Trop J Nat Prod Res, December 2021; 5(12):2074-2078 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Acute Toxicity Studies of a Combination of Ethanol Extracts of *Andrographis paniculata, Centella asiatica,* and *Curcuma heyneana* in Rats

Dadang I. Husori[1]*, Marianne[1], Popi Patilaya[2], Audrey N. Febrika[1]

[1]*Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Padang Bulan, Medan 20155, Indonesia*
[2]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Padang Bulan, Medan 20155, Indonesia*

ARTICLE INFO

Article history:
Received 27 May 2021
Revised 17 June 2021
Accepted 01 November 2021
Published online 03 January 2022

ABSTRACT

Andrographis paniculata, *Centella asiatica*, and *Curcuma heyneana* are beneficial in the treatment of various diseases. A combination of extracts of several medicinal plants increases the potential of the expected effect. The present study was aimed at determining the acute toxicity of a combination of ethanol extracts of *A. paniculata*, *C. asiatica*, and *C. heyneana* in rats. A combination of ethanol extracts of the three plants was prepared. Twenty female rats were divided into 4 groups: the control group, and treatment groups of a combination of extracts at doses of 500, 2,000, and 5,000 mg/kg. The acute toxicity study was carried out on the experimental rats using the Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals number 423. The animals were sacrificed on the 15th day, and biochemical analyses were performed on the collected blood samples. Macroscopic and microscopic examinations of the vital organs were determined. The results revealed that there were no toxic symptoms in the female rats. The combination of extracts does not affect the body weight and the relative organ weight of the rats. Also, the biochemical parameters indicated that alanine transaminase (ALT), aspartate transaminase (AST), and creatinine levels increased at the dose of 5,000 mg/kg BW with no significant difference in AST level. The lethal dose (LD_{50}) of the combination of the extracts is higher than 5,000 mg/kg BW. This study found that combining extracts of *A. paniculata*, *C. asiatica*, and *C. heyneana* is relatively non-toxic, with an LD_{50} greater than 5,000 mg/kg.

Keywords: Acute toxicity, *Andrographis paniculata*, *Centella asiatica*, *Curcuma heyneana*.

Introduction

Several pharmacological studies carried out on *C. asiatica* herb have shown its ability to accelerate wound healing,[1,2] and also possess antioxidant,[3] antiulcerogenic,[4] and neuroprotectants,[5] activities. Meanwhile, *A. paniculata* herb has antibacterial,[6,7] antiulcerogenic,[8,9] antihyperglycemic,[10] and hepatoprotective properties.[11] *C. heyneana* has been reported to be an efficacious gastroprotective agent with antioxidant, antiviral, antiaging, and antibacterial properties.[12–14] The ethanol extract of *A. paniculata* leaves has a gastroprotective effect with the ability to reduce acid secretion in rats induced by pyloric ligation.[15] The ethanol extract of *C. asiatica* leaves also has an anti-secretory effect in rats induced by pyloric ligation.[16] *C. heyneana* rhizome extract was reported as a medicine for digestive tract disorders/stomach ulcers.[17] The combination of bioactive constituents contained in plant extracts tends to increase the effectiveness of many extracts.[18] This provides the potential for the combination and development of three extracts into alternative medicine to treat stomach ulcers.

The acute toxicity test is an important preclinical test carried out by treating rats with chemicals once or several times within a 24-hour period to determine the toxicant's median lethal dose (LD_{50}). This test also indicates possible damage to target organs and their specific toxic effects and provides clues on the dosage that needs to be used in chronic testing.[19] Toxicity studies using animals are useful in determining the biochemical, physiological, and pathological parameters. Previous studies have been carried out to determine the acute toxicity test of *A. paniculata* extract in rats, which showed no sign of toxicity at a dose of 5,000 mg/kg BW.[20] A previous report indicated that the acute toxicity test of *C. asiatica* herb extract yielded an LD_{50} value of above 5,000 mg/kg BW.[21]

This study was conducted to investigate the acute toxicity of a combination of *C. asiatica*, *A. paniculata* herbal, and *C. heyneana* rhizome extracts in rats.

*Corresponding author. E mail: dadang@usu.ac.id
Tel: +62618223558

Citation: Husori DI, Marianne, Patilaya P, Febrika AN. Acute Toxicity Studies of a Combination of Ethanol Extracts of *Andrographis paniculata*, *Centella asiatica*, and *Curcuma heyneana* in Rats. Trop J Nat Prod Res, 2021; 5(12):2074-2078. doi.org/10.26538/tjnpr/v5i12.5

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Materials and Methods

Source of plant samples

Herbs of *C. asiatica* and *A. paniculata*, as well as *C. heyneana* rhizome, were obtained from the Pancur Batu area, Deli Serdang, Sumatra Utara in February 2020. The identification was carried out at the Herbarium Medanense (MEDA), Universitas Sumatera Utara (Voucher Number: 5314/MEDA/2020, 5315/MEDA/2020, and 5316/MEDA/2020 for *C. asiatica*, *A. paniculata*, and *C. heyneana*, respectively). Fresh herbs of C. *asiatica* and *A. paniculata*, as well as *C. heyneana* rhizome, were separated, washed thoroughly, drained, and dried in a drying cabinet at 40^{o}C until the dried leaves were brittle when squeezed. After drying, the leaves were blended into simplicia powder.

Preparation of plant extracts

Dried powder of *C. asiatica* and *A. paniculata* herbs, as well as *C. heyneana* rhizome, were extracted with 96% ethanol solvent using the maceration method. One kilogram of each sample was soaked with 10 L of solvent. The mixture of each sample was protected from sunlight and stirred frequently. After 5 days, the mixture was filtered and

squeezed. The sample residues were rinsed with the solvent and left for 2 days before being re-filtered. Each of the macerated preparations was evaporated with a rotary evaporator, then concentrated in a water bath until a thick extract was obtained.[8]

Experimental animals
The subjects used were healthy female Wistar rats of 8 to 12 weeks, weighing 120 to 140g and were not pregnant. The animals were obtained from the Pharmacology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara. Before testing, the subjects were acclimatized under laboratory conditions for 7 days, and were given a standard diet and drinking water *ad libitum*. A total of 20 rats were randomly divided into 4 groups, each consisting of 5 rats, namely 1 control and 3 treatment groups which were given 0.5% sodium-CMC suspension at a dose of 1% BW and an extract suspension at a dose of 500, 2,000, and 5,000mg/kg.[19]

Ethical approval
The research procedure was approved by the Animal Research Ethics Committee (AREC) of the Universitas Sumatera Utara (Approval number: 00638/KEPH-FMIPA/2020).

Acute toxicity testing and animal grouping
Acute toxicity testing was carried out following the Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals number 423.[19] The animals used were 20 female rats and were divided into 4 groups: the Control Group (given 0.5% Na-CMC suspension at a dose of 1% of BW); Test Group 1 (treated with a combination of extract suspension at a dose of 500 mg/kg BW); Test Group 2 (treated with a combination of extract suspension at a dose of 2,000 mg/kg BW); and Test Group 3 (treated with a combination of extract suspension at a dose of 5,000 mg/kg BW). Before the treatment process, the rats were made to fast for 14 hours, with free access to water. The animals were weighed and administered a single dose of combination extracts. Observations were made in the first 30 minutes after administering the combination of extracts and every 4 hours for the first 24 h. Subsequent observations were made once a day for 14 days. The number of animals that died was calculated and analyzed as an LD_{50}. Furthermore, animals on the verge of dying were sacrificed and counted as dead. On day 15^{th}, the animals were sacrificed for clinical, biochemical examinations, and organ histopathology.

Toxic symptom observation
Toxic symptom manifestations observed were tremors, seizures, salivation, limp, sedation, coma, diarrhea, and animal movements such as walking backward and on the stomach. Observations included the time for the appearance and disappearance of toxic symptoms.[19,22]

Measurement of body weight and relative organ weight
Each animal's body weight was measured before the administration of the combination of extracts. Once a week, the animals' weights were measured, and the survivors were weighed and slaughtered at the end of the study.[22] The excised hearts, livers, kidneys, pancreas, and uterus were washed with sodium chloride and dried with absorbent paper. The organs were immediately weighed to obtain their absolute weight, and the organ-body index was determined.[22]

Biochemical analyses
Kidney function was examined by estimating creatinine and urea levels, while liver function was determined by the quantification of aspartate transaminase (AST) and alanine transaminase (ALT) levels. Measurement of biochemical parameters was carried out using a spectrophotometer. A total of 0.5 ml of blood was collected from the heart and then placed into a microtube and centrifuged at 3,000 rpm to obtain serum. The serum was separated and the levels of urea and creatinine were measured.[19,22]

Macroscopic observation of experimental rat organs
The dead animals were immediately autopsied and followed by macroscopic observations. The heart, liver, kidney, pancreas, and uterus organs were visually observed to determine their color, surface shape, and consistency.[19]

Histopathological analysis of experimental rat organs
At the end of the study, all the living rats were sacrificed for further harvesting of their hearts, livers, kidneys, pancreas, and uterus. Each organ was washed with 0.9% sodium chloride and then placed into a 10% formaldehyde buffer solution. Histological preparations were made by slicing the organs using a cutting machine (microtome) and then placing them on a slide. The staining procedure was carried out using Hematoxylin-Eosin (HE), then covered with a coverslip and glued using entellan. The histological preparations were then observed under a microscope and photographed.[23]

Statistical analysis
The data on the number of animal deaths, blood biochemical analyses, and organ weight were statistically analyzed using the SPSS application. The data were analyzed to determine the normality and homogeneity of the variants. The parametric method used for the data analysis was the one-way analysis of variance (ANOVA), followed by the use of Tukey's post hoc test to determine significant differences at level of confidence of 95%.[23]

Results and Discussion

Toxicity symptoms associated with a combination of plant extracts in experimental rats
The results of the symptoms of acute toxicity are shown in Table 1. The administration of a combination of plant extracts did not lead to toxic symptoms in the control group; however, they appeared in the treatment group. In the 500 mg/kg BW dose group, toxic symptoms were found in the form of tremors, diarrhea, and limp. Meanwhile, in the 2,000 mg/kg BW group, there were toxic symptoms in the form of tremors, salivation, diarrhea, and limp. Also, Table 1 showed that in the 5,000 mg/kg BW dose group, toxic symptoms were found in the form of tremors, salivation, limp, sedation, and walking on the stomach. Evaluation of acute toxicity is not only associated with the LD_{50}, but also linked with the abnormal behaviour, stimulation, and motor activity of test animals.

Effects of a combination of plant extracts on mortality in experimental rats
The administration of a combination of extracts in the control and treatment (500 and 2,000 mg/kg BW) groups did not cause the death of the test animals. Meanwhile, in the dose group of 5,000 mg/kg BW, one rat died (Table 1). Therefore, based on these data, the LD_{50} value of the extract combination cannot be determined absolutely. When the toxicity is low, the LD_{50} does not need to be determined precisely with an estimated number of benefits. If the maximum dose fails to lead to the death of test animals, the LD_{50} is declared "pseudo." Therefore, in this study, the LD_{50} is the pseudo, namely 5,000 mg/kg BB.[23] The 5,000 mg/kg BW is the maximum dose conversion in humans to rats based on the ratio of the body surface area. Compounds with an oral LD_{50} in rats at 5-15 g/kg BW were categorized as "practically non-toxic".[24]

Effects of a combination of plant extracts on the body weight and relative organ weights in rats
The administration of the extract combination did not show a significant difference in the body weight between the control and treatment groups ($p > 0.05$). Toxic symptoms and body weight are sensitive indicators of toxicity. Therefore, the experimental animals were observed daily for toxic symptoms and body weight effects periodically. Significant weight loss is usually a sign of poor health. Weight loss is caused by inadequate consumption of food and drink, disease, or specific toxic symptoms.[19,23] Table 3 shows that there was no significant difference in the relative weight of the heart, liver, kidney, and uterus between the control and treatment groups ($p > 0.05$). However, there was a significant difference ($p < 0.05$) in the relative weight of the pancreas between the control and 5,000 mg/kg BW extract treatment group.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Behavioral responses from the rats treated with a single dose of a combination of *Andrographis paniculata*, *Centella asiatica*, and *Curcuma heyneana* extracts

Symptom	Control group	Treatment group		
		500 mg/kg BW	2000 mg/kg BW	5000 mg/kg BW
Tremor	Not present	Present	Present	Present
Seizure	Not present	Not present	Not present	Not present
Salivation	Not present	Not present	Present	Present
Diarrhea	Not present	Present	Present	Not present
Limp	Not present	Present	Present	Present
Sedation	No effect	No effect	No effect	Present
Coma	Not present	Not present	Not present	Not present
Walk backwards	Not observed	Not observed	Not observed	Not observed
Walk on the stomach	Not observed	Not observed	Not observed	Observed
Death	Alive	Alive	Alive	1 dead

Table 2: Body weight (g) of control and treatment groups of rat

Treatment	Day after treatment		
	0	7	14
Control	148.8 ± 7.5	162.0 ± 7.1	175.6 ± 6.2
Extract Combination 500 mg/kg BW	162.6 ± 5.2	174.8 ± 4.4	186.0 ± 4.3
Extract Combination 2000 mg/kg BW	150.8 ± 6.8	159.6 ± 4.5	170.6 ± 4.0
Extract Combination 5000 mg/kg BW	154.0 ± 5.0	162.0 ± 4.4	169.6 ± 4.0

All the values were expressed as mean ± SEM; *: $p < 0.05$ (significantly different from the control)

Changes in organ weight are a sensitive indicator of toxicity. The difference in organ weight between the control and treatment groups occurs without changes in organ morphology.

Effects of a combination of plant extracts on biochemical parameters in experimental rats

Table 4 shows that there is a significant difference in the mean levels of AST, ALT, and creatinine between the control and treatment groups at a dose of 5000 mg/kg BW ($p<0.05$). The liver plays an important role in metabolism and protein synthesis. This is because it comprises hepatocytes which contain many enzymes. Therefore, when liver damage occurs, these enzymes are released into the blood circulation to increase serum levels.[25] Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are two common markers of hepatocellular damage. Both enzymes catalyze the transfer of amino and carboxylic acids during gluconeogenesis. They are found in the cytoplasm in hepatocytes and mitochondria.

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Table 3: Relative organ weight of rat after 14th day of treatment

Treatment	Relative organ weight of rat (g)				
	Heart	Liver	Kidney	Pancreas	Uterus
Control	0.32 ± 0.01	3.77 ± 0.05	0.33 ± 0.01	0.30 ± 0.04	0.19 ± 0.02
Extract Combination 500 mg/kg BW	0.31 ± 0.01	3.45 ± 0.15	0.34 ± 0.01	0.24 ± 0.03	0.25 ± 0.02
Extract Combination 2000 mg/kg BW	0.35 ± 0.01	3.15 ± 0.15	0.35 ± 0.02	0.21 ± 0.02	0.24 ± 0.03
Extract Combination 5000 mg/kg BW	0.38 ± 0.02	3.58 ± 0.26	0.35 ± 0.01	0.33 ± 0.05*	0.22 ± 0.03

All values were expressed as mean ± SEM; *: $p < 0.05$ (significantly different from the control)

Table 4: Biochemical estimation from blood serum of rat after 14th day of treatment

Treatment	AST	ALT	Ureum	Creatinine
	(U/L)	(U/L)	(mg/dL)	(mg/dL)
Control	213.67 ± 27.64	99.00 ± 10.02	27.33 ± 1.86	0.62 ± 0.10
Extract Combination 500 mg/kg BW	157.33 ± 28.26	97.67 ± 2.85	36.00 ± 5.29	0.60 ± 0.03
Extract Combination 2000 mg/kg BW	156.67 ± 23.51	131.67 ± 5.21	37.67 ± 4.67	0.98 ± 0.06
Extract Combination 5000 mg/kg BW	234.00 ± 22.72*	135.67 ± 10.68*	34.00 ± 3.21	1.28 ± 0.23*

All the values were expressed as mean ± SEM; *: $p < 0.05$ (significantly different from the control); AST: Aspartate transaminase; ALT: Alanine transaminase

AST is also found in the heart and striated muscles, which makes it less sensitive and specific to liver damage. An increase in AST alone leads to a necessary pathological investigation outside the liver.[26] Urea is formed by the liver as an end product of protein metabolism, while protein is broken down into amino acids, and then catabolized in the liver to form free ammonia, which forms urea that is excreted from the blood through the kidneys. Measurement of urea levels is widely used to determine the excretory function of the kidneys. Almost all diseases/damage to the kidneys cause the excretion of very little urea, which results in an increase in the concentration of urea in the blood.[27]. Creatinine is a catabolic product of CPK (creatinine phosphokinase) used in skeletal muscle contraction. It is excreted through the kidneys; therefore, it is used as a parameter in the assessment of renal excretory function.[25,27]

Effects of a combination of plant extracts on the histopathology of rats

The results of histopathological examination of the heart in the control and treatment groups at doses of 500, 2,000, and 5,000 mg/kg BW did not indicate any damage to the heart muscle cells (Figure 1). The heart consists mainly of myocardial cells with less contractile and more mitochondrial material. Mitochondria play an important role in cardiac contractility and are often the target of sub-cell cardiotoxicity.[28–30] The control and 500 mg/kg BW dose groups showed normal kidney tissue without Bowman space widening, tubular lumen dilation, and necrosis. Meanwhile, in the 2,000 and 5,000 mg/kg BW dose groups, there was a visible widening of the Bowman space and the tubular lumen, as shown in Figure 1. Bowman's space widening is due to slow circulatory glomerular atrophy, as well as tissue hypoxia as a result of impaired venous blood circulation from the tissue or severe damage. The dilation of the renal tubular lumen occurs because the extract's administration in high doses causes toxic effects. The toxic effect of xenobiotics leads to dilation of the renal tubules, which directly causes damage to the tubular epithelium, affecting absorption and secretion..[31] The liver in the control rats did not show any damage, as indicated by the central vein. The hepatocytes looked normal, and the sinusoid was arranged radially toward the central vein. Meanwhile, the liver started to experience damage in the treatment group with doses of 500, 2,000, and 5,000 mg/kg BW. The group treated with 500 mg/kg BW of a combination of plant extracts showed a widening of the sinusoids, the cell nucleus was reduced, condensed (pyknotic), and cariorexis, as shown in Figure 1. The 2,000 and 5,000 mg/kg BW caused sinusoidal enlargement accompanied by an irregular sinusoid arrangement, with the occurrence of picnotics and cariorexis. However, the hepatocytes did not experience necrosis. Liver necrosis is the death of hepatocytes due to acute damage.[32,33] The pancreas is a gland with exocrine and endocrine cells. The exocrine part consists of a collection of cells known as pancreatic acinar connected to the duct. Meanwhile, the endocrine consists of endocrine cells known as Langerhans islands.[34,35] Figure 1 shows that the pancreas in the control and treatment groups indicate the Langerhans islands are still normal at all doses. Pancreatic acinar cells around Langerhans Island also did not show any damage. The uterus in the control and treatment groups at doses of 500, 2,000, and 5,000 mg/kg BW indicated no damage with normal epithelial cells and endometrial glands, as shown in Figure 1

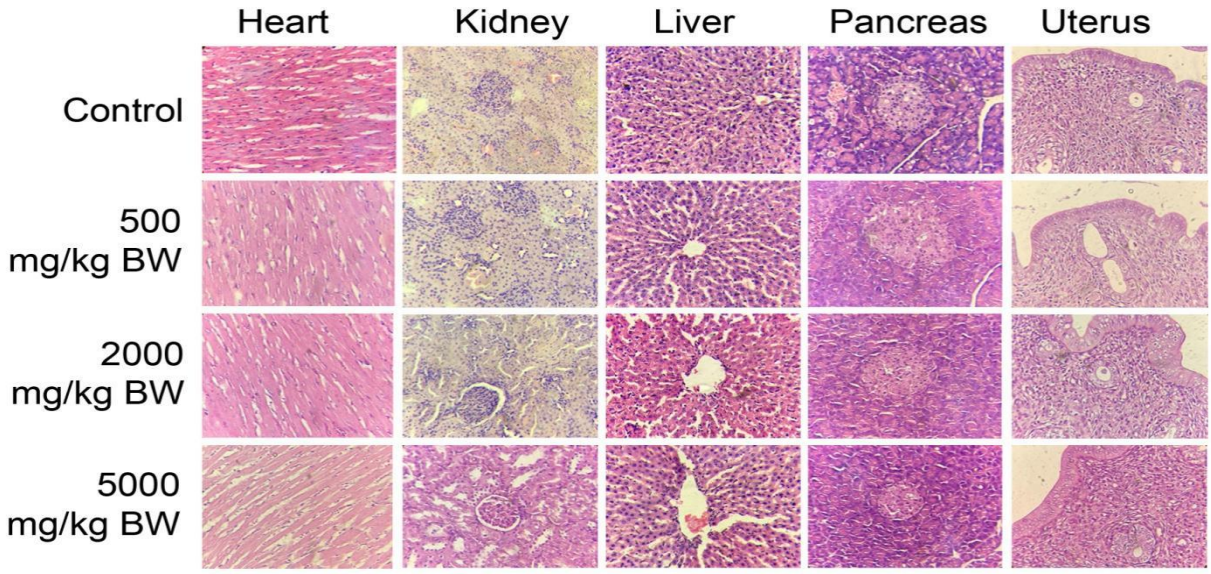

Figure 1: Histology of vital organs of experimental rats.

Conclusion

A combination of ethanol extracts of the herbs of *Andrographis paniculata* and *Centella asiatica*, as well as *Curcuma heyneana* rhizome, is non-toxic at a single dose oral administration with an LD_{50} above 5,000 mg/kg BW.

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.

Acknowledgments

The authors are grateful to the Dikti DRPM Fundamental Research Grant (Nomor: 5/UN5.2.3.1/PPM/KP-DPRP/2020) for supporting this research.

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