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Dose-dependent modulatory effects of Pimpinella alpina *(Purwoceng) root extract on TNF-α and CRP in a CCl_4-induced hepatic injury model*

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Abstract: The liver is highly susceptible to oxidative stress and inflammatory damage, particularly following exposure to hepatotoxic agents such as carbon tetrachloride (CCl_4). Concerns regarding the adverse effects of conventional anti-inflammatory therapies have increased interest in herbal alternatives, including purwoceng (*Pimpinella alpina* Molk), a medicinal plant rich in flavonoids and saponins with potential hepatoprotective activity. This study aimed to evaluate the effects of purwoceng root extract on tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) levels in a CCl_4-induced liver injury model. Twenty-five male Wistar rats were randomly assigned to five groups: a negative control, a positive control receiving curcumin, and three treatment groups administered purwoceng extract at doses of 50, 100, or 150 mg/200 g body weight. Serum TNF-α and CRP levels were quantified using enzyme-linked immunosorbent assay (ELISA). Data were analyzed using the Kruskal–Wallis or one-way ANOVA tests, as appropriate. The purwoceng-treated groups exhibited lower TNF-α levels compared with the negative control; however, these reductions were not statistically significant. CRP levels demonstrated dose-dependent variability, with the lowest concentration observed at the 50 mg dose and a significant increase at the 150 mg dose, indicating a potential shift toward pro-oxidant or pro-inflammatory effects at higher doses. Although a statistically significant dose–response relationship was not established, these findings reflect the complex biological activity of purwoceng extract. In conclusion, purwoceng may exert modulatory effects on inflammatory markers in CCl_4-induced liver injury, but determination of an optimal therapeutic window is essential. Further studies involving bioactive compound isolation, pharmacokinetic and toxicity evaluation, and time-course analysis are warranted to clarify its hepatoprotective potential.

Keywords: anti-inflammatory; CCl4-induced liver injury; CRP; flavonoids; hepatoprotection; purwoceng root; saponins; TNF-α.

INTRODUCTION

The liver plays a crucial role in metabolism and detoxification processes. Exposure to toxic substances and the excessive generation of free radicals in liver cells can lead to oxidative stress and inflammation. Carbon tetrachloride (CCl4) is a well-known hepatotoxic agent that induces liver inflammation, causing hepatocytes to release cytokines such as tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS). This inflammatory process also elevates the levels of C-reactive protein (CRP), a marker of systemic inflammation. Prolonged

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inflammation can lead to severe liver conditions, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[1,2].

Current treatments for inflammation, such as celecoxib and aspirin, are effective but come with adverse effects like gastrointestinal bleeding, ulcers, and gastric perforation[3]. This has led to an increased interest in herbal therapies, which are considered safer alternatives. Purwoceng root (*Pimpinella alpina* Molk), a traditional Indonesian herbal remedy, is rich in flavonoids, saponins, and tannins. Flavonoids inhibit inflammatory mediators such as TNF-α, IL-1β, and COX-2 by blocking NF-κB activation, saponins suppress TNF-α activity, tannins can also reduce inflammatory factors including IL-1β, IL-6, TNF-α, c-fos, c-jun, and caspase 3 through the NF-kB signaling pathway[4–6]. The TNF-α and IFNγ can cause cells to die (apoptosis) by turning on certain genes[7]. The use of some herbal remedies, such as sugar palm fruit (*Arenga pinnata*), snake fruit (*Salacca zalacca* (Gaert.) Voss), and katuk leaves (*Sauropus androgynus*) containing these compounds has been proven to show anti-inflammatory efficacy[8–10]. These compounds make purwoceng root a potential anti-inflammatory and hepatoprotective agent.

Despite the global prevalence of inflammatory diseases as major contributors to mortality, with chronic liver disease ranking as the 12th leading cause of death in the United States, research into the therapeutic effects of purwoceng root remains limited[11,12]. In Indonesia, cirrhosis is one of the top five causes of death, further highlighting the need for effective treatments. Recent studies have demonstrated purwoceng's analgesic and anti-inflammatory properties, but its specific effects on TNF-α and CRP levels in CCl4-induced liver damage have not been thoroughly investigated[13,14].

The objective of this study is to evaluate the effects of purwoceng root extract on TNF-α and CRP levels in male Wistar rats subjected to CCl4-induced liver injury. While previous studies have explored its general anti-inflammatory effects, the potential of purwoceng root to modulate TNF-α and CRP levels in the context of CCl4-induced liver injury has not been established. By focusing on these specific markers, this study seeks to elucidate the mechanisms through which purwoceng root as a hepatoprotective agent, contributes to inflammation reduction and liver protection.

Previous studies have demonstrated the analgesic and anti-inflammatory properties of purwoceng in general inflammatory and toxicological models, but the mechanisms underlying its effect on liver inflammation remain unclear. No previous research has examined how purwoceng modulates both TNF-α and CRP in a CCl_4-induced liver injury model. Evaluating these two biomarkers together provides complementary information, as TNF-α represents local cytokine-mediated inflammation while CRP reflects the systemic acute-phase response. This dual-biomarker approach allows a more comprehensive understanding of purwoceng's potential hepatoprotective activity by capturing both local cytokine-driven inflammation through TNF-α and the systemic acute-phase response through CRP. By evaluating these interconnected pathways, the study provides new insight into how purwoceng may influence inflammatory processes in CCl_4-induced liver injury. Although possible differential effects at varying doses were observed descriptively, the study does not establish a statistically confirmed dose–response relationship, and these patterns should be interpreted with caution within the broader context of herbal-based hepatoprotection.

MATERIAL AND METHOD

Study Design

This study employed a true experimental post-test control group design to evaluate the anti-inflammatory effects of purwoceng root extract on CCl4-induced liver injury in Wistar (*Rattus norvegicus*) rats as illustrated in Figure 1.

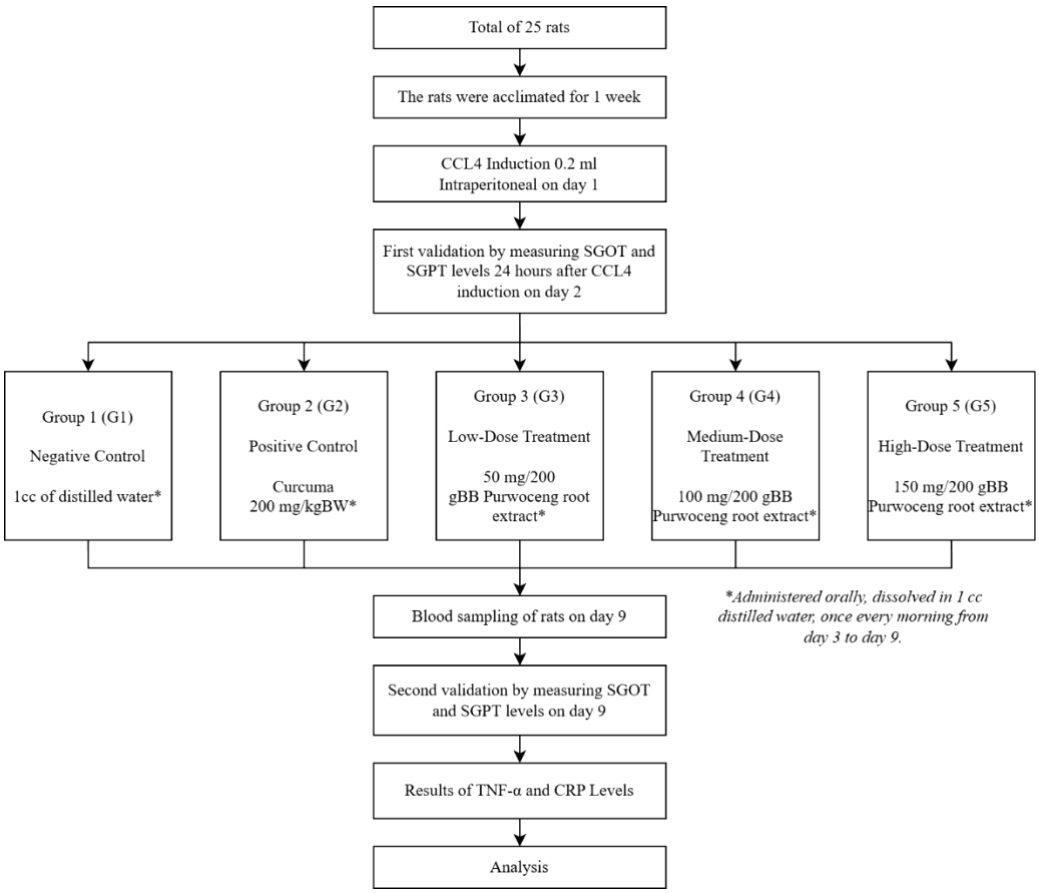

Figure 1. Study Design

Ethical Clearance

The study was approved by the Ethics Committee of the Faculty of Medicine, Sultan Agung Islamic University, under reference number No.402/X/2024/Komisi Bioetik.

Purwoceng Root Extraction Purwoceng root extract was prepared from roots sourced from the Dieng highlands, selected based on their white color, intact structure, and absence of decay or damage. A total of 250 grams of purwoceng roots were pulverized and soaked in 70% ethanol in the Biochemistry Laboratory, Faculty of Medicine, Sultan Agung Islamic University. The mixture was filtered, and the solvent was removed through evaporation to obtain the ethanol extract.

Animal Model

The rats were housed in the Biology Laboratory, Faculty of Medicine, Sultan Agung Islamic University with the polypropylene cages under controlled environmental conditions (temperature: 28–32°C, humidity: 40–70%, and 12-hour light-dark cycles) and provided with standard laboratory chow and water *ad libitum*. Twenty-five healthy male rats, aged 2–3 months, and weighing 150–200 grams were acclimatized for seven days before the experiment and randomly divided into five groups (n = 5 per group):

a) Group 1 (Negative Control): Received 1 mL distilled water daily for seven days.
b) Group 2 (Positive Control): Received curcumin 200 mg/kg BW daily for seven days.
c) Groups 3, 4, and 5**:** Received purwoceng root extract at doses of 50 mg (low-dose), 100 mg (medium-dose), and 150 mg (high-dose) per 200 g BW, respectively, administered orally once daily for seven days.

The dosage range of purwoceng extract (50–150 mg/200 g BW) was selected based on prior studies that investigated its pharmacological and toxicological profiles. Randa[13] reported anti-inflammatory efficacy at moderate doses, while Arjadi et al.[14] identified hepatotoxic effects at subchronic doses exceeding 200 mg/200 g BW. Accordingly, the present study employed lower and mid-range doses to evaluate the therapeutic window while minimizing toxicity risk. This approach also aligns with standard preclinical dose-scaling principles for rodent models[13,14].

CCl4 Induction

CCl4 induction was performed using a dose of 1 mL/kg, effective for inducing liver fibrosis with minimal mortality. For this study, with rats weighing 150–200 grams, a dose of 0.2 mL of CCl4 was administered intraperitoneally as a single injection on day 1 of the experiment. The CCl4 was drawn into a 1 mL syringe and injected into the abdominal cavity of the rats.

Validation of induction was conducted on day 2 by observing behavioral changes (reduced activity and appetite) and confirming increased liver enzyme levels (SGOT and SGPT), indicating successful hepatotoxic induction. After validation, treatments were initiated on day 3 and continued for seven consecutive days according to group allocation (negative control, positive control with curcumin, and three purwoceng extract doses).

Blood Sample Collection

On day 9, blood samples were collected from the rats via the retro-orbital plexus. The procedure involved preparing a microhematocrit (Kimble™ Micro-Hematocrit Capillary Tubes, DWK Life Sciences, Germany) and an EDTA vacutainer tube (BD Vacutainer® 2, BD, USA). The microhematocrit was inserted into the retro-orbital sinus and gently rotated until blood was drawn. The collected blood was transferred to a labeled EDTA vacutainer tube, and the microhematocrit was cleaned after use.

TNF-α and CRP Measurement

Reagents: TNF–α (Chemicon®, Merck, USA), CRP (KHCRP-001, Shanghai Kehua Bio-engineering, China), SGOT (Liqui-UV®, Stanbio Laboratory, USA), and SGPT (ERBA, Erba Mannheim, Germany), standard solutions, and samples were prepared and allowed to reach room temperature before testing. The required number of ELISA strips was placed into frames for use, with any unused strips stored at 2–8°C. A volume of 50 µL of standard solution was added to the designated standard wells. The standard solution already contained biotinylated antibodies, so additional antibodies were not required.

All ELISA measurements were conducted in duplicate wells per sample to ensure reliability and reproducibility. Mean optical density (OD) values were used for analysis. The analyst conducting the ELISA and statistical analysis was blinded to the treatment group allocation to minimize bias. Environmental parameters (temperature, humidity, and light cycle) were monitored daily to maintain consistent experimental conditions. For the sample wells, 40 µL of sample was added, followed by 10 µL of anti-TNF-α antibody and 50 µL of streptavidin-HRP. The plate was gently mixed and sealed before being incubated at 37°C for 60 minutes. Following incubation, the sealer was removed, and the plate was washed five times using 0.35 mL of wash solution per well. Each wash was allowed to soak for 30 seconds to 1 minute, and the excess solution was aspirated, leaving the plate to dry on absorbent material. Next, 50 µL of substrate solution A and 50 µL of substrate solution B were added to all wells. The plate was then incubated at 37°C in the dark for 10 minutes. After incubation, 50 µL of stop solution was added to each well, which caused the color to change from blue to yellow. Optical density (OD) values for each well were immediately measured using a microplate reader (BD™ ELISA Rat TNFA, BD, USA) set to 450 nm within 10 minutes of adding the stop solution. The TNF-α concentrations in the samples were calculated using regression analysis software to fit the curve to the data points.

The CRP measurement procedure followed the same steps as the TNF-α assessment, using specific anti-CRP antibodies and reagents provided in the ELISA kit (BD™ ELISA Rat CRP, BD, USA). Optical density values were measured at 450 nm, and CRP concentrations were calculated based on a standard curve. Both TNF-α and CRP were measured because they represent complementary stages of the inflammatory cascade. TNF-α acts as an early pro-inflammatory cytokine that triggers downstream acute-phase responses, while CRP is a hepatocyte-derived protein synthesized under the influence of cytokines such as TNF-α and IL-6. Measuring both biomarkers provides integrated insight into local cytokine activation and systemic inflammatory response, allowing for a more comprehensive assessment of the hepatoprotective mechanism of purwoceng extract[11,15].

Data Analysis

The normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated with Levene's test. TNF-α levels showed non-normal distribution in certain groups, thereby warranting the use of the Kruskal–Wallis test as a nonparametric alternative to one-way ANOVA. Conversely, CRP data met assumptions of normality and homogeneity; thus, one-way ANOVA followed by post hoc LSD testing was applied. All statistical analyses were performed using SPSS version 26.0, with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

After seven days of treatment, the mean TNF-α level was highest in the negative control group (G1, 187.53 ± 32.32 pg/mL) and lowest in the positive control group (G2, 128.00 ± 29.75 pg/mL). Among the purwoceng-treated groups, TNF-α levels varied, with the lowest value observed in G5 (141.89 ± 37.23 pg/mL), followed by G3 (159.56 ± 56.44 pg/mL) and G4 (162.54 ± 53.83 pg/mL). Although these numerical differences suggest a possible pattern, they did not form a consistent or statistically supported trend across doses.

Normality testing using the Shapiro–Wilk test indicated that TNF-α data were not normally distributed in G1 and G2 ($p < 0.05$), while G3–G5 showed normality ($p > 0.05$). Levene's test demonstrated homogeneity of variance ($p = 0.336$). Therefore, a Kruskal–Wallis test was used, revealing no significant difference among the five groups ($H = 9.62$, $p = 0.233$). Although numerical differences were observed, the variation was not statistically significant.

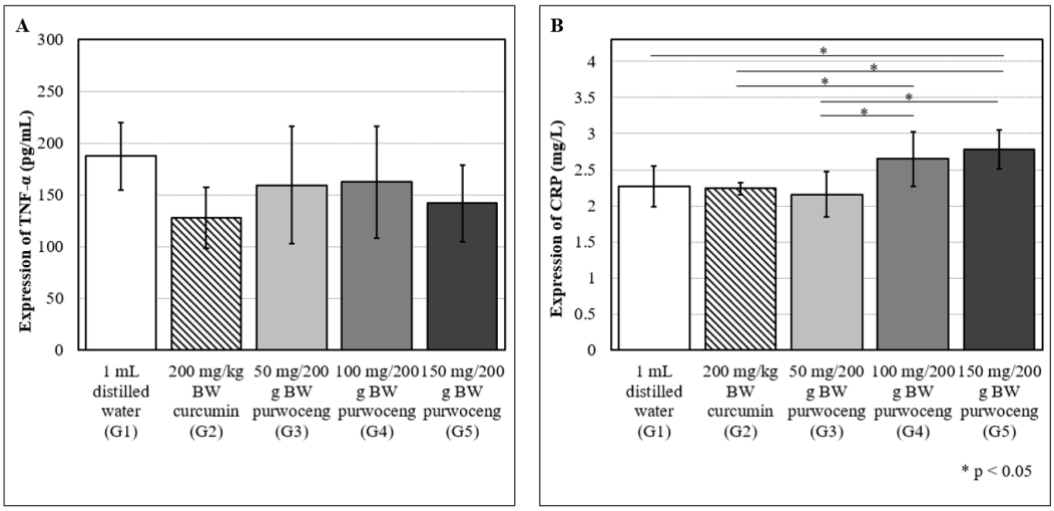

Figure 1. Expression of TNF-α and CRP in different treatment groups.

*Note: (A) TNF-α expression was decreased in all groups with no significant difference. (B) CRP expression was significantly increased in the high-dose treatment group (G5). Bars represent mean ± SD; * indicates significant difference between groups (*p < 0.05).*

Table 1. Mean expression and statistical tests of TNF-α and CRP levels across groups.

Expression/ statistical test	Negative control/ 1mL distilled water (G1)	Positive control/ 200 mg/kg BW curcumin (G2)	Low-dose/ 50 mg/200 g BW purwoceng (G3)	Medium-dose/100 mg/200 g BW purwoceng (G4)	High-dose/150 mg/200 g BW purwoceng (G5)	P-Value
TNF-α (pg/mL)	187.53 ± 32.32	128.00 ± 29.75	159.56 ± 56.44	162.54 ± 53.83	141.89 ± 37.23	
Shapiro wilk (p)	0.019	0.008	0.818†	0.067†	0.782†	
Lavene test (p)						0.336‡
Kruskal-Wallis test (p)						0.233
CRP (mg/L)	2.27 ± 0.28	2.24 ± 0.08	2.16 ± 0.31	2.65 ± 0.38	2.78 ± 0.27	
Shapiro wilk (p)	0.507†	0.669†	0.672†	0.577†	0.405†	
Lavene test (p)						0.058‡
Oneway ANOVA (p)						0.008*
Post hoc LSD (p)						
Negative control/1 mL distilled water (G1)		0.864	0.524	0.050	0.010*	
Positive control/200 mg/kg BW curcumin (G2)	0.864		0.640	0.035*	0.007*	
Low-dose/ 50 mg/200 g BW purwoceng (G3)	0.524	0.640		0.013*	0.002*	
Medium-dose/100 mg/200 g BW purwoceng (G4)	0.050	0.035*	0.013*		0.466	

Note:
† Normal (p>0.05)
‡ Homogenous (p>0.05)
* Significant difference (p < 0.05)

The mean CRP level was highest in the high-dose treatment group (G5, 2.78 ± 0.27 mg/L) and lowest in the low-dose treatment group (G3, 2.16 ± 0.31 mg/L). The negative control (G1) and positive control (G2) showed similar CRP levels of 2.27 ± 0.28 mg/L and 2.24 ± 0.08 mg/L, respectively.

Normality and homogeneity assumptions were met (Shapiro–Wilk $p > 0.05$; Levene $p = 0.058$), allowing parametric analysis. One-way ANOVA revealed a significant difference in CRP among groups ($p = 0.008$). Post hoc LSD tests indicated significant differences between G1 and G5 ($p = 0.010$), G2 and G4 ($p = 0.035$), G2 and G5 ($p = 0.007$), G3 and G4 ($p = 0.013$), and G3 and G5 ($p = 0.002$), whereas other pairwise comparisons were not significant ($p \geq 0.05$). The data indicate that purwoceng at high doses (150 mg/200 g BW) significantly increased CRP levels, suggesting a possible pro-oxidant or hepatotoxic response at elevated concentrations.

The theoretical framework illustrated in Figure 3 outlines the complex interplay between purwoceng root extract, CCl_4-induced hepatic injury, and the downstream inflammatory pathways involved. Within this framework, curcumin functions as a well-established reference compound that suppresses inflammation by inhibiting NF-κB signaling and reducing pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[16]. These regulatory actions are essential for preventing the progression of acute damage into chronic inflammation[17]. TNF-α, a critical pro-inflammatory cytokine, plays a pivotal role in promoting hepatic inflammation and fibrosis by activating signaling pathways like NF-κB and MAP kinase[18].Purwoceng root extract, which contains flavonoids, saponins, and tannins, is thought to modulate similar pathways and thus may contribute to hepatoprotection[4,5,7]. In the present study, all treated groups showed numerically lower TNF-α levels compared with the negative control, although these reductions were not statistically significant. This lack of significance may be attributed to variability in bioavailability, differences in extract composition, or limitations in the absorbed concentrations of active constituents.

CRP, a hepatocyte-derived acute-phase protein regulated primarily by IL-6 and influenced by NF-κB activation, exhibited a different pattern[15]. At low doses, purwoceng produced the lowest CRP levels, but at the highest dose CRP increased unexpectedly. This paradoxical elevation is biologically plausible. Many flavonoids exhibit a biphasic (hormetic) dose–response, functioning as antioxidants at low concentrations but becoming pro-oxidant at higher concentrations[19,20]. Mechanistically, excessive flavonoid exposure can promote auto-oxidation and the formation of semiquinone radicals, which participate in redox cycling and generate reactive oxygen species[21]. Such oxidative stress may activate NF-κB and subsequently elevate acute-phase proteins like CRP, thereby counteracting the expected anti-inflammatory effect[21–23]. This dual behavior has been documented in several phenolic compounds, suggesting that purwoceng may similarly exhibit a narrow therapeutic window where benefits diminish or reverse at supra-physiological doses[14,24].

In addition to the hormetic dose-response effects, the bioavailability and metabolism of purwoceng's constituents may further influence its biological activity. Flavonoids often have limited oral absorption and undergo rapid metabolic conjugation, reducing their systemic availability[25,26]. Saponins are similarly characterized by poor intestinal permeability and are extensively transformed by gut microbiota before entering the circulation[27]. These pharmacokinetic constraints may explain the modest magnitude of purwoceng's systemic anti-inflammatory effects at tolerated doses and the inconsistent biomarker responses observed at higher doses.

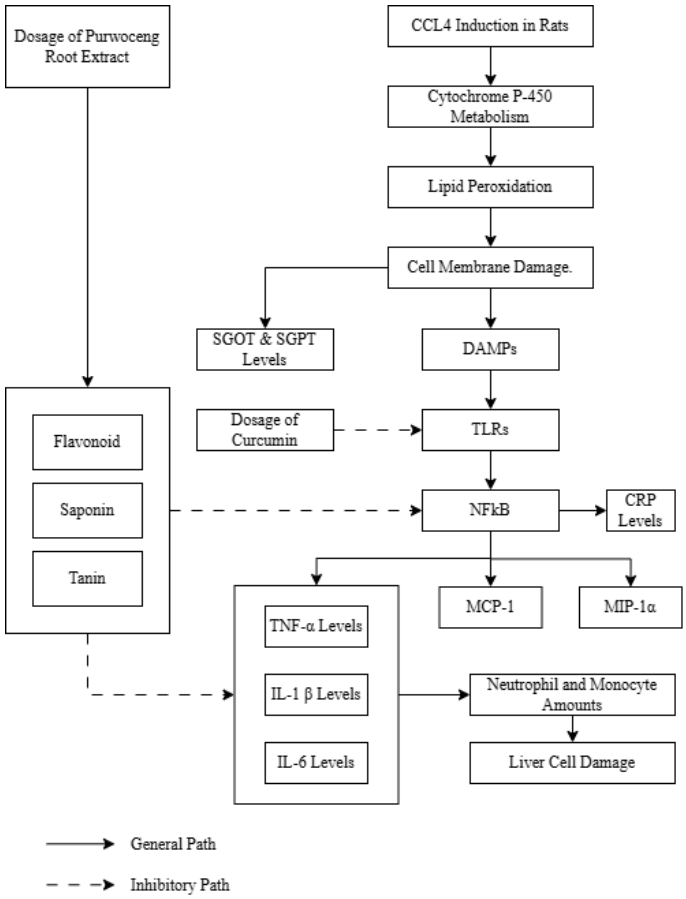

Figure 2. Theoretical framework of purwoceng in the complex anti-inflammatory mechanism in this study.

Compared to these results, standardized Indonesian hepatoprotective herbs behave somewhat differently. *Curcuma xanthorrhiza* (Java turmeric) and its active component xanthorrhizol consistently show anti-inflammatory action across doses. For example, administration of *C. xanthorrhiza* extract in murine models significantly suppressed CRP, IL-1β, IL-6 and TNF-α in liver and other tissues[28]. Curcumin, a well-established anti-inflammatory agent, also demonstrates robust efficacy in modulating inflammatory cytokines and protecting against oxidative stress[29]. Similarly, *Andrographis paniculata* (bitter leaf), whose main diterpene andrographolide is used clinically, robustly inhibits NF-κB signaling and downstream TNF-α/IL-6 production in inflammation models[30]. In contrast to the apparent hormetic response we observed with purwoceng, these standardized extracts tend to produce monotonic anti-inflammatory effects within their therapeutic range. The difference likely reflects their distinct phytochemical profiles and the relative ratios of active constituents. These comparisons suggest that, while purwoceng contains anti-inflammatory flavonoids and saponins, its optimal dosing may be narrower or more complex than for the reference herbs, underscoring the need for careful dose-finding. This study's findings partially align with previous research on purwoceng's efficacy in pain and inflammation models but reveal gaps in dose optimization, underscoring the need for further investigation[13].

From a translational perspective, the findings underscore both the promise and the caution required in developing purwoceng as a hepatoprotective agent. The extract's ability to influence TNF-α and CRP suggests biologically meaningful activity, yet the lack of statistically confirmed dose–response relationships and the potential for pro-oxidant effects at higher doses underscore the importance of

determining a safe and effective dosing range. Further investigations that incorporate pharmacokinetic profiling, standardized extract preparation, and mechanistic assays, such as direct measurements of oxidative stress or NF-κB activation, will be required to clarify purwoceng's therapeutic potential.

This study has several limitations. First, individual-level biomarker data were not retained, which prevented the use of formal trend analyses such as regression or monotonic tests. As a result, potential dose-related patterns in TNF-α and CRP could only be interpreted descriptively and cannot be considered statistically confirmed. Second, natural variability in the composition of purwoceng extract may influence the consistency of biological effects, and the study did not include acute or chronic toxicity assessments to determine the safety margin of the extract. Additionally, the absence of compound-specific profiling limits the ability to identify which active constituents contribute most to the observed responses. Future research should focus on standardizing extract composition, performing comprehensive toxicity testing, isolating key bioactive compounds, and evaluating their pharmacokinetic and bioavailability characteristics to better understand purwoceng's therapeutic potential and optimize its dosing strategy.

CONCLUSION

The findings of this study indicate that purwoceng root extract exerts measurable effects on inflammatory biomarkers in CCl_4-induced liver injury, with a modest reduction in TNF-α across treatment groups and a variable CRP response that was lowest at the low dose and elevated at the highest dose. This pattern suggests a complex biological profile in which purwoceng may demonstrate beneficial anti-inflammatory activity at certain concentrations while potentially eliciting pro-oxidant or pro-inflammatory effects at higher levels. Although these observations do not constitute statistically confirmed dose-dependent effects, they highlight the importance of identifying an optimal therapeutic window and understanding the extract's biphasic potential.

Future research should focus on isolating and characterizing purwoceng's active metabolites, performing comprehensive pharmacokinetic and toxicity profiling, and evaluating time-course changes in inflammatory biomarkers to determine the temporal dynamics of its effects. Standardization of extract composition and mechanistic studies examining oxidative stress pathways, NF-κB activity, and hepatocyte responses will also be essential to clarify purwoceng's therapeutic potential and safety profile.

AUTHORS' CONTRIBUTIONS

Viki Dwi Randa: Conceptualization, Methodology, Software, Data curation, Writing-Original draft preparation, Visualization **Setyo Trisnadi**: Supervision, Validation, Writing- Reviewing and Editing **Titiek Sumarawati**: Supervision, Validation, Writing-Reviewing and Editing.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the

authors. The data is the result of the author's research and has never been published in other journals.

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