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Original Article

Subchronic Toxicity Studies of a Combined *Andrographis paniculata* (Burm.f.) Nees, *Syzygium cumini* (L) Skeels, and *Caesalpinia sappan* L Extract in *Sprague-Dawley* Rats

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ABSTRACT

Introduction: *Andrographis paniculata, Syzygium cumini* and *Caesalpinia sappan* (ASC) are plants that are widely used as traditional medicines in treating diabetes. The acute toxicity test results of the combination of these three plants were safe up to 5000 mg/Kg BB. **Objectives:** To evaluate subchronic toxicity of a combined ASC extract. **Methods:** Male and female *Sprague Dawley* rats were acclimatized for 14 days and then fed a normal diet with ASC extract at doses of 150, 575 and 1000 mg/kg BW daily for 135 days. At the end of the study, the rats were sacrificed and then blood, heart, pulmonary, liver, kidneys, spleen and pancreas were collected. **Result:** The results showed no abnormality in the experimental group compared with the control group. All values of other parameters assessed remained within the normal range. **Conclusions:** The combination of ASC extract given orally for 135 days to male and female rats did not show any subchronic toxicity.

Key words: Subchronic yoxicity, *Andrographis paniculata, Syzygium cumini, Caesalpinia sappan,* Rats.

INTRODUCTION

Diabetes is one of the chronic diseases many people suffer in the world. By 2021, 537 million people will have diabetes. This number is predicted to be 643 million by 2030 and 783 million by 2045.[1] *Andrographis paniculata, Syzygium cumini and Caesalpinia sappan (ASC)* are plants that are widely known and used traditionally for the treatment of diabetes.[2-4] The content of andrographolide in *Andrographis paniculata*,[5-7] quercetin in *Syzygium cumini*[8] and brazilin in *Caesalpinia sappan*[9,10] are substances that have potential antidiabetic effects. The combination of the extracts of these three plants is expected to be an alternative therapy in the treatment of diabetes. In the acute toxicity test, the combination of ASC extract (1:1:1) up to a dose of 5000 mg/kg BW in rats presented no toxic effects.[11] However, subchronic toxicity studies of combined ASC extracts have not been investigated. Therefore, this study aimed to evaluate the subchronic toxicity of the combined ASC extract in rats.

MATERIALS AND METHODS

Materials

ASC dried extract, CMC Na 0,5% solution, normal diet (4% fat, 23% protein, and 5% carbohydrates), formalin 10% solution from Sigma, rats oral probe, digital scale, magnetic stirrer, syringe (Terumo), beaker glass (Pyrex), urine pot, minor surgical tools.

Experimental animals

Sprague Dawley rats were obtained from the National Agency of Drug and Food Control. Animals were maintained on a 12:12 h light-dark cycle at constant temperature (22 ± 3 °C), humidity (70 ± 10%), with food and water ad libitum. This study was approved by the Faculty of Medicine Ethics Committee, Universitas Indonesia (KET-136/UN2.F1/ETIK/PPM.00.02/2020).

Subchronic toxicity test

Sprague Dawley rat's male and female, 4 - 5 weeks of age and weighing 100-120 grams, were given normal diet and ASC extract that was administrated peroral daily for 135 days. Rats were divided into 4 groups where each group consisted of 10 males and 10 females: normal control (NC) treated with CMC Na 0,5% solution; group A (A) treated with ASC extract 1000 mg/kg BW in CMC Na 0,5% solution; group B (B) treated with ASC extract 575 mg/kg BW in CMC Na 0,5% solution; group C (C) treated with ASC extract 150 mg/kg BW in CMC Na 0,5% solution. At the end of the study, the rats were sacrificed after fasting for 12 hours and then blood was taken from cardiac puncture for Hematology analysis: Hemoglobin (HB); Red Blood Cells (RBC); White Blood Cells (WBC); differential leukocyte (basophil, eosinophil, neutrophil, segmented neutrophil, lymphocyte, monocyte); hematocrit; platelets; Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC), blood chemistry analysis: glucose; total-cholesterol (CHL); triglycerides (TG); urea nitrogen; creatinine; alanine aminotransferase (ALT); aminotransferase aspartate (AST) while heart, pulmonary, liver, kidneys, spleen and pancreas were collected. Then the organs that have been taken are then weighed and their relative weights are calculated. All of organs were washed with buffered saline and then stored in 10% formalin solution for 1 week. Hematoxylin-eosin staining was used for all organs.

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Statistical analysis

Statistics were analyzed using GraphPad Prism eighth version and expressed as the mean ± SEM. A normality check was done by the Shapiro-Wilk test. The data was analyzed using ANOVA, followed by the least significant difference (LSD) or Kruskal Wallis test, which was observed using Dunn's test. At p<0,05, the distinction was considered statistically significant.

RESULT

At the end of the experiment, 80 male and female rats were sacrificed with each group's distribution being 10 males and 10 females. All rats that received daily oral administration of ASC extract for 135 days exhibited normal general behavior, breathing patterns, cardiovascular signs, motor activity and responses, and normal skin and coat changes.

The results of blood hematology measurements can be seen in Table 1. Several parameters in the group administered the ASC extract were significantly different as compared to the control group, according to hematological blood test data on male and female rats. The value, on the other hand, is not excessive.

The results of blood chemistry measurements can be seen in Table 2. It can be seen that there are several parameters whose values are significantly different when compared to normal controls. However, these differences did not reveal a value that differed significantly from the standard control.

The data on the relative organ weight measurement results can be seen in Table 3. Several organ weights are significantly different from normal controls but the values are not too far off. The results of relative lung weight showed a decrease in lung weight in male and female rats at all doses compared to normal controls. However, no abnormal changes were seen on microscopic examination of the organ.

After the organ weights were weighed and measured, the organs were subjected to HE staining (figure not shown). Tissue observation at 40X magnification, all organs in male and female rats showed normal anatomy or no abnormalities. Rats treated with ASC extract showed normal structure, size, shape, color and texture of internal organs (heart, lungs, liver, kidneys, spleen, pancreas), both macroscopically and microscopically.

DISCUSSION

In a 135-day sub-chronic toxicity study, female and male rats were treated with ASC extract at all three doses, 150, 575 and 1000 mg/kg BW. All rats at each dose did not show any changes in behavior, toxic signs and mortalities. Furthermore, the health of male and female rats showed normal behavior in the respiratory system, cardiovascular system, motor activity and normal changes in skin and fur.

The hematological examination observed signs of toxicity and bone marrow function, while blood cell count evaluated the immune system. Hematological parameters are sensitive to toxic substances that invade the biological system and can be used as toxicity markers. Damage to the blood system of humans and animals can impair organ function.[12] The

Table 1: Effect of combination ASC extract on the hematological values of male and female in the subchronic toxicity test.

Hematological Values	Unit	Control			Combination ASC Extract								
					A (1000 mg)			B (575 mg)			C (150 mg)		
Male													
RBC	million cell/ul	8,32	±	0,232	7,91	±	0,160	7,89	±	0,135	8,44	±	0,115
HB	g/dL	15,51	±	0,473	14,18	±	0,308*	14,24	±	0,16*	14,77	±	0,178
Hemaocrit	%	44,4	±	1,586	40,3	±	0,978*	41,4	±	0,581	42,7	±	0,518
MCH	pg	18,7	±	0,300	18	±	0,149	18	±	0,258	17,6	±	0,163*
MCV	fl	53,1	±	0,623	50,9	±	0,547*	52,4	±	0,562	50,5	±	0,373*
MCHC	g/dl	35,1	±	0,674	35,4	±	0,221	35,1	±	0,690	34,8	±	0,200
Platelets	10^3/ul	686,3	±	31,250	650,3	±	19,390	634,2	±	32,420	590,9	±	15,16*
WBC	10^3/ul	7,533	±	0,762	8,11	±	0,704	7,37	±	0,733	7,533	±	0,446
Basophil	10^3/ul	0	±	0	0	±	0	0	±	0,000	0	±	0
Eosinophil	10^3/ul	0,073	±	0,020	0,058	±	0,017	0,036	±	0,015	0,122	±	0,016
Neutrophil	10^3/ul	0,074	±	0,017	0,100	±	0,019	0,050	±	0,015	0,126	±	0,023
Segmented Neutrophil	10^3/ul	3,789	±	0,578	5,142	±	0,475	4,409	±	0,449	4,757	±	0,413
Lymphocyte	10^3/ul	2,735	±	0,563	2,686	±	0,223	2,689	±	0,292	2,083	±	0,186
Monocyte	10^3/ul	0,406	±	0,078	0,133	±	0,039*	0,187	±	0,030	0,098	±	0,042*
Female													
RBC	million cell/ul	7,44	±	0,117	7,49	±	0,055	7,37	±	0,118	7,31	±	0,144
HB	g/dL	14,45	±	0,276	14,41	±	0,088	13,86	±	0,155	14,01	±	0,296
Hematocrit	%	42,6	±	0,833	41,4	±	0,267	41	±	0,615	41,2	±	0,867
MCH	pg	19,3	±	0,213	19,1	±	0,100	18,7	±	0,213	19,1	±	0,180
MCV	fl	56,9	±	0,690	55,6	±	0,306	55,8	±	0,467	56,3	±	0,616
MCHC	g/dl	33,8	±	0,291	34,8	±	0,291	33,5	±	0,167	34,2	±	0,133
Platelets	10^3/ul	718,8	±	45,19	739,7	±	24,75	686,6	±	25,45	776,5	±	23,08
WBC	10^3/ul	8,111	±	0,893	5,729	±	0,589	5,278	±	0,597*	5,067	±	0,273*
Basophil	10^3/ul	0	±	0,000	0	±	0	0	±	0	0	±	0
Eosinophil	10^3/ul	0,091	±	0,020	0,025	±	0,010*	0,036	±	0,011*	0,064	±	0,017
Neutrophil	10^3/ul	0,110	±	0,031	0,066	±	0,010	0,072	±	0,020	0,084	±	0,025
Segmented Neutrofil	10^3/ul	4,726	±	0,627	3,758	±	0,775	3,315	±	0,334	3,161	±	0,276
Lymphocyte	10^3/ul	2,35	±	0,42	2,39	±	0,571	1,56	±	0,244	1,39	±	0,072
Monocyte	10^3/ul	0,174	±	0,054	0,105	±	0,052	0,111	±	0,025	0,13	±	0,029

*Significantly different from NC ($p < 0.05$).

Table 2: Effect of combination ASC extract on the blood chemical values of male and female in the subchronic toxicity test.

Blood Chemical Values	Unit	Control			Combination ASC Extract								
					A (1000 mg)			B (575 mg)			C (150 mg)		
Male													
SGPT	U/L	89,4	±	8,393	78,9	±	7,397	108,3	±	4,551*	69,3	±	6,554
SGOT	U/L	116	±	9,540	101	±	6,400	136	±	4,240	100	±	7,320
Cholesterol	mg/dl	76,8	±	5,781	58	±	2,124*	78	±	3,756	81,6	±	2,99
Triglyserilida	mg/dl	98,3	±	3,572	55,6	±	2,32*	81,1	±	1,882*	82,5	±	1,939*
Urea	mg/dl	64,21	±	3,318	62,8	±	2,84	57,49	±	1,263	57,28	±	3,161
Creatinine	mg/dl	0,84	±	0,052	0,73	±	0,039	0,88	±	0,033	0,81	±	0,038
Glukose	mg/dl	88,44	±	3,245	90,9	±	3,420	74,2	±	3,518*	68,8	±	4,292*
Female													
SGPT	U/L	74,3	±	3,739	84,38	±	4,637	88,6	±	4,308	69,3	±	5,669
SGOT	U/L	108	±	5,86	135	±	6,84*	121	±	8,26	109	±	4,73
Cholesterol	mg/dl	66,3	±	2,696	82,2	±	4,117*	88,78	±	2,592*	102,3	±	5,26*
Triglycerides	mg/dl	85,9	±	5,05	81	±	2,31	89,3	±	3,16	93,4	±	3,24
Urea	mg/dl	64,57	±	1,686	67,54	±	3,379	50,04	±	1,456*	61,48	±	3,948
Creatinine	mg/dl	0,87	±	0,021	0,69	±	0,023*	0,8	±	0,037	0,71	±	0,028*
Glukose	mg/dl	96	±	2,418	85,9	±	1,888	80,4	±	3,142*	88,9	±	4,857

*Significantly different from NC ($p < 0.05$).

Table 3: Effect of combination ASC extract on the organ weight of male and female in the subchronic toxicity test.

Organ Values	Unit	Control			Combination ASC Extract								
					A (1000 mg)			B (575 mg)			C (150 mg)		
Male													
Heart	%	0,299	±	0,013	0,264	±	0,006*	0,280	±	0,003	0,276	±	0,007
Pulmo	%	1,05	±	0,094	0,516	±	0,025*	0,525	±	0,016*	0,643	±	0,038*
Liver	%	3,46	±	0,306	2,98	±	0,052	3,11	±	0,204	3,1	±	0,119
Pancreas	%	0,159	±	0,014	0,237	±	0,021*	0,234	±	0,010*	0,25	±	0,011*
Kidney Right	%	0,343	±	0,013	0,333	±	0,005	0,346	±	0,017	0,332	±	0,008
Kidney Left	%	0,347	±	0,011	0,327	±	0,006	0,335	±	0,010	0,337	±	0,006
Spleen	%	0,200	±	0,009	0,241	±	0,019	0,206	±	0,010	0,211	±	0,008
Female													
Heart	%	0,339	±	0,010	0,311	±	0,005*	0,290	±	0,004*	0,306	±	0,005*
Pulmo	%	1,039	±	0,110	0,679	±	0,033*	0,593	±	0,018*	0,703	±	0,033*
Liver	%	3,523	±	0,121	3,061	±	0,047*	3,175	±	0,077*	3,206	±	0,088*
Pancreas	%	0,271	±	0,020	0,326	±	0,008	0,249	±	0,022	0,340	±	0,017*
Kidney Right	%	0,365	±	0,014	0,332	±	0,011	0,348	±	0,008	0,349	±	0,009
Kidney Left	%	0,362	±	0,011	0,328	±	0,008*	0,338	±	0,007	0,337	±	0,005
Spleen	%	0,275	±	0,027	0,28	±	0,011	0,245	±	0,007	0,267	±	0,017

*Significantly different from NC ($p < 0.05$).

calculates results of hematology values show that several parameters are significantly different from normal controls, but the values are not far away and are still within the normal range.[13] So this result shows that ASC extract has no hematological and immunological effects.

The changes in blood biochemical parameters were used to assess any toxic outcomes on the liver, kidney and pancreas. Organs such as the liver and kidneys play an important role in the poison elimination mechanism.[14] Several parameters were significantly different from normal controls in the study results, but when viewed numerically, they were still within normal limits.[13] Especially when viewed macroscopically and microscopically the organ does not show any abnormalities. These results recommend that ASC extract has no impact on liver, kidney and pancreas features.

Macroscopic and microscopic changes in the organ were aimed to see whether there is organ damage that will later cause toxicity.[15] This study showed no macroscopic or microscopic changes in the internal organs of rats given with the extract, which indicates the absence of toxic effects of the test results.

CONCLUSION

The combination of ASC extract given orally for 135 days to male and female rats did not show any subchronic toxicity. These data confirm that this extract is safe for use by rodents. However, chronic toxicity studies are still needed to prove the safety of the combination ASC extract if taken for a longer period of time. Further research on the toxicity of this extract should be performed in non-rodent or humans to increase safety reliability for future drug development.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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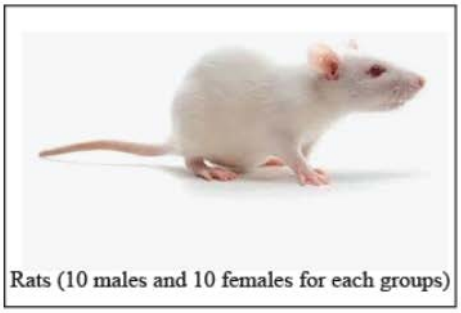
GRAPHICAL ABSTRACT



Rats (10 males and 10 females for each groups)

Acclimatization for 2 weeks

ASC extract given for 135 days

Treatment rats were sacrificed

Blood, heart, lung, liver, kidneys, spleen and pancreas were taken

Group	Sex	Treatment
Control	Male	CMC 0,5%
	Female	CMC 0,5%
A	Male	ASC Extract 1000 mg/ kg BB in CMC 0,5%
	Female	ASC Extract 1000 mg/ kg BB in CMC 0,5%
B	Male	ASC Extract 575 mg/ kg BB in CMC 0,5%
	Female	ASC Extract 575 mg/ kg BB in CMC 0,5%
C	Male	ASC Extract 150 mg/ kg BB in CMC 0,5%
	Female	ASC Extract 150 mg/ kg BB in CMC 0,5%

The combination of ASC extract given orally for 135 days to male and female rats did not show any subchronic toxicity.

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Heri Setiawan received Bachelor of Pharmacy from Universitas Indonesia in 2007, Master of Medical Science and Ph.D.in Medical Science from Okayama University in 2012 and 2016 respectively. He joined Faculty of Pharmacy Universitas Indonesia in 2019 as Lecturer in the field of Pharmacology and Toxicology. He is currently engaged in pharmacodynamics and toxicology study of compound to treat diabetes mellitus and gonadal hormones dysfunction.

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