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Simultaneous use of *Morinda citrifolia* fruit extract and amlodipine: Antihypertensive activity and sub-chronic toxicity in rats

Nur Azizah[1], Irma Melyani Puspitasari[1]*, Aliya Nur Hasanah[2], Eli Halimah[1]

[1]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia.
[2]Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia.Najran, Saudi Arabia.

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ABSTRACT

This study investigates the effects of the simultaneous use of *Morinda citrifolia* fruit extract (MCFE) and amlodipine (AML) on rats' antihypertensive activity and sub-chronic toxicity. The antihypertensive activity was assessed in 25 male Wistar rats divided into 5 groups: 1. normal, 2. negative control (NaCl 8%), 3. positive control [NaCl 8% + AML 1 mg/kg body weight (BW)], 4. Treatment-1 [NaCl 8% + MCFE (45 mg/kg BW)], and 5. Treatment-2 [NaCl 8% + MCFE (45 mg/kg BW) + AML (1 mg/kg BW)]. MCFE and AML were given orally on days 22–35. The blood pressure was measured on days 0, 7, 21, 28, and 35. The sub-chronic toxicity study was conducted with a repeated dose 28-day oral toxicity test. The results showed that the blood pressure reduction in the treatment-2 group was not significantly different from those in the positive control or treatment-1 group ($p > 0.05$). In addition, it changed aspartate aminotransferase, alanine transaminase, Blood urea nitrogen, creatinine levels, and histological parameters in the liver and kidneys. The simultaneous use of MCFE and AML might have the same activity as either AML or MCFE alone but could cause toxic effects on liver and kidney function. Therefore, simultaneous use should not be considered for hypertension therapy.

INTRODUCTION

Herbal medicines are widely used in developed and developing countries to treat various diseases, including hypertension. This is attributable to the ease of obtaining herbal medicines, as well as their low costs and minimal side effects, and many herbal medicines contain phytochemicals that are effective against hypertension [1,2]. As such, herbal medicines are commonly used to improve health. Although herbal medicines are considered safe, they can have side effects that sometimes result in life-threatening consequences when used simultaneously with other medicines [3,4].

A study in a rural community of West Java, Indonesia, reported that hypertensive patients combined their pharmacological antihypertensive medication with *Morinda citrifolia* (14.1%) almost daily [5,6]. According to previous reviews on the communities of patients with hypertension in several countries, 80% of such patients use herbal medicines simultaneously with antihypertensive drugs, including the fruit of *M. citrifolia* [7].

Amlodipine (AML) is a widely used antihypertensive drug for treating hypertension [8]. Based on the results of previous studies, among the monotherapy categories, the various classes of prescribed antihypertensive medicines are calcium channel blockers (CCBs, 15%), followed by diuretics (8%), angiotensin receptor blockers (4%), and angiotensin-converting enzyme inhibitors (ACEIs, 2%), and this indicates that AML, either as monotherapy or in combination with other treatments, is the drug of choice for patients with hypertension [9].

**Corresponding Author*
Irma Melyani Puspitasari, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia. E-mail: irma.melyani @ unpad.ac.id

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The simultaneous use of herbal medicines and certain prescription drugs often leads to herb-drug interaction (HDI) with clinically significant outcomes, including mortalities. This largely depends on the nature of the herb, drug, and individual, as their synergistic or antagonistic interactions may reduce drug efficacy or cause organ toxicity [10]. Another research states that these interactions demonstrate the potential effects of herbal medicines on the bioavailability and pharmacokinetic (PK) parameters of antihypertensive drugs, synergistic or antagonistic effects may result from pharmacodynamic interactions [11,12]. In general, the severity of PK HDIs is determined by the toxicity of the co-administered drug when its plasma concentration exceeds the minimum toxic concentration or by the consequences when its therapeutic plasma concentration is not reached [13].

A study by Zhang *et al.* [14] reported that a multiherbal Chinese formula could reduce the pharmacological effects of AML when used simultaneously. Another study stated that *Enantia chlorantha* and lisinopril co-administration significantly increased hematocrit (HCT) levels in Wistar rats [15]. A study on the interaction of AML with herbs found that both rosella and ginger have lowered blood pressure better when used simultaneously with AML but had a significant impact on PK parameters of AML such as C_{max}, AUC_{0-t}, and T_{max} [16]. In addition, based on previous studies, *M. citrifolia* can induce hepatotoxicity and kidney injury [17,18], and AML can cause liver enzyme elevations in idiosyncratic ways [19]. Consequently, simultaneously administered medications that regulate cytochrome (CYP) enzyme activity can lead to drug-herbs interactions [10,20]

To the best of our knowledge, no study has been performed on the simultaneous use effect of AML and *Morinda citrifolia* fruit extract (MCFE). Therefore, this study investigated the effects of simultaneous MCFE and AML treatment on antihypertensive activity and their toxic effect on hematologic, biochemistry parameters, and organs in rats.

MATERIAL AND METHODS

Materials

Morinda citrifolia extract is a standardized extract obtained from PT. Semarang Indo Plant (Central Java, Indonesia), batch number SLACG100, and the data analysis from the certificate of analysis received in Table 1.

We re-analyzed physicochemical parameters using the procedure in the subsection physicochemical analysis. AML besylate tablets were obtained from PT. Kimia Farma (Jakarta, Indonesia), and NaCl (Merck KGaA, Darmstadt, Germany). The phytochemical study used methanol, hydrochloric acid, sulfuric acid, and ferric chloride were purchased (Sigma–Aldrich, Germany), and all chemicals used are pro-analysis grade.

Animals

The Wistar rats were obtained from the animal house at Universitas Gadjahmada's Integrated Research and Testing Laboratory, Yogyakarta, Indonesia. The rats were grouped and kept in acrylic cages under controlled conditions, including a 12-hour:12-hour light-dark cycle, constant temperature (23°C–25°C), and relative humidity (50%–55%). All animals were fed standard laboratory food and water ad libitum throughout the experiment and acclimatized for 2 weeks before experimentation [21]. At the end of the experiment, the animals were humanely euthanized through chamber delivery of carbon dioxide [22]. The experimental procedures were approved by the Research Ethics Committee, Universitas Padjadjaran, West Java, Indonesia (No. 565/UN6.KEP/EC/2020), and we followed the guidelines of the European Directive 2010/63/EU.

Physicochemical analysis

The various physicochemical parameters such as moisture content; ash values including total ash, acid-insoluble ash, and water-soluble ash levels; and ethanol-soluble extractive value were determined by the methods described in the World Health Organization (WHO) guidelines [23,24].

Determination of heavy metal content

Heavy metal analysis was conducted by atomic absorption spectroscopy (Perkin Elmer-400) using argon as the carrier gas, and the flow rate was maintained at 1 ml/2 minutes. 500 mg of air-dried powder (accurately weighed) was used to determine the major heavy metal content. The protocol added 5 ml of concentrated nitric acid, and the mixture was refluxed for 30 minutes at 60°C–80°C before cooling. Five milliliters of concentrated nitric acid were added, and the mixture was heated in a water bath. Two milliliters of 30% hydrogen peroxide solution were added to the mixture, which was warmed until a clear solution was obtained. The mixture was then cooled

Table 1. Analysis data from certificate of analysis of MCFE.

Parameters	Reference	Result
Characteristic		
Appearance	Powder	Complies
Color	Dark brown	Complies
Odor	Specific	Complies
Physicochemical		
Moisture content	Max.10%	2.61%
Acid in soluble ash content	Max.4%	2.46%
Flavonoid	Positive	0.0630%
Tannin	Positive	1.85%
Microbiological		
Total plate count	Max. 1 × 10^4/g	<1 × 10^1/g
Mold	Max. 1 × 10^3/g	5 × 10^1/g
Yeast	Max. 1 × 10^3/g	<1 × 10^1/g
Escherichia coli	Negative/g	Negative/g
Shigella	Negative/g	Negative/g
Salmonella	Negative/g	Negative/g
Enterobacteriaceae	Max. 1 × 10^3/g	<1 × 10^1/g
Clostridium perfringens	Negative/g	Negative/g
Residual alcohol	Ethanol Max. 1%	0.00%

and filtered through Whatman 42 filter paper and diluted with deionized water to a volume of 100 ml in a volumetric flask [25,26].

Phytochemical analysis

A qualitative phytochemical test of filtrates of MCFE was performed to clarify the presence of phytochemicals such as tannins, flavonoids, saponins, phenolic acid, alkaloids, and terpenoids using a standard procedure [27,28]. The qualitative phytochemical tests were carried out using Mayer's test, Alkaline reagent test, foam layer test, Chloroform + Sulfuric acid test, and Ferric chloride test [29,28,30,31].

Experimental procedure

Antihypertensive test activity

The antihypertensive activity was assessed in this study using a test developed by Wigati *et al*. [32] and Mulyati *et al*. [33]. Twenty-five Wistar rats (weight between 200 and 250 g) were randomly divided into five groups of five animals, each group as follows: normal, negative control (NaCl 8%), positive control [NaCl 8% + AML 1 mg/kg body weight (BW)], treatment-1 (NaCl 8% + MCFE 45 mg/kg BW), and treatment-2 (NaCl 8% + MCFE 45 mg/kg BW + AML 1 mg/kg BW). NaCl 8% 3 ml/kg BW induction was performed for 21 consecutive days and continued throughout the test. The AML dose was determined by converting the human dose to the rat dose and previous study [14]. The MCFE dose was determined by converting the human dose to the rat dose from The Indonesian herbal medicine formulary [34]. MCFE and AML were given orally from day 22 to 35. Blood pressure parameters, including systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MABP), were measured using the CODA™ Non-Invasive Blood Pressure System version 4.1 (Kent Scientific Corporation, Torrington, CT) on days 0, 7, 14, 21, 28, and 35 [32,33].

Sub-chronic toxicity study

The sub-chronic oral toxicity study was conducted according to OECD Test Guideline 407: repeated dose 28-day oral toxicity test [35,36], with minor modification. Forty Wistar rats aged 8–10 weeks of both sexes were randomly divided into four groups ($n = 20$, 5 males and 5 females), one control group, and three treatment groups. The control group was given CMC-Na 0,5% as a vehicle, the positive control group was given AML 1 mg/kg BW, and the treatment groups were treatment-1 [MCFE 45 mg/kg BW (MCFE)], and treatment-2 [MCFE (45 mg/kg BW) + AML (1 mg/kg BW)]. An additional ten rats (five males and five females) were assigned to the satellite MCFE + AML group for the recovery period. The test substance was suspended in 0.5% CMC-Na and administered orally for 28 consecutive days. Every 5 days, the test material was blended with a vehicle. All animals were observed daily for clinical signs and food and water consumption. Every week, individual BWs were recorded. At day 28, rats in the control and dose groups were sacrificed. Throughout the recovery period of 14 days, satellite groups were continuously observed without treatment and then sacrificed. Rats were euthanized with an overdose of aesthetic ether. Kidney and liver tissues were removed to prepare hematoxylin and eosin-stained tissue sections.

Clinical observation and BW

Once per day, general clinical observations were made on all animals (morbidity and mortality). Behavioral patterns, physical appearance, and other toxicity-related symptoms were observed. Each rat was weighed once per week and immediately before the necropsy.

Hematological analysis

Blood samples were collected from retro-orbital sinus veins under light ether anesthesia, and the hematological analysis was conducted using whole blood collected in EDTA-treated tubes. The assessed hematological parameters included the WBC, differential leukocyte, RBC, and PLT counts; HB and HCT levels; MCV; MCH level; and MCHC. All analyses were performed using the BC-2800VET hematology analyzer (Mindray Bio-Medical Electronics, Shenzhen, China) [37,38].

Biochemical analysis

Blood samples were obtained in anticoagulant-free sterile tubes for biochemical testing (serum). Using spectrophotometric assay kits (DiaSys Diagnostic System, Germany), the levels of alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine, and urea were determined.

Organ weight and histological analysis

The relative liver and kidney weights were determined by dividing organ weight by BW. To facilitate slicing, the organs were washed in an isotonic solution, fixed in 10% formalin, and embedded in paraffin. Hematoxylin–eosin dye was used to stain samples, which were then examined microscopically.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.5.1 statistical software (GraphPad Software, San Diego, CA). Antihypertensive outcomes were expressed as the mean, SD, and differences between treatment groups for each blood pressure were examined using two-way ANOVA followed by Tukey's multiple comparison testing and Dunnett's. All sub-chronic analysis data were reported as mean ± SD and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons and Dunnett's. Statistical significance was considered at $p < 0.05$.

RESULTS

Physicochemical analysis

Table 2 presents the results of the physicochemical analysis of MCFE. The ethanol-soluble, total, water-soluble, and acid-insoluble ash contents were 46.82, 9.67, 96.55, and 2.14% *w/w*, respectively. The moisture content was 4.07% *w/w*. The results of all physicochemical analyses are in accordance with the Herbal Pharmacopeia and Indonesian Materia Medica [39].

Table 2. Physicochemical parameters of MCFE.

Parameters	Result (% *w*/*w*)
Moisture content	4.07
Total ash	9.67
Acid-insoluble ash	2.14
Ethanol-soluble ash	46.82
Water-soluble ash	96.55

Table 3. Concentrations of heavy metals in MCFE.

Heavy metals	Values (µg/g)
Lead	<0.001
Cadmium	<0.001
Arsenic	<0.001
Mercury	<0.001

Table 4. Qualitative phytochemical analysis of MCFE.

Metabolites	Results
Phenol	+
Tannins	+
Flavonoids	+
Saponins	+
Triterpenoids	+
Alkaloids	+

Heavy metal analysis

Table 3 presents the heavy metal content in MCFE. The lead, cadmium, mercury, and arsenic concentrations were meager (0.01 µg/g). The concentrations of all analyzed heavy metals were within the permissible limits of the WHO and Joint FAO/WHO Expert Committee on Food Additives [40,41].

Qualitative phytochemical test

Table 4 presents the results of the qualitative phytochemical screening performed using MCFE. The results revealed the presence of flavonoids, alkaloids, tannins, saponins, steroids, terpenoids, and phenols.

Antihypertensive activity

Table 5 presents the antihypertensive activity of simultaneous use of MCFE and AML. On day 0, no significant difference in blood pressure was detected between the groups. After being administered NaCl for 21 days, the rats' SBP, DBP, and MABP increased significantly in the intervention groups compared with the values in the normal group (all $p < 0.05$). On day 35, the treatment-1 and treatment-2 groups exhibited decreased SBP, DBP, and MABP. The reductions significantly differed from those in the negative control group (all $p < 0.05$). The most significant percentage decreases in blood pressure were recorded in the treatment-2 group, as SBP, DBP, and MABP decreased by -31.40 ± 2.39, -13.00 ± 2.55, and -25.20 ± 2.68 mmHg, respectively. However, these decreases were not significantly different from those in the positive control ($p > 0.05$) or treatment-1 group ($p > 0.05$).

Sub-chronic toxicity study

Clinical observation and BW

No mortality nor severe clinical signs were observed in any treated groups after 28 days of repeated oral administration. During the first 2 days after dosing, minor clinical signs (mild diarrhea) were observed in a few rats across all study groups. These minor symptoms did not affect the animals' overall health and were considered typical for Wistar albino rats.

After 28 days of treatment, there were no significant differences ($p > 0.05$) in the BWs of the treated groups compared to the control group, as shown in Figure 1. At the oral doses administered, the positive control, treatment-1, and treatment-2 groups did not affect the normal growth of rats, as both the control and treatment groups appeared equally healthy.

Hematological parameters effect

Table 6 shows the hematological parameters after 28-day treatment. There were no significant differences in the hematological parameters of the positive control, treatment-1, and treatment-2 groups compared to the control group ($p > 0.05$). The results indicate that the positive control, treatment-1, and treatment-2 groups did not affect the production or circulation of blood cells.

Biochemical parameters effect

Figure 2 shows biochemical analysis results on male and female rats. There was a significant difference in both sexes between all group treatments and the control group ($p < 0.05$). In the male treatment-1 and treatment-2 groups, there were significant differences with the positive control male groups in AST and ALT levels ($p < 0.05$). Blood urea nitrogen (BUN) levels in the treatment-1 male group show no significant difference with the positive control male group, but there is a significant difference in the treatment-2 groups ($p < 0.05$). Meanwhile, there were no significant differences in creatinine levels in the treatment-1 and treatment-2 male groups compared with the positive control group ($p > 0.05$). Furthermore, in the female treatment-1 group, there was a significant difference in AST and ALT levels ($p < 0.05$) compared with the control and positive control group. In contrast, in the treatment-2 group, there was no significant difference in AST and ALT levels ($p > 0.05$). The BUN and creatinine levels in the treatment-1 and treatment-2 female groups significantly differ from the positive control group ($p > 0.05$). The results indicate that AML, MCFE, and MCFE + AML may affect liver and kidney function through increased AST, ALT, BUN, and creatinine levels.

Organ weight and histological analysis

Figure 3 shows the organ liver and kidney weight of positive control, treatment-1, and treatment-2 male and female groups. After 28 days of treatment, a macroscopic examination revealed no organ abnormalities in any treatment or control group, regardless of gender. The liver and kidney's absolute and relative organ weights in all treatment groups did not differ

Table 5. Antihypertensive activity of simultaneous use of MCFE and AML.

Group	Day-0	Day-7	Day-14	Day-21	Day-28	Day-35	Blood pressure change (mmHg)	Percentage change (%)
Sistolic								
Normal	115 ± 1.58	116 ± 2.24	114 ± 2.30	114 ± 1.58	115 ± 2.41	116 ± 2.24	2.00 ± 3.61	−1.79 ± 3.20
Negative control	116 ± 1.92	131 ± 4.34[*a]	139 ± 2.30[*a]	149 ± 1.92[*a]	159 ± 3.70[*a]	164 ± 3.91[*a]	14.80 ± 5.02[*a]	−9.97 ± 3.50[*a]
Positive control	115 ± 1.58	129 ± 5.26[*a]	138 ± 1.92[*a]	148 ± 2.07[*a]	125 ± 4.97[*a b]	119 ± 2.92[*b]	−29.40 ± 1.52[*ab]	19.82 ± 1.13[*ab]
Treatment-1	114 ± 2.30	130 ± 4.09[*a]	140 ± 1.52[*a]	150 ± 1.95[*a]	128 ± 3.11[*ab]	122 ± 1.92[*ab]	−27.80 ± 2.39[*ab]	18.58 ± 1.45[*ab]
Treatment-2	114 ± 1.92	129 ± 4.09[*a]	140 ± 2.59[*a]	149 ± 1.58[*a]	122 ± 1.67[*ab]	118 ± 1.67[*b]	−31.40 ± 3.05[*ab]	21.06 ± 1.83[*ab]
Diastolic								
Normal	89 ± 3.27	88 ± 3.44	90 ± 2.39	89 ± 3.21	91 ± 3.27	90 ± 3.36	0.80 ± 4.22	−1.00 ± 2.99
Negative control	87 ± 3.54	98 ± 2.07[*a]	104 ± 1.92[*a]	110 ± 2.86[*a]	117 ± 3.03[*a]	120 ± 1.52[*a]	9.40 ± 4.16[*a]	−8.61 ± 3.58[*a]
Positive control	88 ± 3.96	95 ± 2.24[*a]	102 ± 2.07[*a]	109 ± 2.39[*a]	100 ± 2.28[*ab]	94 ± 2.17[*b]	−15.40 ± 3.11[*ab]	13.92 ± 2.40[*ab]
Treatment-1	89 ± 3.96	96 ± 2.77[*a]	103 ± 1.14[*a]	111 ± 1.64[*a]	105 ± 4.49[*ab]	99 ± 5.05[*ab]	−11.60 ± 1.52[*ab]	10.59 ± 1.31[*ab]
Treatment-2	89 ± 5.81	98 ± 1.30[*a]	103 ± 2.28[*a]	109 ± 2.59[*a]	103 ± 3.11[*ab]	98 ± 2.51[*ab]	−13.00 ± 2.55[*ab]	12.02 ± 2.09[*ab]
MABP								
Normal	98 ± 2.05	97 ± 2.83	97 ± 3.11	97 ± 1.96	99 ± 2.52	98 ± 2.05	2.40 ± 2.07	−2.55 ± 2.20
Negative control	97 ± 3.05	109 ± 2.28[*a]	116 ± 2.00[*a]	123 ± 1.32[*a]	131 ± 2.82[*a]	134 ± 1.96[*a]	11.40 ± 2.97[*a]	−9.29 ± 2.52[*a]
Positive control	97 ± 3.11	106 ± 2.97[*a]	114 ± 1.64[*a]	122 ± 2.25[*a]	108 ± 2.62[*ab]	102 ± 2.06[*b]	−22.80 ± 1.48[*ab]	18.67 ± 1.33[*ab]
Treatment-1	98 ± 2.49	107 ± 1.52[*a]	115 ± 1.10[*a]	124 ± 1.06[*a]	109 ± 3.05[*ab]	107 ± 3.98[*ab]	−23.20 ± 3.70[*ab]	18.73 ± 2.93[*ab]
Treatment-2	98 ± 4.04	108 ± 1.64[*a]	115 ± 2.45[*a]	123 ± 2.14[*a]	103 ± 2.56[*ab]	105 ± 1.56[*ab]	−25.20 ± 2.68[*ab]	20.53 ± 1.84[*ab]

SBP, Systolic blood pressure; DBP, diastolic blood pressure; MABP, mean arterial blood pressure; MCFE, *Morinda citrifolia* fruit extract; AML, amlodipine; Negative control: (NaCl 8%); Positive control: (NaCl 8% + AML), Treatment-1: (NaCl 8% + MCFE); Treatment-2: (NaCl 8% + MCFE + AML).

Data are expressed as the mean ± SD. Blood pressure change = Blood pressure at day-35 − Blood pressure at day-21; Percentage change = (Blood pressure at 21 − Blood pressure at 35) / Blood pressure at 21 × 100%.

*Significant ($p < 0.05$).

[a]Compared to the normal group.

[b]Compared to the negative control group (NaCl 8%).

[c]Compared to the positive control group (NaCl 8% + AML).

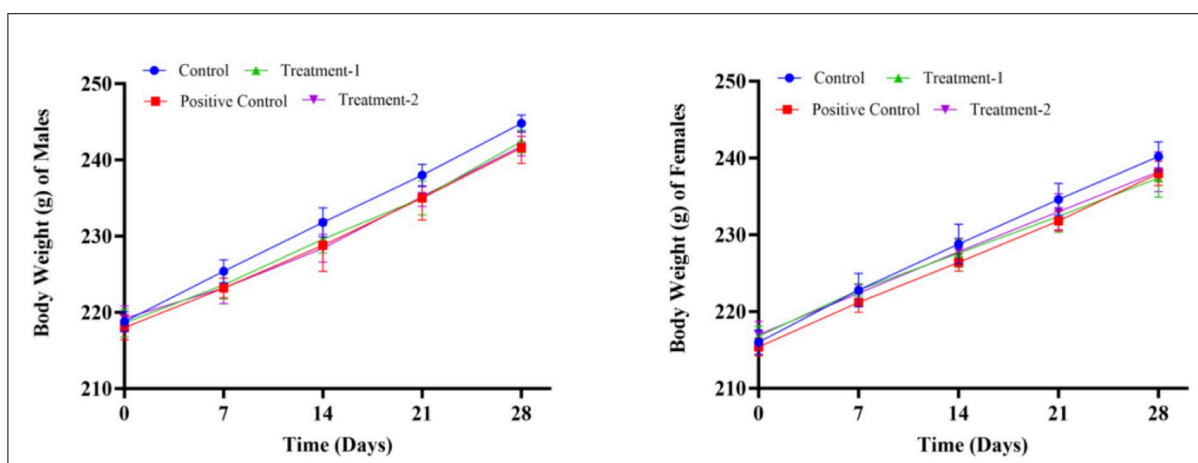

Figure 1. In the sub-chronic toxicity study, the BW of male and female rats after AML, MCFE, and AML + MCFE administration. Values are expressed as mean ± SD; $p < 0.05$, the significant difference compared to the control group using the one-way ANOVA continued by Tukey's multiple comparison test. Abbreviations: MCFE, *Morinda citrifolia* fruit extract; AML, amlodipine; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML).

Table 6. Hematological parameters of male and female rats in sub-chronic toxicity.

Parameters	Control	Positive control	Treatment-1	Treatment-2
Male				
WBC (10^3/µl)	7.06 ± 0.68	7.13 ± 0.63	7.35 ± 0.78	7.55 ± 0.81
RBC (10^6/µl)	8.12 ± 0.39	8.21 ± 0.39	8.28 ± 0.35	7.96 ± 0.52
HGB (g/dl)	15.08 ± 0.25	15.17 ± 0.75	15.02 ± 0.57	15.22 ± 0.65
HCT (%)	46.67 ± 0.93	47.22 ± 0.44	46.63 ± 0.77	47.19 ± 0.57
MCV [fl (µm^3)]	56.00 ± 0.56	55.90 ± 0.48	56.11 ± 0.56	55.93 ± 0.64
MCH (pg)	18.92 ± 0.80	18.81 ± 0.66	19.01 ± 0.50	18.93 ± 0.53
MCHC (g/dl)	35.08 ± 0.56	35.05 ± 0.60	34.93 ± 0.43	35.10 ± 0,68
Platelets (10^3/µl)	1,053 ± 8.32	1,056 ± 6.83	1,058 ± 7.52	1,061 ± 4.49
Female				
WBC (10^3/µl)	6.43 ± 0.46	6.84 ± 0.51	7.01 ± 0.56	7.00 ± 0.17
RBC (10^6/µl)	8.08 ± 0.10	8.29 ± 0.45	8.14 ± 0.26	7.88 ± 0.17
HGB (g/dl)	14.61 ± 0.83	14.94 ± 0.22	15.10 ± 0.44	15.01 ± 0.26
HCT (%)	44.18 ± 0.41	43.85 ± 0.62	44.23 ±0.46	43.82 ± 0.24
MCV [fl (µm^3)]	55.84 ± 0.31	55.85 ± 0.29	54.75 ± 0.76	55.96 ± 0.12
MCH (pg)	18.39 ± 0.53	18.09 ± 0.24	18.43 ± 0.48	18.65 ± 0.38
MCHC (g/dl)	34.18 ± 0.41	35.17 ± 0.61	34.80 ± 0.73	34.62 ± 0.35
Platelets (10^3/µl)	1,037 ± 4.16	1,042 ± 5.15	1,039 ± 5.39	1,044 ± 2.74

MCFE, *Morinda citrifolia* fruit extract; AML, amlodipine; WBC, White Blood Cell; RBC, Red Blood Cell; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin, MCHC, Mean Corpuscular Hemoglobin Concentration; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML).

Data are expressed as the mean ± SD.

*Significant ($p < 0.05$).

[a]Compared to the control group.

[b]Compared to the positive control group (AML).

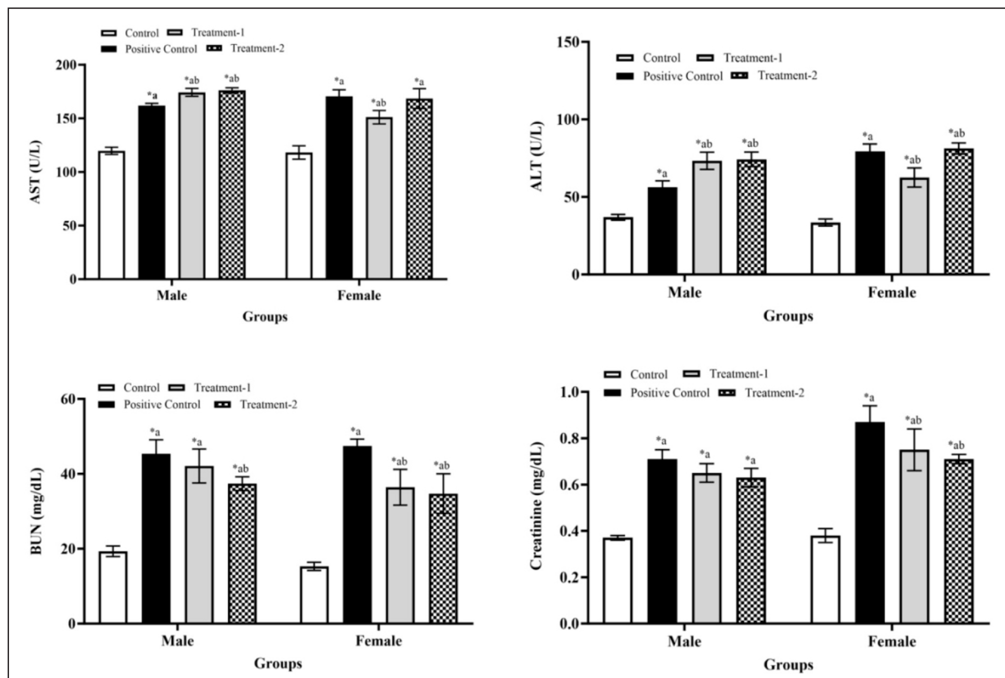

Figure 2. Biochemical parameters of male and female rats in sub-chronic toxicity. Values are expressed as the mean + SD. Compared to the control group; Compared to the AML group; *significant ($p < 0.05$). Abbreviations: MCFE, *Morinda citrifolia* fruit extract; AML, amlodipine; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; BUN, Blood urea nitrogen.

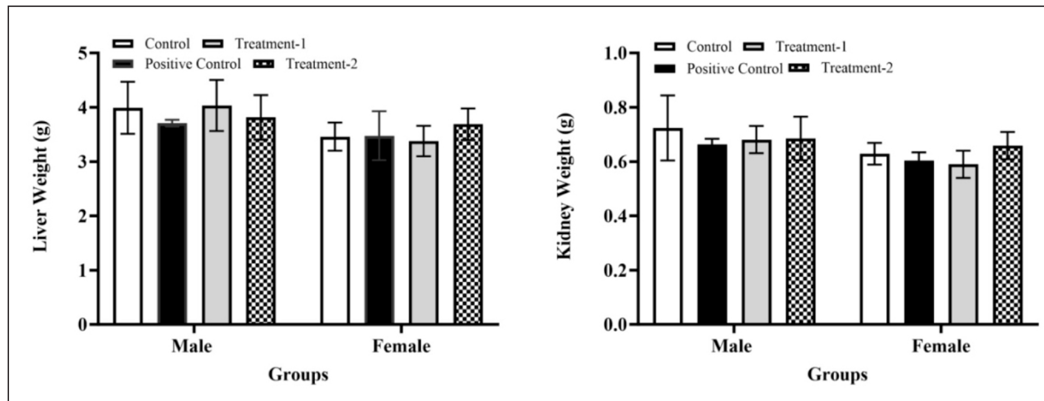

Figure 3. The relative organ weight (liver and kidney) of male and female rats in the sub-chronic toxicity study. Values are expressed as the mean ± SD. Compared to the control group; Compared to AML group; *significant ($p < 0.05$). Abbreviations: MCFE, *Morinda citrifolia* fruit extract; AML, amlodipine; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML).

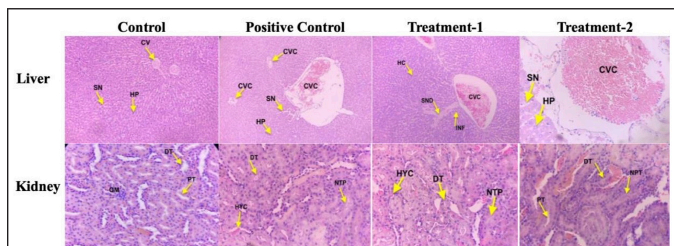

Figure 4. Histopathology of the male liver and kidney in sub-chronic toxicity study (Hematoxylin and eosin stained). Normal histology showed on the liver and kidney of a control group. The liver of the posotive control group showed slight congestion of the central vein. Mild central vein congestion, sinusoidal dilatation, and inflammation in the treatmen-1 group were seen. The treatment-2 group showed moderate central vein congestion. The kidney positive control, treatment-1, and treatment-2 groups showed narrowing of proximal tubules, epithelium necrosis of proximal tubules, and hyalin cast. CV, Central vein; SN, Sinusoidal; HP, Hepatocyte; CVC, Central Vein Congestion, INF, Inflammation; GM, Glomerulus; DT, Distal Tubules; PT, Proximal Tubules; NPT, Narrowing, and Necrosis Proximal Tubules, HYC, Hyaline Cast; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML).

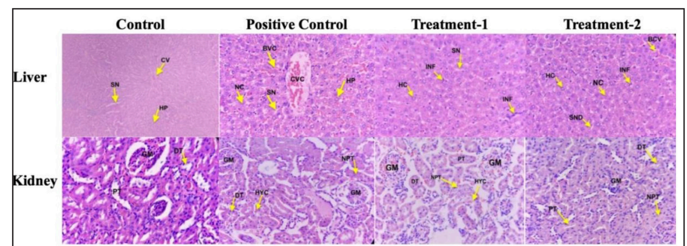

Figure 5. Histopathology of the female liver and kidney in sub-chronic toxicity study (Hematoxylin and eosin stained). Normal histology showed on the liver and kidney of the control group. The liver of the positive control group showed moderate congestion of the central vein, bleeding around of central vein, and necrosis of hepatocytes. Mild inflammatory cell infiltration in the treatment-1 group was seen. The treatment-2 group showed moderate central vein congestion, bleeding around the central vein, inflammation cell infiltration, and necrosis of hepatocytes. In the kidney of positive control and treatment-1, groups showed moderate to severe narrowing of proximal tubules, epithelium necrosis of proximal tubules, and hyalin cast. Mild necrosis of epithelium and narrowing proximal tubules were seen in the treatmen-2 group. CV, Central vein; SN, Sinusoidal; HP, Hepatocyte; CVC, Central Vein Congestion; BVC, Bleeding around Central Vein; INF, Inflammation; GM, Glomerulus; DT, Distal Tubules; PT, Proximal Tubules; NPT, Narrowing, and Necrosis Proximal Tubules, HYC, Hyaline Cast; NC, Necrosis Hepatocyte; Positive control: (AML); Treatment-1: (MCFE); Treatment-2: (MCFE + AML).

significantly ($p > 0.05$) from the control group among males and females. These findings indicated that administering AML, MCFE, and MCFE + AML once daily for 28 days did not alter the liver's and kidneys' absolute or relative weight.

The histological analysis of the positive control, treatment-1, and treatment-2 groups confirmed the treatment-related changes in the liver and kidney (male) of rats compared to the control group (Fig. 4). In the liver, mild to moderate central venous congestion was observed in 3/5 of males in the positive control, treatment-1, and treatment-2 groups. The treatment-2 group showed moderate central vein congestion. At the same time, sinusoidal dilatation and infiltration of inflammatory cells in the MCFE male group were also seen. In the kidney, hyaline cast material in the lumen of the proximal tubule and necrosis of the proximal tubular epithelium was observed in 3/5 males in positive control, treatment-1, and treatment-2 groups with percentage damage of 6%, 5%, and 4%, respectively.

Figure 5 presented the histological analysis of the positive control, treatment-1, and treatment-2 female group showing the change in the liver and kidney compared to the control group. The liver of the positive control group showed hepatocyte necrosis, central venous congestion, and bleeding around the central veins, with mild damage. The mild infiltration of inflammation cells was indicated in the treatment-1 and treatment-2 groups, while hepatocyte necrosis, central vein congestion, and bleeding around the central veins in the treatment-2 group were also seen. In the female kidney, hyaline cast material in the lumen of the proximal tubule and necrosis of the proximal tubular epithelium with percentage damage of 80%, 47%, and 20%, respectively, were observed in 2/5 females in positive control, treatment-1, and treatment-2 group. Toxic effects on the liver and kidneys were found in both male and female groups, and gender differences did not affect the apparent toxic results.

DISCUSSION

This study demonstrated that the simultaneous use of MCFE and AML or MCFE alone could decrease SBP, DBP, and MABP in NaCl-induced rats. The percent decrease in blood pressure was higher in the treatment-2 (MCFE + AML) group than in the other groups. Still, the change was not significantly different from that in rats treated with treatment-1 (MCFE) or positive control (AML). We may assume that simultaneous treatment with AML and MCFE has the same effect on blood pressure as either AML or MCFE alone. Our study also demonstrated that the simultaneous use of AML of MCFE could cause toxic effects on liver and kidney function through AST, ALT, BUN, Creatinine levels, and histology organs of rats.

We used the dose for AML 1 mg/Kg BW in our study based on Zhang *et al.* [14] study in 2019 and based on converting human dose to rat dose calculation. The Zhang study investigated the effects of the simultaneous use of danshen tablets, a traditional Chinese herbal medicine, and AML tablets in rat's PKs. The result showed HDIs between danshen tablets and AML, whereas the dose of MCFE used in this study was based on the dose listed on the Indonesian herbal medicine formulary (500 mg/kg BW), which was then converted into a rat dose (45 mg/kg BW) [32].

Morinda citrifolia fruit exerts hypotensive effects through a vasodilatory mechanism involving its smooth muscle relaxant activity, acting as a nonspecific spasmolytic agent, and it could have an angiotensin-I converting enzyme-inhibitory effect [34]. Meanwhile, AML is an effective first-line option among several available antihypertensive medications and long-acting CCB inhibiting calcium entry into vascular smooth muscle cells and cardiac cells, decreasing peripheral vascular resistance [8]. A study revealed that AML could lower blood pressure by reducing malondialdehyde levels and increasing Na^+ K^+ ATPase, superoxide dismutase, and endothelial nitric oxide (NO) levels [42,43]. According to other research, *M. citrifolia* juice administration increased vasodilation in patients with hypertension and induced vasorelaxation in the aorta via NO generation by endothelial cells [44] Based on these findings, *M. citrifolia* could have the same antioxidant action as AML, and it may have the same activity as AML, in line with the results of this study.

The higher percent decrease in blood pressure in the MCFE + AML (treatment-2) group was caused by their combined antioxidant activities. This is in line with the results of previous studies stating that the combination of *Carthamus tinctorius* extract and captopril could reduce blood pressure by increasing NO bioavailability and reducing oxidative stress [45]. Another study reported that combining garlic or garlic oil and carvedilol exerts antihypertensive and cardioprotective effects by increasing lactate dehydrogenase, creatinine phosphokinase, superoxide dismutase, and catalase activities [46,47]. In addition, the combination of AML and ACEIs is more effective than AML alone in controlling blood pressure [48].

Combining herbal medicine and prescription drugs may provide a more desirable effect than either treatment alone [49]. When herbal and prescription drugs are used together, drug–herbal interactions may occur. Drug–herbal interactions are considered additive if the combined effect is the same as the sum of the effect of each drug alone [50]. The simultaneous use of herbal medicines and drugs can cause pharmacodynamic interactions such as additive, synergistic, or antagonistic effects [49,51]. Previous studies stated that simultaneously administering *Zingiber officinale* or *Hibiscus sabdariffa* with AML improves its pharmacodynamic response [16]. On the other hand, HDIs have traditionally focused on metabolic enzyme and/or transporter-mediated PK changes in a drug induced by concomitant herbal products because PK changes of a drug can result in the alternation of efficacy and toxicity [52,53]

According to sub-chronic toxicity (28 days) in the current study, clinical observation and the rat's BW showed no abnormal animal behavior, nor did mortality appear in the positive control, treatment-1, and treatment-2 groups until the end of the study. The average BW of male and female rats between the first and fourth week increased in all group treatments. This indicates that neither AML, MCFE nor the combination of MCFE + AML reduced the BW of the test animals after 28 days of administration. An increase in BW is one of the indicators of the health status of the experimental animals [54,55] because BW changes can be attributed to adverse drug effects [21,56]. In this study, both male and female rats in the treatment and control groups gained BW because they were in a growing phase.

The most vital tissue is the blood, which may reflect changes in metabolic processes. As a result, significant changes in blood parameters are markers of pharmacological, chemical, and disease-related toxicity [57,58]. In this study, the simultaneous use of MCFE and AML did not significantly affect rats' hematological variables (WBC, RBC, HB, HCT, MCV, MCH, MCHC, and platelets), in line with previously reported findings that MCFE administration in rats at a dose of 2,000–5,000 mg/kg BW for 13 weeks had no adverse effect on the hematological profile of rats [59].

In the present study, AST, ALT, BUN, and creatinine levels were significantly elevated ($p < 0.05$) in positive control, treatment-1, and treatment-2 groups compared to the control group of both sexes. Organ anatomic-pathological alterations frequently accompany the biochemical alterations of the serum. Since injured organs discharge their contents into circulation, this may alter the normal biomarker concentrations in the blood plasma [60,61]. The liver and kidney, which are involved in eliminating xenobiotics, are sensitive organs susceptible to alteration by substances, such as plants and drugs [62].

AST and ALT are serum enzymes that indicate hepatocellular toxicity and liver injury [63]. In this study, the levels of AST and ALT were significantly elevated ($p < 0.05$) in the positive control, treatment-1, and treatment-2 groups when compared with the values of the control group in both sexes. An increase in serum AST and ALT, the effects of using AML have been reported. Although the mechanism of the hepatotoxic effect is not widely known, this must still be considered [19]. Furthermore, there is a potential risk of HDIs leading to adverse side effects, including adverse hepatic reactions such as hepatotoxicity or liver injury, which is an increase in serum aminotransferase (ALT/AST) levels by at

least two times the normal upper limit (2N) or increase over 2–4× normal/control limits [64,63]. Increased levels of AST and ALT in the group of male and female rats that were given MCFE + AML simultaneously showed an interaction between AML and MCFE. This aligns with previous research stating that herbal-drug interactions can induce hepatotoxicity [10,50]. Several plant secondary metabolites, including terpenes, alkaloids, and anthraquinone, can affect liver enzymes [65,66]; these compounds are present in MCFE and, when combined with pharmaceuticals, can modulate various cytochrome P450 enzymes, particularly CYP3A4 [67,68]. AML was metabolized by cytochrome P450 CYP3A4 isoenzymes; modulators of CYP3A4 could enhance the intrinsic hepatotoxicity of other substances by increasing their conversion to toxic metabolites [69,70]. Therefore, plant metabolites with a given pharmacological property/metabolizing enzyme should not be combined with drugs that have the same pharmacological property/metabolizing enzyme [71].

Furthermore, kidney dysfunction was evaluated by measuring creatinine and BUN. Creatinine and urea are nitrogenous nonprotein byproducts of protein metabolism that must be continuously eliminated. Therefore, an increase in these kidney function indices indicates that kidney dysfunction is predominantly caused by injury [72]. This study showed a significant ($p < 0.05$) increase in serum creatinine and BUN levels in all group treatments compared to the control group in both sexes. The simultaneous use of MCFE + AML (treatment-2 group) on male rats shows significantly elevated BUN levels compared to the AML (positive control) group. Meanwhile, in creatinine levels, there is no significant difference. In the female group of MCFE + AML (treatment-2), there was a significant increase in creatinine and BUN levels. Due to their inherent properties, plant extracts can have renal toxicity, and this effect is not only related to the presence of contaminants in the extract; herbal-drug interactions with a compound present in the herb must also be considered [73,74]. The kidney can be regarded as an important target organ for exogenous toxins. Nephrotoxicity is a kidney-specific condition in which toxic chemicals, herbs, or drugs disrupt excretion [75,76]. In general, nephrotoxicity is associated with numerous factors, such as direct nephrotoxic effects of the herbal product or its compound, herbal-drug interactions that enhance nephrotoxicity, the insolubility of substances and their metabolites in urine, and protracted exposure at high doses [77].

This study examined the liver and kidney sections for histopathological alterations. For liver histological changes in male rats caused by AML, MCFE, and the simultaneous use of MCFE + AML, we found mild damage, such as central venous congestion, sinusoidal dilatation, and inflammation. Meanwhile, in female positive control, treatment-1, and treatment-2 group livers showed histological changes, including necrosis hepatocyte, sinusoidal dilatation, congestion of central veins, bleeding around central veins, and infiltration of inflammation cells. The xenobiotic-induced hepatic lesions are diverse and heterogeneous. All liver cells (hepatocytes and endothelial cells) are potentially implicated. Consequently, xenobiotics can be responsible for all liver lesions, affecting all liver vascular system levels. Conventional medications, medicinal plants, and industrial agents are involved in vascular lesions. This lesion can be identified by sinusoidal dilatation without obstruction of the liver's efferent vessels [78–80]. Sinusoids regulate hepatic microcirculation, transporting oxygen, nutrients, and toxins between the vascular space and hepatocytes. Sinusoidal dilatation and congestion are common due to obstructed hepatic venous outflow and elevated venous pressure [79].

We also observed histological changes in male and female positive control, treatment-1, and treatment-2 kidney groups through narrowing of the proximal tubule, necrosis of the proximal tubule epithelium, and hyaline casts. The kidney's proximal tubules play a vital role in the reabsorption and excretion of substrates from the body into the urine, resulting in elevated local concentrations of xenobiotics. Impaired endocytosis, receptor/transporter inhibition, lysosome disruption, oxidative stress and depletion of antioxidant defenses, and mitochondrial dysfunction are the xenobiotic-induced causes of proximal tubule dysfunction [73,81]. The nephrotoxicity of xenobiotics depends on the drug's intrinsic reactivity with subcellular or molecular targets. Both cytochrome P450 and cysteine conjugate-lyase are almost exclusively localized in the proximal tubule, and bioactivation contributes at least partially to proximal tubular lesions. Finally, proximal tubular cells appear more susceptible to ischemic damage than distal tubular cells [82,83]. Hyaline casts are the most common cast encountered in renal biopsy specimens [84]. They are associated with increased glomerular permeability and granular (necrotic cellular debris) casts indicative of previous tubule cell necrosis associated with chemicals that induce α2u-globulin nephropathy [85]. In this study, the satellite rats of both sexes showed the same histological results as the treatment group, indicating that organ damage can be irreversible. Therefore, more studies with a prolonged exposure time (90 days) are required to determine whether organ recovery or repair is delayed after drug administration.

The limitations of this study included the inability of the study design to provide specific information about the mechanisms behind the interactions. In addition, mechanism-based research can be conducted, such as *in-vitro* microsomal studies to confirm the function of particular drug-metabolizing enzymes and the PK profiles of simultaneous use of these herbs with antihypertensive drugs.

CONCLUSION

The simultaneous use of MCFE and AML may have the same effect in reducing high blood pressure as AML or MCFE alone. The treatment of MCFE and simultaneous use of MCFE + AML did not affect the hematological parameters of rats but affected AST, ALT, BUN, and creatinine levels. This treatment also caused histological changes in the rat's organs, including the liver and kidneys, that led to toxic effects. Therefore, the simultaneous use of *M. citrifolia* and AML to treat hypertension should not be considered.

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AUTHOR CONTRIBUTIONS

Conceptualization, NA, IMP, and ANH; methodology, NA; validation, NA, ANH, IMP, and EH; formal analysis, NA; investigation, NA; writing—original draft preparation, NA, IMP, and ANH.; writing—review and editing, NA, IMP, ANH; supervision, EH, IMP, and ANH.; All authors have read and agreed to the published version of the manuscript

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

The experimental procedures were approved by the Research Ethics Committee, Universitas Padjadjaran, West Java, Indonesia (No. 565/UN6.KEP/EC/2020), and we followed the guidelines of the European Directive 2010/63/EU.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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