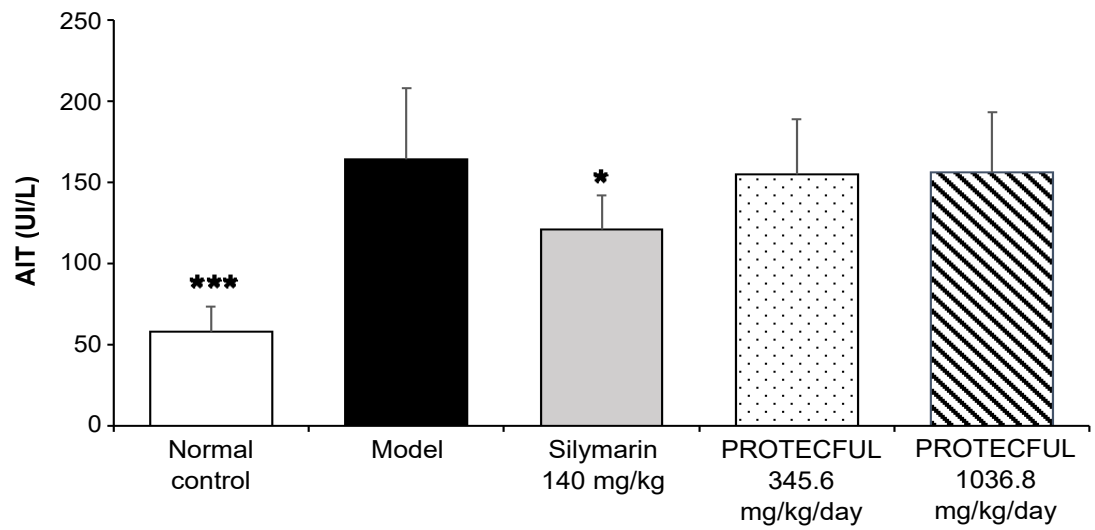

Chart 3. Effects of PROTECFUL on ALT in the blood of mice

**p < 0.05, ***p < 0.001: compared to model group*

The findings from Table 2 reveal significant insights into the effects of the treatments on oxidative stress markers in the liver. It is shown that the MDA concentration significantly increased in the model group compared to the normal control ($p < 0.05$). Treatment with silymarin significantly reduced MDA levels compared to the model group ($p < 0.05$). Similarly, PROTECFUL at both doses exhibited a trend toward lowering MDA concentrations, although these reductions were not statistically significant ($p > 0.05$). Additionally, silymarin significantly increased the concentration of GSH compared to the model group ($p < 0.05$). While PROTECFUL also increased GSH levels at both doses, these increases were not statistically significant ($p > 0.05$).

Table 2. Effects of PROTECFUL on the MDA, GSH concentrations

Group	MDA concentration (nmol/100 mg liver)	GSH concentration (µg/100 mg liver)
Normal control	30.34 ± 4.20*	954.26 ± 110.91
Model	40.62 ± 12.38	867.43 ± 224.45
Silymarin 140 mg/kg	30.26 ± 6.68*	1127.01 ± 214.41*
PROTECFUL 345.6 mg/kg/day	30.49 ± 8.34	1098.13 ± 292.24
PROTECFUL 1036.8 mg/kg/day	33.12 ± 6.55	1095.03 ± 261.55

**p < 0.05: compared to the model group*

Figure 2 showed that the level of microscopic liver damage in PROTECFUL-treated groups also tended to decrease slightly compared to the model group, with a lower level of damage accompanied by images mainly of scattered foci of inflammation in the periportal and internal spaces lobule, no necrosis.

IV. DISCUSSION

Drug-induced liver injury is a rising cause of liver dysfunction worldwide, especially acute liver failure cases in elderly populations, and varying widely in incidence across different regions and drug types. The incidence of drug-induced liver injury has increased over the past decade, with Asia showing the highest rates at 17.82 per 100000 person-years, compared to America at 1.72 per 100000 person-years.[14] PROTECFUL is acknowledged for hepatoprotective support of each ingredient, but it requires evaluation for toxicity and its total effectiveness in protecting the liver. Thus, the present study investigated the acute toxicity in mice and evaluated the hepatoprotective effects of PROTECFUL hard capsules on mice with paracetamol-induced acute hepatitis.

Studies on acute toxicity serve to determine the dose resulting in mortality, often characterized by lethal doses or serious toxicological events after a single administration. Examining acute toxicity thus provides essential information for subsequent research on herbal extract applications.[9] The acute toxicity study showed no abnormal sign in mice across the doses from 10 capsules/kg to 17 capsules/kg. This suggests a high tolerance level of PROTECFUL in these experimental animals. Moreover, the calculated maximum tolerated dose was found to be 35.4 times, which is higher than the expected human dose, showing a substantial safety margin for potential human consumption. As defined by WHO, PROTECFUL is a safe herbal medicine.

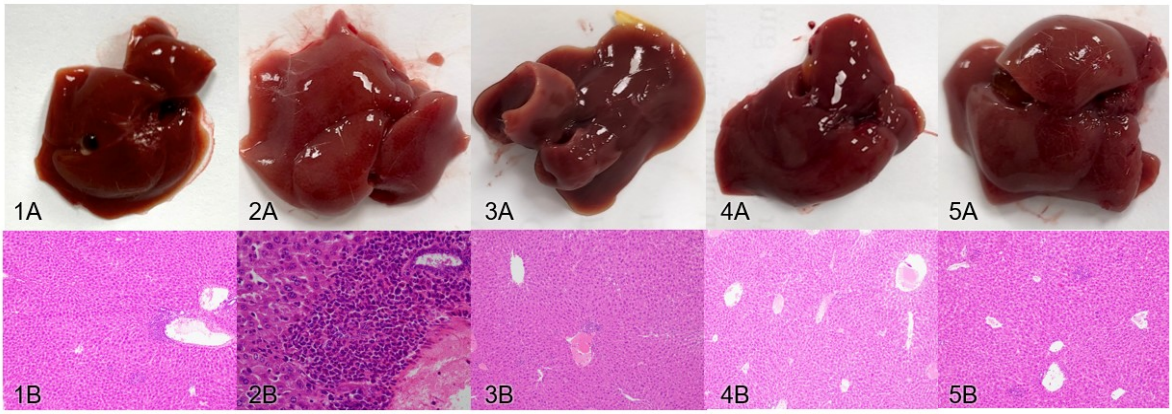

Figure 2. Effects of PROTECFUL on macroscopic and microscopic images of mice liver

(1A-1B) Liver tissue has a clear histological structure with scattered foci of inflammation in periportal spaces and lobules. No cirrhosis or necrosis. No cholestasis. (2A-2B) Liver tissue inflammation occurs in most of the periportal spaces, inflammation in the lobules, and increased infiltration of eosinophils. No cirrhosis or necrosis. No cholestasis. (3A-3B) Liver tissue has a clear histological structure with scattered foci of inflammation in periportal spaces and lobules. No cirrhosis or necrosis. No cholestasis. (4A-4B) Liver tissue has a clear histological structure with scattered foci of inflammation in the lobules. No cirrhosis or necrosis. No cholestasis. (5A-5B) Liver tissue has a clear histological structure, scattered foci of inflammation in the lobules, and scattered periportal inflammation. No cirrhosis or necrosis. No cholestasis

These findings highlight the promising safety profile of PROTECFUL and suggest its potential for further preclinical and clinical investigations.

Paracetamol hepatotoxicity primarily arises from the metabolism of paracetamol into a toxic substance: N-acetyl-p-benzoquinone imine (NAPQI), by cytochrome P450 enzymes, predominantly CYP2E1, 1A2, and 3A4. In normal conditions, NAPQI is detoxified by glutathione. However, excessive paracetamol consumption can decrease the amount of glutathione, accumulating NAPQI, which can subsequently bind to cellular proteins, causing oxidative stress, mitochondrial dysfunction, and cell death. This process is further exacerbated by the activation of inflammatory responses and the release of damage-associated molecular patterns from necrotic cells, leading to acute liver injury and potentially progressing to acute liver failure.[15] The dosage of 400 mg/kg of paracetamol has been established in prior research as inducing acute liver injury; therefore, we utilize this dosage as a determinant for inducing acute hepatitis in mice.[16] Our study used ALT and AST assessments, as these are commonly used to evaluate liver damage in paracetamol-induced hepatitis due to their ability to reflect hepatocellular injury. Besides, measuring MDA and GSH levels offers insights into oxidative stress and antioxidant capacity. MDA is a marker of oxidative stress, indicating cell membrane damage due to lipid peroxidation. GSH is a potent antioxidant, protecting cells from free radicals and detoxifying reactive

oxygen species.[17] The results showed that liver weight, AST, ALT, macroscopic and microscopic liver images in our study, and the experiment of acute hepatitis were successfully modeled. The model group showed significantly higher liver weight and enzyme activity (AST and ALT) than others.

The paracetamol-induced acute hepatitis study also had promising results with PROTECFUL in hepatoprotection. PROTECFUL-treated groups in both dosages tended to decrease liver weight and lower liver enzyme activity than the model group. The microscopic evaluation of PROTECFUL-treated groups' liver damage slightly reduced the level of microscopic liver damage compared to the model group. This reduction in total damage score, accompanied by images predominantly showing scattered foci of inflammation in the portal and internal spaces of the lobule without necrosis, suggests a potential protective effect of PROTECFUL against hepatocellular damage induced by paracetamol. However, in this context, PROTECFUL showed no significant effect on MDA and GSH concentration in paracetamol-induced acute hepatitis mice, indicating that this drug does not demonstrate effective antioxidant functions over a short period. Each ingredient in PROTECFUL was combined to achieve the mentioned pharmacological effects. This study's findings align with previous research showing the hepatoprotective function of compounds. Silymarin, the major ingredient, controls the free radicals generated during the metabolism of toxic substances like paracetamol, preventing cellular damage and inflammation in the liver.[4] Meanwhile, choline influences phospholipid synthesis, lipoprotein secretion, mitochondrial function, and endoplasmic reticulum stress, thereby mitigating the development of liver damage.[6]there have been significant advances in our understanding of the mechanisms that influence choline requirements in humans and in our understanding of choline's effects on liver function. These advances are useful in elucidating why nonalcoholic fatty liver disease (NAFLD Though present in smaller amounts in PROTECFUL, other ingredients play crucial roles in hepatoprotection. Artichoke leaf extract contains phenolic compounds that enhance bile production, prevent lipid peroxidation in liver cell membranes, and provide cellular protection against oxidative damage induced by harmful substances like paracetamol.[5] L-Ornithine L-Aspartate facilitates ammonia removal through urea synthesis and glutamine production and also targets mechanisms related to oxidative stress and lipid peroxidation.[7] Besides, curcumin can effectively regulate oxidative stress and inflammation pathways by modulating the expression of nuclear factor-kappa B (NF-κB) in liver cells, which is critical in protecting against oxidative and inflammatory damage caused by paracetamol overdose.[8] PROTECFUL administered at 345.6 mg/kg/day and 1036.8 mg/kg/day is equivalent to silymarin doses of 75.6 mg/kg/day and 226.8 mg/kg/day, respectively. Therefore, the silymarin content in PROTECFUL capsules at 1036.8 mg/kg/day is higher compared to the active control group. When treated with Legalon®, the ALT level significantly decreased compared to the model group. In contrast, the PROTECFUL groups, despite showing a trend toward reduced ALT levels, did not achieve statistically significant differences compared to the model group. Moreover, there was no difference in the reduction of ALT activity between the groups treated with Legalon® and those with PROTECFUL. This may be explained by differences in the formulation processes of the

two products, leading to variations in silymarin absorption and, consequently, different effects on ALT levels.

The results of our study underscore the potential hepatoprotective effects of PROTECFUL, particularly evident in paracetamol-induced acute hepatitis. This drug illustrated mitigating liver damage, enzyme activity, and a reduction in microscopic liver damage. Besides, when considering the translation of experimental findings from mice to humans, it's crucial to acknowledge potential differences in dosage and metabolism between the two species. In this study, the dose of PROTECFUL administered to mice was determined based on body weight and ranged from 10 to 17 capsules/kg; the maximum tolerant dose of this capsule is calculated to be 35.4 times higher than the expected human dose. The recommended dosage is notably lower, with children over 6 years old and adults advised to take just one tablet twice daily. Additionally, while mice provide valuable insights into the initial safety and efficacy of PROTECFUL, extrapolating these findings to humans requires careful consideration of potential variations in drug metabolism, absorption, distribution, and excretion. Therefore, clinical trials are necessary to determine the optimal dosage and assess the safety and efficacy of PROTECFUL in human populations. A subchronic toxicity study is also warranted to determine any potential adverse effects from repeated exposure over a moderate period.

V. CONCLUSION

Our research reveals PROTECFUL's potential hepatoprotective effects in paracetamol-induced acute hepatitis and its acute toxicity. The acute toxicity study confirms its safety profile. Our findings indicate that PROTECFUL tends to have a hepatoprotective effect against paracetamol-induced hepatitis.

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