Pharmacogn J. 2021; 13(6)Suppl: 1549-1556
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Analyzing of Urine 3-Hidroxy Propil Mercapturic Acid on Cyclophosphamide Induced Rat to Determine Ameliorating Effect of *Leucaena leucocephala* (Lam.) de Wit Seed Extract

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History

- Submission Date: 26-09-2021;
- Review completed: 06-10-2021;
- Accepted Date: 14-10-2021.

DOI : 10.5530/pj.2021.13.197

Article Available online

http://www.phcogj.com/v13/i6

ABSTRACT

Background: A 70% ethanolic extract of *Leucaena leucocephala* (Lam.) de Wit seeds contain a certain amount of sulfhydryl active compounds and potential for ameliorated cyclophosphamide side effects. **Objective:** The aim of this study was to analyze of urine 3-hidroxy propil mercapturic acid (3-HPMA) in a rat model for cyclophosphamide-induced hemorrhagic cystitis to determine the effect of *Leucaena leucocephala* (Lam.) de Wit seeds extract. **Materials and methods:** The levels of urine 3-hidroxy propil mercapturic acid was measured using LCMS/MS. Urine preparation was carried out by dilution (1:5) with mobile phase and contained a deuteurated internal standard. The LC separation was performed using a C_{18} column (1.7 µm; 2.1 mm × 100 mm). The mobile phase consisted of formic acid (0.1%) and formic acid (0.1%) in acetonitrile (90:10 v/v), as a starting gradient at flow rate of 0.2 ml/min with total run 7 min. **Results:** The lower limit of quantification was 40 ng/ml and the linear extended to 20,000 ng/ml. The method has been successfully to be applied in determined of rat urine 3-HPMA. The results showed that the 70% ethanolic extract of seeds can increased the amount of 3-HPMA in rat urine. **Conclusion**: The 70% ethanolic extract of seeds displayed a potential ameliorating effect against cyclophosphamide side effects.

Key words: *Leucaena leucocephala* (Lam.) de Wit, Cyclophosphamide, 3-Hidroxypropil mercapturic acid, Urine.

INTRODUCTION

Cyclophosphamide is a chemotherapy that is widely used to fight various types of cancers.[1,2] However, its administration often causes side effects, including hemorrhagic cystitis.[2-5] Approximately 10%–25% of patients develop hemorrhagic cystitis 1 week to several months after cyclophosphamide administration.[6] Hemorrhagic cystitis occurs in 10% of patients receiving normal doses and 40% of patients receiving high doses of cyclophosphamide, despite precautions.[7,8] Acrolein is a metabolite of cyclophosphamide, and it was identified as being responsible for causing hemorrhagic cystitis.[1,2]

The incidence of hemorrhagic cystitis can be reduced by stopping treatment or reducing the dose of the drug, and it can also be decreased by 6%–50% with bladder irrigation and mesna administration.[1,4,9,10] Mesna, amifostine, and N-acetylcysteine are sulfhydryl compounds that are administered to avoid hemorrhagic cystitis.[1,2,11] Consumption of nutrients that induce glutathione S-transferase, diallyldisulfide, and S-allylcysteine compounds can reduce the effect of hemorrhagic cystitis in mice.[12-17] *Leucaena leucochepala* (Lam) de Wit seeds contain sulfhydryl compounds at 1.5 mmol/100g dry seeds.[18] The content of sulfhydryl compounds in seeds can potentially be used as an alternative medicine to avoid the occurrence of hemorrhagic cystitis. *Leucaena leucochepala* (Lam) de Wit seeds are easy to find and occur in abundance; their use as a source of nutrients to produce sulfhydryl compounds can increase value.[17,18]

The potential of natural ingredients in improving the side effects of cyclophosphamide therapy has been widely investigated, but their association with metabolite levels has not been studied.[19] This study was conducted to analyze the metabolites produced from cyclophosphamide by measuring 3-hydroxypropyl mercapturic acid in rat urine after administration of *Leucaena leucochepala* (Lam.) de Wit seed extract and cyclophosphamide. Acrolein analysis was determined for 3-hydroxy propyl mercapturic acid because acrolein has a small half-life and is easily metabolized and excreted in the urine in the form of 3-HPMA, so analysis as 3-HPMA is more accurate than analysis in the form of acrolein.[20-22]

MATERIALS AND METHODS

Chemical and extract

First, 3-HPMA (95% purity) and internal standard (IS) 3-hydroxypropyl mercapturic acid-d3 (3-HPMA-d3, chemical purity, 98%; isotopic purity, 95%) were obtained from Toronto Research Chemicals (Canada) as dicyclohexylammonium salts. Furthermore, creatinine kit (Greiner Diagnostic GmbH, Germany), HPLC grade-acetonitrate, formic acid, and ethanol were purchased from Merck Indonesia. All other chemicals used were analytical grade. Cyclophosphamide injection (Dankos, Indonesia) were purchased from Dharmais, Cancer Hospital, Jakarta, Indonesia, *Leucaena leucocephala* (Lam.) de Wit seed extract was prepared according to the procedure detailed in Wardatun *et al.*[24].

Cite this article: Wardatun S, Harahap Y, Sutandyo N, Mun'im A. Analyzing of Urine 3-Hidroxy Propil Mercapturic Acid on Cyclophosphamide Induced Rat to Determine Ameliorating Effect of *Leucaena leucocephala* (Lam.) de Wit Seed Extract. Pharmacogn J. 2021;13(6)Suppl: 1549-1556.

Animal and experimental design

Thirty male Sprague-Dawley rats were obtained from PT Medical Technology Indonesia, Indonesia. Animals were maintained at 24^{0}C ± 1 at a 12:12 h dark/light cycle. The animals have give food and water *ad libitum* but 8 h before tested, the food were deprived. All experimental procedures were approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia (No. KET.298a/UN2.FI/ETIK/PPM.00.02/2020).

The rats were acclimatized in a 1-week and were divided into three groups randomly. The first group is a negative control and was treated with aquadest orally for 1 week. The second group received aquadest orally for 1 week and 1 h prior to being treated intraperitoneally with cyclophosphamide (50 mg/kg) on the last day, according to El-Sebaey *et al.*[17] The third group was received ethanolic extract seeds once daily for 7 consecutive days at a dose of 300 mg/kg and 1 h prior to injection of cyclophosphamide on the last day.[17]

Urine sample collection

After treatment, all rats were placed in a standard methabolic cage for urine collection. Urine was collected within 24 h. Only regular water was supplied during the experiments. Occasionally, urine was collected by manually pressing the bladder. The urine were stores at -20^{0}C.

Specimen kidney collection

After collecting urine within 24 hours, the rats were sacrified using ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Specimen from kidney was fixed with 10 % neutral buffer solution of formaline for histhopathological examination.

Analysis of creatinine

Creatinine levels were determined using a creatinine kit (Greiner Diagnostic GmbH, Germany), which was analyzed with Photometer 5010. Urine creatinine was analyzed after 20 dilutions. The alkaline picrate solution was prepared according to the manual procedure by adding one part of NaOH (160 mmol/L) with 4.0 mmol/L picric acid. The creatinine standard contained 2.0 mg/dL. Fifty µl of urine sample solution and standard were added to each sample with 1.0 mL of reagent solution. The solution was mixed and stored at 37°C for 1 min and then measured to 492 nm with a distilled water blank solution. The second measurement was carried out 2 min after the first measurement. The difference between the first and second absorption was used to analyze the sample.

Calibration standard and quality control samples

A stock solution of 3-HPMA was prepared by diluting 10 mg 3-HPMA in aquadest to obtain 1,000 µg/ml, which was further diluted with authentic urine to obtain the following concentrations: 20,000; 10,000; 5,000; 1,000; 500; 100 and 40 ng/ml. Furthermore, 3-HPMA-d3 was prepared at 1,000 µg/ml in aquadest and further diluted to obtain 10 µg/ml. Quality control samples were prepared at 200; 8,000 and 16,000 ng/ml in authentic urine for QCL, QCM, and QCH, respectively. It was used for method validation.

Liquid chromatography–mass spectrometry instrument and conditions

The LC-MS/MS system consisted of a binary pump, an autosampler, and a C_{18} column (1.7 µm, 100 mm × 2.1 mm, Waters Milford, MA, USA). The mass spectrometry detection method was quadrupole (Xevo TQD, Waters) in ESI positive. The optimum chromatographic conditions was achieved by gradient elution. Eluent A consisted of 0.1% formic acid and eluent B consisted of 0.1% formic acid on acetonitrile. At time 0, 90% eluent A and 10% eluent B were flushed through the column. After 2 min, eluent A was decreased to 10% and was maintained for 2 min. Furthermore, the composition of the mobile-phase was set back at 90% eluent A at 0.1 min, remaining as such for 7 min of the run. The flow rate was 0.2 ml/min, and the column temperature was 40°C. The mass transition of 3-HPMA was monitored at m/z 221.968 > 90.993, and it was labeled 3-HPMA at m/z 225.032 > 117. The injection volume was 10.0 µl. The mass spectrum conditions are shown in Table 1. The data were processed using Masslynx version 4.1 software (Waters, USA).

Sample preparation

The urine samples were filtrated with a nylon syringe filter measuring 0.2 µm. Then 100 µl of urine sample had added to it 10 µl of formic acid and 50 µl of labelled 3-HPMA at 10 µg/ml. Mobile phase (Phase A:B; 90:10) was added to the resulting solutions until 500 µl. The mixture was vortex mixed for 2 min and centrifuged at 5,000 rpm for 10 min. Furthermore, 10 µl of aliquot was injected into the LC-MS/MS.

Validation procedure

Validation of the quantitative LCMS/MS method was performed by means of linearity, accuracy, precision, selectivity, carry over, integrity dilution, matrix effect, and stability.[24,25] A partial validation was performed by means of linearity, accuracy, precision, and selectivity with the rat urine matrix. Furthermore, the assay application was demonstrated.

Statistical analysis

Statistical analyses were conducted using SPSS 26 (BMI, New York) to test for significant differences among treatments. $P < 0.05$ was chosen as the level of significance.

RESULTS

Analytical method

The results of the detection using a mass spectrometer showed fragmentation of m/z 221.968 >90.993 for 3-HPMA and 225.032>117 for 3-HPMA-d3. Analytical conditions of mass spectrometry (Table 1). The analytical method for 3-HPMA was developed by Harahap *et al.*[26] Urine samples were prepared using a dilution system. The direct dilution method is a procedure for diluting a urine sample with a number of solutions. This method is easy and economical and is the main choice for analyzing urine with high analyte concentrations. The compounds 3-HPMA and 3-HPMA-d3 are water-soluble compounds, so there was no need for preparation using liquid–liquid extraction.

Validation assay

Selectivity tests were conducted to determine the interference caused by endogenous matrix compounds or other compounds in the sample. These tests were carried out on blank samples and samples at LLOQ concentrations, both for analyte and internal standards. The interference in the urine matrix meets the predetermined requirements if interference in analyte (3-HPMA) is not more than 20% and 5% for the internal standard (3-HPMA-d3). Chromatogram ions of 3-HPMA and IS of blank urine are depicted in Figures 1. The calibration curve was obtained by dilution of 3-HPMA 1,000 µg/ml, and it had a calibration curve in concentrations of 40, 100, 500, 1,000, 5,000, 10,000, and 20,000 ng/mL, as well as for blank and zero samples. Intraassay and interassay accuracy and precision were determined within 2 days using a quality control sample solution at low, medium, and high concentrations (Table 2).

The carry-over test was implemented to ensure that there was no interference in the retention time of the analyte after analysis of a high concentration sample. This test was carried out by injecting a sample

Table 1: Analytical Conditions for Mass Spectrometry.

Compound	Ion fragment (m/z)	Ionization mode	Capillary voltage (KV)	Temperature of gas desolvation	Flow rate of gas desolvation	Orifice voltage	Collision voltage (V)
				(°C)	(L/hour)	(V)	
3-HPMA	221.968 > 90.993	ESI +	3.5	350	650	20	22
3-HPMA-d3	225.032 > 117.00					18	14

Table 2: Accuracy and Precision Data.

Analyte	Concentration		Precision (%CV)		Accuracy (%bias)	
	QC	ng/ml	intra assay	inter assay	intra assay	inter assay
3-HPMA	LLOQ	40	1.647	2.977	14.487	14.971
	QCL	200	6.618	7.273	-6.351	-6.763
	QCM	8000	9.672	10.610	-0.606	-3.510
	QCH	16000	5.052	8.431	4.140	-5.493

Table 3: Sample Stability Data for Short- and Long-Term Storage.

		Concentration	Measured		
		ng/ml	Mean ± SD (n=3)	%Bias	RSD (%)
short term stored at room temperature (20°C)	QCL	200	212.824±3.024	6.412	1.421
	QCH	16000	16464.451±796.955	2.902	4.84
Long term stored at -20°C at 30 days	QCL	200	191.963±11.610	6.412	6.048
	QCH	16000	15342.963±697.619	-4.106	4.547

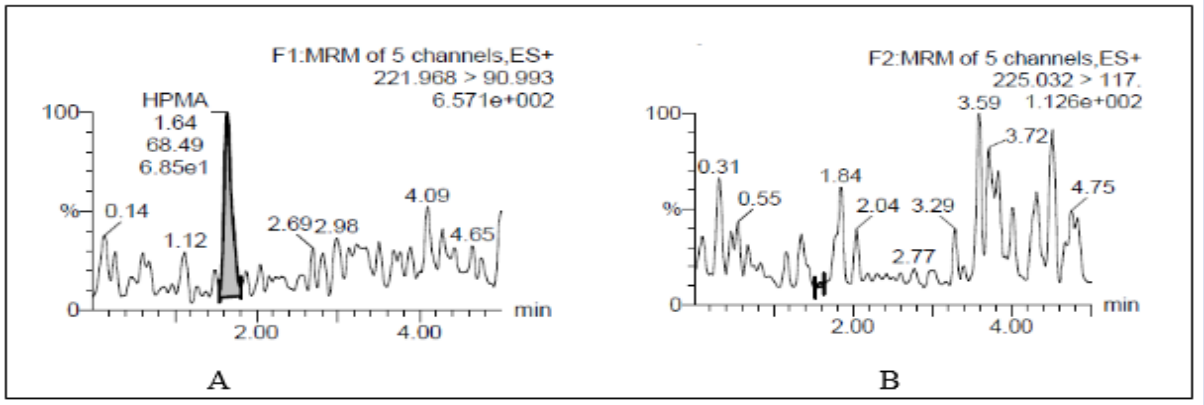

Figure 1: Chromatogram ion 3-HPMA (A) and 3-HPMA-d3 (B) in blank urine.

with a concentration of ULOQ and then observing interference in the retention time of the next injected blank sample.[24] The results of the carry-over test on the sample showed that the carry over in the blank and LLOQ after being injected with ULOQ was an average of 6.973%, while the average was 0.008% in the internal standard. This results showed that there was no carry over in this analysis system. The matrix effect is determined in analyses involving mass spectrometry, especially when using the ESI mode, and is observed as an increase or decrease in the ion response. The test results revealed that the standard normalized matrix effects for 3-HPMA on QCL and QCH were 0.868 ± 0.028 and 0.957 ± 0.015, respectively.

Stability tests were implemented to ensure that every step taken from sample preparation to analysis, including storage, did not affect the analyte concentration in the sample. Stability test were determined for stock solution of analyte and internal standard as well as working solution at low and high concentration. The results of stability test indicated that all solution were stable during preparation and storage period (Table 3). The results of partial validation using rat urine demonstrated that the validation assay results fulfilled the requirements.

Creatinine levels

The results of the determination of creatinine levels showed that creatinine levels in Groups 1, 2, and 3 were 6.46 ± 1.61, 5.76 ± 1.88, and 5.51 ± 1.50 mg/dL, respectively (Figure 2). The results of statistical analysis revealed that these data were homogeneous and that there were no significant differences in creatinine levels in the three groups of rats ($p > 0.05$).

Analysis of 3-HPMA levels

The results for the determination of 3-HPMA levels in rat urine normalized by creatinine levels can be seen in Figure 3. Statistical analysis also showed that the data of each group were homogeneous and displayed significantly different effects ($p < 0.05$). The analyte in urine sample can be seen in Figure 4.

The levels of 3-HPMA in the urine of the negative control group (Group 1) were lower than in other groups. These results were indicated that 3-HPMA naturally forms in the urine of rats in very low amounts.

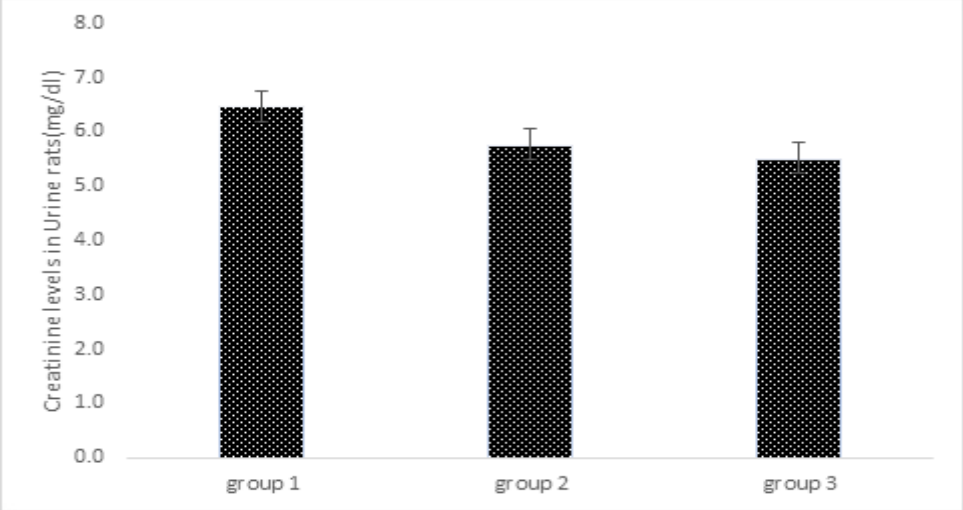

Figure 2: Comparison of creatinine levels in urine rats (mg/dL).

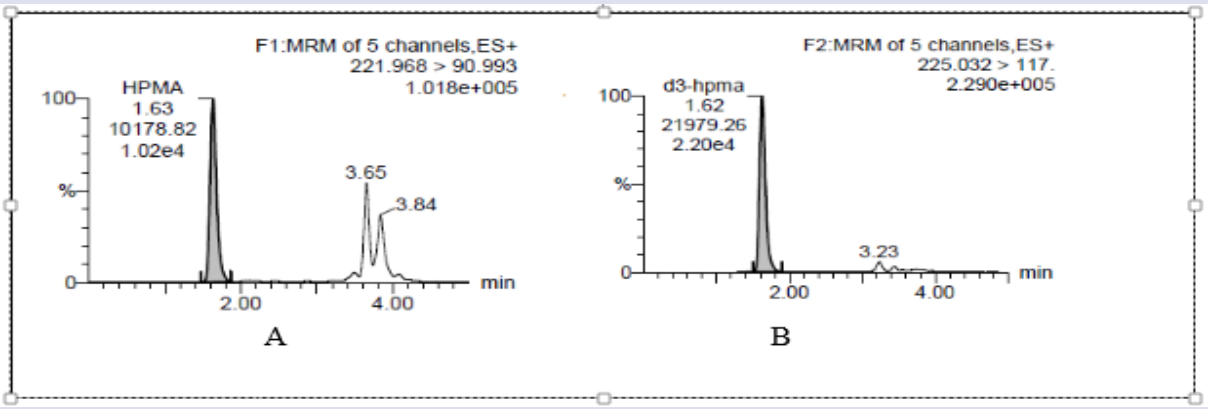

Figure 3: Chromatogram ion 3-HPMA (A) and 3-HPMA-d3 (B) in urine rat sample.

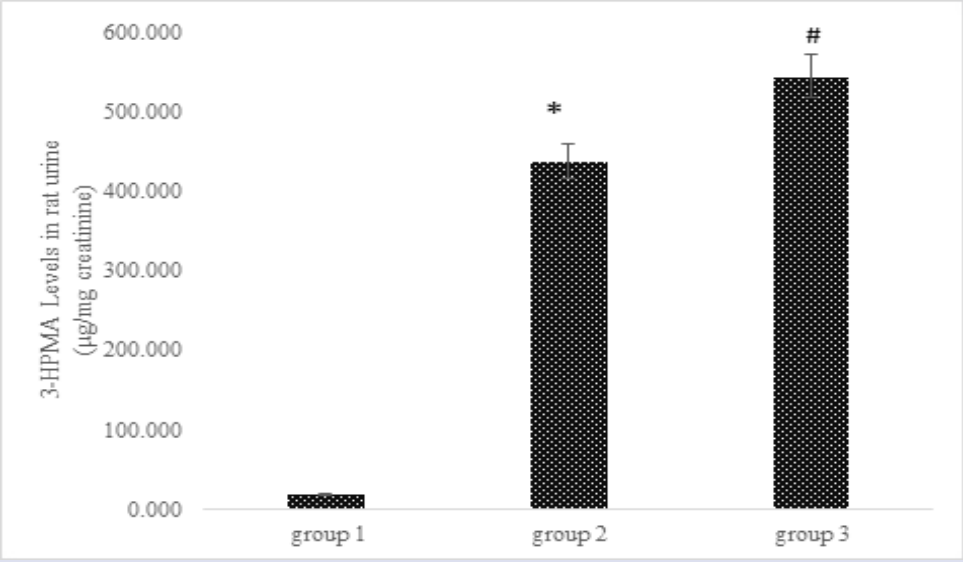

Figure 4: Comparison of 3-HPMA levels in rat urine (µg/mg creatinine). *significantly different with group 1 (P > 0.05), # significantly different with group 2 (P > 0.05).

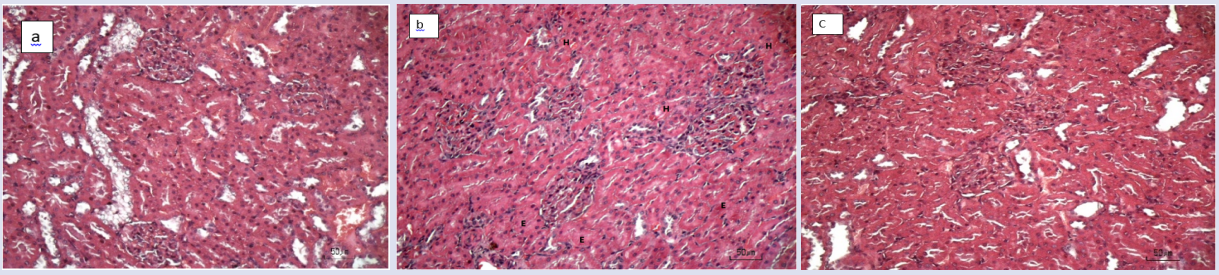

Figure 5: H&E stained kidney section (H&E, 20x), a. the control group showed normal condition b. cyclophosphamide induced rats showed glomerular lesion, tubular lesion, edema and haemorrhage in interstitium c. cyclophosphamide induced rats with *Leucaena leucocephala* (Lam.) de Wit extract showed similar with control group.

The amount of urine 3-HPMA in Group 2 increased significantly compared to Group 1, which indicates the effect of cyclophosphamide on the amount of 3-HPMA produced[16]. Acrolein produced from the metabolism of cyclophosphamide is neutralized by the body through a reaction with glutathione, so that 3-HPMA levels increase. The levels of urine 3-HPMA in Group 3 were significantly different with the levels of urine 3-HPMA in Group 2 ($p < 0.05$). These results indicate that administering *Leucaena leucochepala* (Lam) de Wit seed extract in Group 3 had a significant effect.

Histhopathological examination

As demonstrated in Figure 5. cyclophosphamide induced rat showed glomerular lesion, tubular lesion, as well as edema and haemorrhage in interstitium. Moreover, the cyclophosphamide induced rat with *Leucaena leucocephala* (Lam.) de Wit extract show the normal condition. Similar to the control group.

DISCUSSION

In summary, 3-HPMA analysis was performed on rat urine collected within 24 h. The volume of urine collected differed among the various rat groups. Therefore, 3-HPMA levels in urine were normalized to urine creatinine values. Creatinine can used to correct hydration status, because the production is constant and eliminated at stable rate between individuals. Therefore, dividing the chemical compound level by the creatinine value can eliminate variations between individuals due to urine dilution.[27] Gender, body mass index, age, race, free fat mass and kindey failure were influenced urinary creatinine levels.[27] The treatment of rats led to no significant effects in terms of creatinine levels in the various groups. The rats were homogeneous in age and weight, so the results revealed that biological factors had no effect on urine creatinine levels. The differences in creatinine levels in the urine of individual rats were more influenced by hydration factors. The lower the total volume of urine excreted per day, the higher the urinary creatinine levels.[27] Therefore, creatinine levels can be used to correct the volume of urine excreted.

Moreover, 3-HPMA levels in rat urine were analyzed using a validated method; 3-HPMA is a metabolite from the reaction of acrolein with glutathione, which is excreted in the urine.[21] Acrolein is naturally found in food and is produced by heating organic matter, cigarettes, engine exhaust combustion, and steam due to the excessive heating of oil. In vivo acrolein is a metabolite product of the anticancer drug cyclophosphamide.[20]

The sulfhydryl compound reacts by reducing acrolein, which leads to the elimination of the side effects of acrolein.[17] The 70% ethanolic extract of seeds in this study contained a sulfhydryl concentration equivalent to 29.878 mg/100g. The sulfhydryl compounds contained in the *Leucaena leucochepala* (Lam) de Wit seeds are expected to act similarly to garlic extract in improving the side effects of cyclophosphamide.[17]

The activity of thiol/sulfhydryl compounds is associated with their ability to induce the signaling of the Nrf2 pathway (*nuclear factor* 2) and can affect glutathione metabolism in the body.[28,29] Glutathione in the body functions as an antioxidant that can reduce reactive oxygen compounds derived from acrolein. Glutathione with glutamyl transpeptidase cysteineylglycine N-acetyltransferase enzyme will form 3-HPMA metabolites, which are unreactive and water soluble and are excreted in urine.[30] The amount of glutathione in the body can be increased through the use of supplements, especially those containing cysteine ester compounds.[16] Based on the study results, *Leucaena leucochepala* (Lam) de Wit seed extract plays a role in increasing the amount of 3-HPMA.

Based on histophatological examination, cyclophosphamide induced cause degeneration in kidney jar. Cyclophosphamide in the body will produce acrolein and can causes oxidative stress.[29] The oxidative stress state in the body will activate *nuclear factor kaffa* B (Nf-ƙB) and will increase inflammatory cytokine factors such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and cyclooxygenase-II (COX-II). These changes lead imbalance between inflammatory factors and antioxidants.[29] When a state of oxidative stress was identified, Nrf2 will play a role in cell protection through ARE activation and affect the glutathione synthesis pathway, glutathione peroxidase activity and enzymes and can play a role in phase 1 and 2 metabolism in the detoxification process, antioxidant and anti-inflammatory effects.[29] Natural ingredients containing antioxidants and sulfhydryl compounds have been shown to increase the expression and activity of Nrf2.[28] *Leucaena leucocephala* (Lam.) de Wit seed contain sulfhydryl compounds and may increase Nrf2 expression, and can protect from cell damage even though they were given cyclophosphamide injection.

CONCLUSION

In short, 3-HPMA as an analysis method for rat urine was valid and can be applied in assays as demonstrated. Levels of creatinine can be used to correct urine volume. The 70% ethanolic extract of *Leucaena leucochepala* (Lam) de Wit seed can increase the amount of 3-HPMA in rat urine and possess potential to ameliorate the side effects of cyclophosphamide. Concentrations of 4-hydroxycyclophophamide in plasma must be assayed to determine the interaction of the active metabolite cyclophosphamide with the extract.

ACKNOWLEDGEMENTS

We thank to Universitas Indonesia for providing financial support for Doctoral Grant number NKB-3189/UN2.RST/HKP.05.00/2020.

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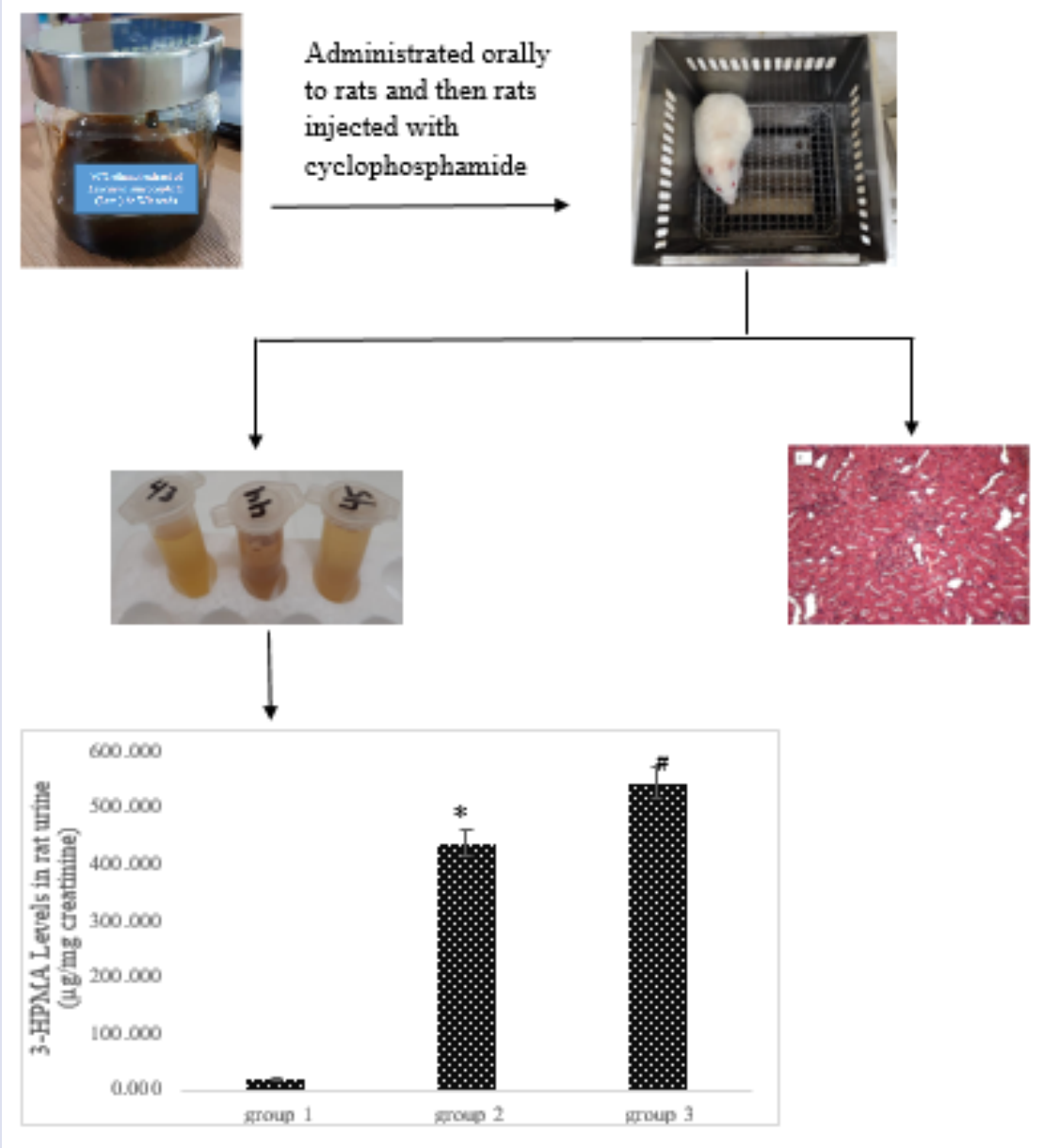
GRAPHICAL ABSTRACT



SUMMARY

A 70% ethanolic extract of *Leucaena leucocephala* (Lam.) de Wit seeds contain a certain amount of sulfhydryl active compounds and potential for ameliorated cyclophosphamide side effects. The extract was administered to a rat model for cyclophosphamide-induced hemorrhagic cystitis. The rats urine were collected for 24 hours and analyzed for the amount of 3-HPMA, furthermore histhopathological examination were performed on the kidneys. The results showed 70% ethanolic extract can increase the amount of 3-HPMA in rat urine and there was ameliorated in hemorrhagic cystitis kidney tissue. The 70% ethanolic extract of seeds displayed a potential ameliorating effect against cyclophosphamide side effects.

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Cite this article: Wardatun S, Harahap Y, Sutandyo N, Mun'im A. Analyzing of Urine 3-Hidroxy Propil Mercapturic Acid on Cyclophosphamide Induced Rat to Determine Ameliorating Effect of *Leucaena leucocephala* (Lam.) de Wit Seed Extract. Pharmacogn J. 2021;13(6)Suppl: 1549-1556.