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Original Research Article

The Evaluation of the Acute Toxicity Of MCH2 Syrup Formulations on the Liver Organs of Male Wistar Strain Rats Entailed Histopathological Observations and the Examination of Symptoms of Toxicity

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ABSTRACT

In this study, acute toxicity tests and liver histopathology in male white rats were used to explore the immunomodulatory potential of MCH2 syrup, which was prepared with a 2:2 ratio of *Phyllantus niruri* L. and *Morinda citrifolia* L. The study, which used a posttest-only control group design, entailed giving different dosages of MCH2 syrup orally and then assessing behavioral changes, body weight changes, and liver histological characteristics like degeneration, congestion, and necrosis. The results validated the immunomodulatory activity of MCH2 syrup by showing notable effects on the liver histology of male white rats, demonstrating modest and quickly regenerating damage.

Keywords: *Phyllantus niruri L., Morinda citrifolia L.,* Acute Toxicity, Histopathology

Introduction

The global prevalence of traditional medicine, derived from plant, animal, or mineral sources, is attributed to its historical effectiveness, with approximately 60% of the world's population, spanning developed and developing countries, embracing herbal remedies.[1] In Indonesia, renowned for its rich biodiversity, traditional medicine remains integral to society due to its proven efficacy across generations.[2] The country's diverse flora, exemplified by *Phyllanthus niruri* L. and *Morinda citrifolia* L., offers the potential for developing traditional medicines.[3] Given that diseases are influenced by various factors, such as environmental conditions, there is a growing emphasis on bolstering the immune system or utilizing immunomodulatory substances.[4]

The study formulates MCH2 syrup, combining *P. niruri* herb and *M. citrifolia* fruit extract, to assess immunomodulatory activity, employing test animals to monitor resultant symptoms. *M. citrifolia*, historically recognized for its use in tropical regions, contains diverse active compounds, while *P. niruri* is acknowledged for its immunostimulant properties.[5] Immunomodulators, encompassing compounds like curcumin, flavonoids, and vitamins, aim to either stimulate or suppress the immune system.[6]

The transition of traditional medicines into Standardized Herbal Medicines necessitates pre-clinical testing and raw material standardization.[7] Despite their natural origin, herbal medicines are not inherently completely safe, necessitating assessments of safety, including toxicity tests.[8]

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In this study, an acute toxicity test of the MCH2 syrup, comprising *P. niruri* herb and *M. citrifolia* fruit extract in a 2:2 ratio, was conducted to ascertain intrinsic toxicity, identify target organs, assess species sensitivity, and determine initial dose levels, providing essential hazard information post-acute exposure.[9] The test involved repeated oral administration of the substance to observe toxicity symptoms, with subsequent histopathological examination focusing on the liver, a pivotal organ in detoxification.[10] The study aimed to contribute insights into identifying toxic chemicals and associated hazards by evaluating symptoms of toxicity in test animals.

Materials and Methods

Materials

The study utilized the following materials: *P. niruri*, *M. citrifolia*, purified water, methyl paraben, sucrose, sorbitol, sodium benzoate, tutty fruity flavor, rattus norvegicus male rat, diethyl ether, buffer neutral formalin 10%. The equipment employed included an ohaus analytical balance, I-2000 taffware scales, binder brand oven, infusion pan, memmert WNB 10 waterbath, ultra turrax IKA T25 digital brand, microscope light brand miconos MCX50LED series, digital camera brand optilab plus, beaker glass, dropper pipette, funnel glass, scissors network, tweezers, obsidi oral probe, and Syringe 1 cc. The herbal plants *P. niruri* and *M. citrifolia* were gathered in December 2023 at the Herbal Materia Medica Laboratory in Batu, East Java, Indonesia. The collection locations are situated between the latitudes of S 11° 09' 41" and S 11° 18' 52", and the longitudes of E 182° 72' 32" and E 182° 62' 11". The city of Batu experiences a substantial amount of rainfall each year, with an average of 3,290 mm. The mean relative humidity is 77%, while the mean temperature stands at 23.5°C. Botanist Dr Budi Sumarta authenticated the plant samples and placed voucher specimens (FA:054-MACHUNG-2023) and (FA:055-MACHUNG-2023) in the Pharmacognosy Laboratory of the Department of Pharmacy at Ma Chung University.

Extraction
The ripe *M. citrifolia* fruits were carefully peeled and cleaned with purified water before their seeds were segmented and air-dried at room temperature to reduce moisture. The dried fruit was then ground using a hammer mill and further dehydrated in an oven at 50°C until its moisture content dropped below 10%. As for the *P. niruri* herb, it underwent initial wet sorting, extensive washing with purified water, and subsequent drying in an oven at 50°C[10]. Once dried, the meniran herbs were homogenized to create a fine powder. For the extraction process, the infusion method was employed. A ratio of 500 grams of simplicia powder to 3 liters of purified water was used, with the mixture stirred until it reached boiling point, typically taking 30-60 minutes. The mixture was then filtered using a filter cloth, and the resulting extract underwent concentration through evaporation, using a water bath set at 80°C, resulting in a dense extract.[11]

Syrup preparations
Initially, 87.5 mL of purified water was heated to 90°C in a 400 mL beaker glass, followed by the addition of 0.3 grams of methyl paraben, stirring until fully dissolved. This solution was then combined with 150 grams of sucrose and stirred for 10 minutes while maintaining a solution temperature of 40°C. In another container, 214 mL of purified water and 50 grams of sorbitol were dissolved through mixing. The two solutions were then blended using an ultraturax for 5 minutes at 40°C. Afterwards, 10 grams of *M. citrifolia* condensed extract were incorporated into the mixture and homogenized for 5 minutes using the ultraturax. Subsequently, 10 grams of condensed *P. niruri* extract were added to the mixture and homogenized with the ultraturax for 15 minutes. Following this, 0.5 grams of sodium benzoate and 1.25 grams of tutty fruity flavor were added and homogenized with the ultraturax for 5 minutes. Finally, purified water was added to bring the total volume to 500 mL, and the mixture was homogenized with the ultraturax for an additional 5 minutes.[12]

Toxicity test
The research has obtained approval from the Research Ethics Commission of Brawijaya University under the reference number 829-KEP-UB. The study included adult male Rattus norvegicus white rats, with a weight range of 150 to 250 grams and an age range of 4 to 5 months. The rats were given a one-week time to acclimate to their surroundings before the study began. During this acclimation phase, the rats were kept in a controlled environment and given unlimited access to food. The housing facilities were kept in a state of ideal cleanliness, with the temperature set at 22 ± 3 °C and the relative humidity maintained between 30% and 70%. Furthermore, a steady 12-hour cycle of light and darkness was also upheld. Prior to commencing the experimental procedures, the rats experienced a fasting period of 14 hours, during which they were allowed to continue drinking water. Afterwards, the rats were weighed individually and then given the test chemicals through oral gavage delivery. After a 7-day period of adjustment, the rats were randomly allocated into 5 groups, with each group including 5 rats. Over the course of the 14-day experiment, the rats were given the investigational medications orally. Systematic monitoring of the rats was carried out at certain time intervals of 30 minutes, 4 hours, and 24 hours after the test material was given, during the whole 14-day study period.[13]

Histopathological analysis
Histopathological investigation was performed on all experimental animals throughout the entire duration of the study. The liver histopathology preparations were analyzed using a light microscope to evaluate any apparent alterations. The visual field, which is observed as a circular area under the microscope, was divided into four sections. Each section was assessed using the Knodell scoring system. The scores from each section were summed and then divided by the number of sections to calculate the average. In order to guarantee a thorough evaluation, every preparation underwent a minimum of five examinations, and the resulting results were then averaged. This score, which was calculated by taking the average, represents the data for a single preparation. The histopathological index or scoring value, which quantifies the extent of damage in each sample, was calculated by summing the scores of all observed lesions. The scoring system used was a modified iteration of the Knodell method, which involved combining scores for different histopathological abnormalities. The data on the histopathological changes in rat livers, which were evaluated and scored, were collected, ranked, and analyzed statistically using one-way ANOVA and non-parametric Kruskal-Wallis tests to discover any significant differences between the groups.[14]

Statistical analysis
A one-way analysis of variance (ANOVA) was employed to examine differences in the means across multiple samples in a single direction. Normality of the data was assessed using the Shapiro-Wilk test, which was conducted on the raw data without any preprocessing, such as frequency distribution table creation. The Kruskal-Wallis test, a non-parametric test based on ranks, was utilized to determine if there were statistically significant differences among two or more groups of independent variables with respect to a dependent variable. This test is particularly suited for assessing differences in numerical data (interval/ratio) and ordinal scales.[15]

Results and Discussion

Rat body weight
The results of our observations have revealed a notable trend: an increase in weight observed across all treated groups in our experimental study involving rats. This intriguing outcome prompts an exploration into the underlying factors contributing to this observed weight gain. Upon closer examination, it becomes evident that the rats in our study were not subjected to the typical stressors often associated with laboratory environments. This absence of stress proved to be a critical factor, allowing the mice to adapt more effectively to their experimental surroundings. As a result, the test animals experienced a heightened sense of comfort within their environment. [16]
This newfound comfort played a pivotal role in influencing the physiological responses of the rats, particularly in relation to their body mass. When animals are under stress, their metabolic processes can become dysregulated, potentially impacting their weight. However, in our study, the alleviation of stress enabled the mice to regulate their metabolism more efficiently, leading to a gradual increase in weight over the course of the experiment. Furthermore, the absence of stress likely prompted behavioral changes in the rats, further contributing to their weight gain. Without the distractions and anxieties associated with stress, the rats may have been more inclined to engage in behaviors that promote weight gain, such as increased food intake and reduced energy expenditure.[17] In summary, our findings underscore the profound influence of stress and environmental factors on the physiological responses of experimental animals. By providing a comfortable and stress-free environment, we observed a consistent increase in weight across all treated groups, highlighting the intricate relationship between stress, adaptation, and metabolic regulation in laboratory rats.

Toxicity symptoms in rats
Throughout our experimental study involving male white rats, spanning a duration of 14 days, meticulous observations were conducted at various intervals: within the first 30 minutes, the first 4 hours, and the first 24 hours following treatment administration.[18] These observations aimed to assess the presence of any signs indicative of toxicity across three distinct groups: the treatment group, the positive control group, and the negative control group. Remarkably, our findings revealed an absence of any discernible signs of toxicity within all three groups throughout the duration of the observation period. This absence of toxicity was consistent across all time intervals examined, indicating that the experimental animals did not exhibit any noticeable changes in behavior suggestive of toxic effects.[19]
In the treatment group, where the experimental intervention was administered, no adverse reactions or toxic symptoms were observed within the specified time frames. Similarly, in both the positive control group, which served as a reference for expected toxic effects, and the negative control group, which received no treatment, there were no indications of toxicity or abnormal behavior.[19] This remarkable lack of toxicity observed in our study suggests that the experimental treatment

did not elicit any harmful effects on the male white rats under the conditions tested. Furthermore, the absence of toxicity in the positive and negative control groups reinforces the reliability of our experimental observations, indicating that any changes in behavior observed were not attributable to toxicological effects but rather to other factors. Overall, our findings provide reassuring evidence of the safety profile of the experimental intervention in male white rats, as demonstrated by the absence of toxicity-related symptoms and changes in behavior throughout the observation period. These results lay a solid foundation for further investigations into the efficacy and potential therapeutic applications of the experimental treatment.[20]

Liver weight

Observing the liver across different treatment groups revealed no notable differences, although subtle changes may exist beyond visible detection, possibly due to the study's short duration. The ANOVA test requires normally distributed data with homogeneous variance and independent samples. However, Shapiro-Wilk Normality test results showed non-normal distribution (sig ≤ 0.05), while the homogeneity test indicated homogeneous variance (sig ≥ 0.05). Consequently, the Kruskal-Wallis test was employed due to these unmet assumptions, yielding a non-significant result (sig ≥ 0.05), suggesting no significant difference in liver weight among treatment groups. Subsequently, a Post Hoc test was conducted to identify specific group differences. While the Tukey HSD test typically follows One-Way ANOVA, here the Dunn test was used as a continuation of the Kruskal-Wallis test. Significance was determined by comparing sig values between group pairs, with ≤ 0.05 indicating a difference and ≥ 0.05 indicating no difference.[21]

Liver histopathological observation

Congestion observation results

In this study, liver damage in the tested rats primarily manifested as hemorrhage and intrasinusoidal congestion, indicative of moderate hepatic injury. Histopathological examination utilizing a modified Knodell scoring method revealed congestion levels below 25% of the visual field in the P1, P3, and P5 treatment groups. Notably, the positive control group, which included *P. niruri* known for its antihepatoxic properties, showed no congestion, along with the negative control group (placebo treatment). However, when compared to other forms of liver damage such as degeneration and necrosis, it is speculated that the additives in the MCH2 formulation may either lack toxicity, leading to congestion, or be excessively toxic, causing direct hepatocyte necrosis (Figure 1). Statistical analysis using Kruskal-Wallis test indicated non-normal distribution (sig ≤ 0.05) and lack of homogeneity (sig ≤ 0.05) in congestion data. Hence, Kruskal-Wallis test was chosen, revealing a significant effect of treatment groups on congestion (sig = 0.002, p < 0.05). Subsequent Dunn Test showed the positive control group differed significantly from all other treatment groups except the negative control. Additionally, the negative control group exhibited no difference compared to the P3 group, but significantly differed from P1 and P5 groups. Conversely, no statistically significant differences were observed among the P1, P3, and P5 treatment groups. Differences were deemed significant when sig ≤ 0.05.[22]

Degeneration observation results

Histopathological examination of the livers of male white rats, utilizing the Knodell scoring method, revealed inflammatory cell infiltration across all treatment groups. Surprisingly, even the negative control group exhibited such infiltration, despite the absence of *P. niruri* and *M. citrifolia* extracts, indicating potential influence from active and additional ingredients present in the MCH2 syrup formulation. Notably, sorbitol, a sugar alcohol in the syrup, is recognized for its impact on liver damage due to its challenging digestibility, with a minimal portion being metabolized by the pancreas during digestion.[23]

Similarly, inflammatory cell infiltration was observed in the positive control group due to the absence of *M. citrifolia* fruit extract in the stimuno test preparation. Conversely, while the scoring results were consistent among the P1, P3, and P5 treatment groups, variations were anticipated given the differing active substances within the syrup. Nevertheless, the presence of *P. niruri* in each preparation likely contributed to mitigating hepatic damage, though scoring outcomes ideally should have been lower for the P1, P3, and P5 groups owing to the inclusion of *M. citrifolia* extract (Figure 2).

Statistical analysis using Kruskal-Wallis analysis was conducted on degeneration observation data. Despite normally distributed data (sig ≤ 0.05) and homogeneous variation (sig ≥ 0.05) in degeneration, ANOVA testing revealed a significant effect of treatment groups (sig ≤ 0.05) on degeneration. Subsequent Tukey HSD test indicated significant differences between the positive control group and all other treatment groups, with only the negative control group differing significantly from the P5 group. However, no statistically significant differences were observed among the P1, P3, and P5 treatment groups (sig ≤ 0.05).

Necrosis observations result

The liver cell damage observed in the rats under study presented as karyolytic necrosis, characterized by nucleus dissolution and reduced color absorption. The histopathological examination using the Knodell scoring method revealed varying degrees of necrosis across treatment groups. The P1 group exhibited necrosis with bridging necrosis spanning 25-50% of visual fields, whereas the positive control group showed >50% bridging necrosis. Intriguingly, the negative control group also displayed >50% necrosis throughout the visual field. Conversely, the P3 and P5 groups demonstrated multilobular necrosis, indicating necrosis across multiple tissue blocks.

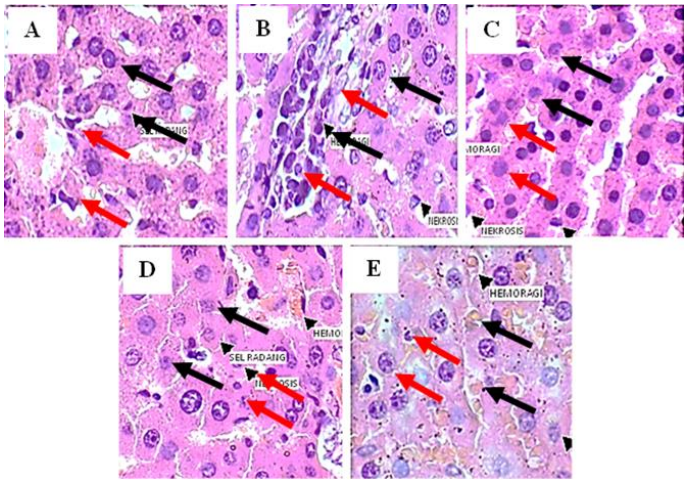

Figure 1: Histopathology of the congested liver. Description: A (positive control); B (negative control); C (once a day); D (twice a day); E (three times a day); necrosis (red arrow); and inflammation (black arrow).

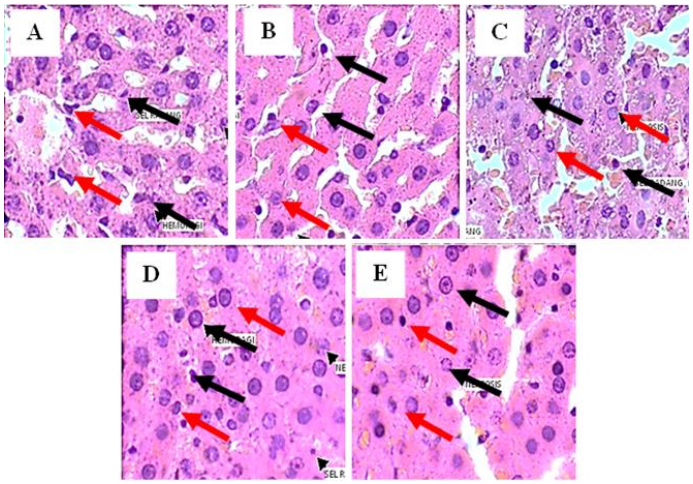

Figure 2: Histopathology of the degenerated liver. Description: A (positive control); B (negative control); C (once a day); D (twice a day); E (three times a day); necrosis (red arrow); and inflammation (black arrow).

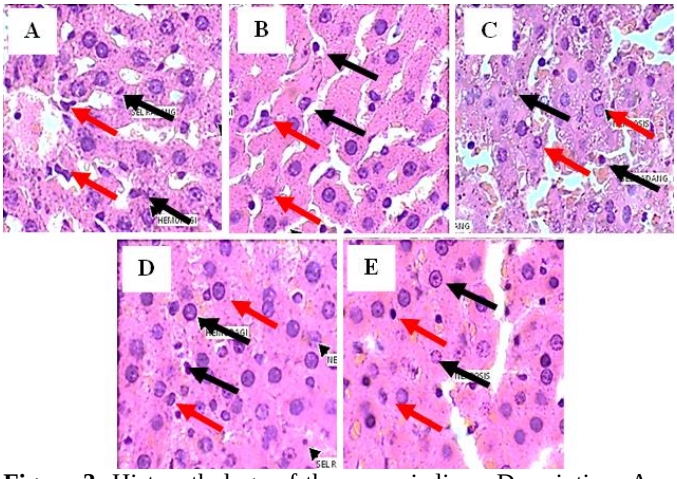

Figure 3: Histopathology of the necrosis liver. Description: A (positive control); B (negative control); C (once a day); D (twice a day); E (three times a day); necrosis (red arrow); and inflammation (black arrow).

The positive control group, solely comprising meniran extract, exhibited higher levels of bridging necrosis, indicating limited efficacy in liver cell regeneration. In contrast, the P1 group, containing active compounds from MCH2 syrup such as phyllanthin and hypophyllanthin from *P. niruri*, and scopoletin, flavones, xeroin, and terpenoids from *M. citrifolia* fruit extract, displayed bridging necrosis at a lower percentage, suggestive of their roles in aiding liver cell regeneration. The occurrence of multilobular necrosis in the P3 and P5 groups may stem from excessive active compounds in MCH2 syrup, leading to antagonistic interactions or toxic effects with additional ingredients like sorbitol. Notably, the negative control group, devoid of active substances, still exhibited significant necrosis, potentially attributed to the inclusion of sorbitol, known for its limited digestibility (Figure 3).

The statistical analysis revealed that the data for necrosis followed a normal distribution (p-value ≥ 0.05), however there was evidence of non-homogeneous variance (p-value below 0.05). The Kruskal-Wallis test was used to analyze the data, and it showed a significant impact of treatment groups on the occurrence of necrosis ($p < 0.05$). The subsequent Dunn Test revealed significant disparities between the positive control group and all other treatment groups, with the exception of P1. On the other hand, there were no notable distinctions found between the P1, P3, and P5 treatment groups, as well as between the negative control group and these treatment groups (p-value ≤ 0.05).

Conclusion

No symptoms of toxicity were observed in the examined animals following the administration of MCH2 syrup. Significantly, all of the test individuals did not display any symptoms such as reduction in body weight, lack of strength, abnormal bowel movements, involuntary muscle contractions, or fluid retention. Nevertheless, the injection of MCH2 syrup had noticeable impacts on the liver histology of male white rats, leading to degeneration, congestion, and necrosis. Significantly, the increased frequency of administration worsened the damage to the liver. Among the treatment groups, the P1 group exhibited the lowest level of damage in comparison to the P3 and P5 groups, suggesting a considerably more advantageous state for the regeneration of hepatocytes.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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