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The Combination of Belimbing Wuluh Fruit (Averrhoa bilimbi L.) and Leaves of Tapak Dara (Catharanthus roseus G.) from Indonesia as a Candidate Hypoglycemic Agents and Thin Layer Chromatography Profiles

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ABSTRACT

The aim of this study was to evaluate the combination of 70% ethanol extract of Averrhoa bilimbi L. (A bilimbi L.) fruit & Catharanthus roseus G. (C. roseus G) leaves extracts in lowering blood glucose levels. Twenty-five male Wistar rats were divided into 5 groups. All rats were weighed on day 0, then induced by alloxan 150 mg/KgBW (ip). Group I was treated with 0.126 mg/ 200 gBW glibenclamide (po); Group II was treated by CMC Na 2 ml/200 gBW po; Group III; IV; and V were treated by the extract combination of A. *bilimbi* L and C. *roseus* G 40:40; 40:80; and 80:40 mg/200 gBW respectively, for 15 consecutive days (po). The results showed that the combination *A. bilimbi* L and C. *roseus* G doses 40:80 and 80:40 mg/200 gBW had the effect of lowering blood glucose levels in 7 days, but couldn't prevent renal damage by induction of alloxan.

Key words: Belimbing wuluh (*Averrhoa bilimbi* L.), Tapak dara (*Catharanthus roseus* G.), candidate hypoglycemic.

INTRODUCTION

Diabetes mellitus is defined as a group metabolic disease by hyperglycemia, resulting in a defective secretion of insulin, action of insulin, or both[1]. Diabetes is one of the most common endocrine diseases found in Indonesia. By the year 2030, diabetes prevalence is estimated at 8 million Indonesians, the world's fourth largest affected population after India, China, and the U.S.[2]

There are many Indonesian native plants, which are used empirically to treat diabetes. These crops include bawang umbi, bawang prei, bawang pule, bawang putih, tapakdara, dandanggula, jentik manis, salam, mindi, cincin hitam, bidara upas, pare, mengkudu, lampes, kumis kucing, petai kulit, ceplukan, jengkol, urat, bidara laut, mahoni, duwet, brotowali, seledri, and jambu biji.[3]

Averrhoa bilimbi L. (A. *bilimbi* L.), commonly known as belimbing wuluh in Indonesia, belongs to the oxalidaceae family.[4] The various extracts of fruit and leaves have many uses, including as antidiabetics[5], hepatoprotective[6] and as an antithrombotic and antioxidant.[7] *Catharanthus roseus* G.(C. *roseus* G) belongs to the Apocynaceae family.[8] Many researchers state that C. *roseus* G is effective for treatment as antidiabetics[9,10,11,12,13,14] anticancer[,15,16,17], antispermatogenic[18], antihy pertensive[19], and antifungal[,20,21,22].

In our previous study, A. *bilimbi* L and leaves of Tapak dara were effective in lowering the

blood glucose level in male rats induced by alloxan. The 70% of ethanolic extract of belimbing wuluh fruit, with doses of 20 mg/200 gBW , 40 mg/200 gBW, and 80 mg/200 gBW were able to lower blood glucose levels by a percentage of reduction of blood glucose level of 42.7, 43.3 and 58.95%, respectively. The 70 % ethanolic extract of tapak dara leaves (*C. roseus* G.), dose of 20 mg/200 gBW, 40 mg/200 gBW, 80 mg/200 g BW were able to lower blood glucose levels by 43.56%, 53.7%, and 58.8% respectively.[5].

Based on the potential hypoglycemic effects of the plant, researchers tested the combination of both extracts in lowering blood glucose levels on male rats that were induced by alloxan. This method refers to previous studies by other researchers.[23,24,25,26,27,28,29,30,31]

MATERIALS AND METHODS

Plant Materials

A.*bilimbi* L fruit and tapak dara extract were collected from Boyolali, central Java, Indonesia, and stored in a laboratory of pharmacology of Faculty of Medicine at Muhammadiyah University of Surakarta (March 2013).

Experimental animal

Healthy Wistar male rats aged 2-3 months, weighing 150-250 g. All rats were housed in aluminum cages, placed in a room with a temperature of 28-30,5^{0}C and fairly light. The animals were acclimatized for 10 days before the experiment. The study was approved by Ethics Committee, Moewardi Hospital of Surakarta with no 35/1/HREC/2014.

Preparation of extract

Ethanolic extract of *A. bilimbi* L. fruit & *Catharanthus roseus* G. leaves were prepared by cold maceration. Then extracts were dried with a rotary evaporator.

Induction of Diabetes

After overnight fasting, all rats were made diabetic by an injection of fresh solution of 150 mg per kg of alloxan monohydrate intra peritoneal. Blood glucose levels were measured on days 0, 5, 7, 9, 13, and 19.

Experimental Design

Rats were randomly placed into 5 groups, with each group consisting of 5 white rats that consisted of a negative control group (-) and a positive control group (+). Groups III, IV, and V were given a combination of extract I, II, and III, respectively. All groups were induced by alloxan 150 mg / kg BW given intraperitoneal (IP). This dose was conducted by other researchers (Dhanabal *et al*., 2008; Kumar *et al*., 2010 & Viswanathaswamy *et al*., 2011). All rats were fed pellets at 20 g/day. On day 5, the blood glucose levels of all rats were measured. On days 5 through 19, the negative control group (-) was treated by aquadest, and the positive control group (+) was treated by glibenclamide 0.126 mg/200g BW peroral.

Group III was treated with a combination of ethanolic extract of *A. bilimbi* L. fruit 40 mg/200 gBW and *C. roseus* G. leaves 40 mg/200 g BW. Group IV was treated with a combination of ethanolic extract of A bilimbi L. fruit 40 mg/200 gBW and C. roseus G. leaves at 80 mg/200 g BW. Group V was treated with a combination of ethanolic extract of *A. bilimbi* L. fruit at 80 mg/200 gBW and *C. roseus* G. leaves at 40 mg/200 g BW. All treatments were done for 15 consecutive days. All groups were given 40 % of glucose solution every day. Blood glucose levels were measured on days 0, 5, 7, 9, 13, and 19. Blood Urea Nitrogen and creatinine levels were measured on days 0 and 19.

Method of Blood Collection

A total of 0.5 ml blood samples were taken from the rats' tail vein. It was collected in Eppendorf. Blood glucose, Blood Urea Nitrogen and creatinine were estimated by DiaSys kit reagent.

Method of Thin Layer Chromatography

Analysis of chemical constituents in the ethanolic extract of A. bilimbi L fruit and C. roseus G were conducted using thin-layer chromatography. TLC for C. roseus G was done by silica gel GF_{254} plate and mobile phase chloroform:Methanol (9.5: 0.5), was then examined by UV_{254} and $UV_{366.}$ TLC for A. bilimbi L was done using silica gel GF254

plate and mobile phase toluene: ethyl acetate: methanol: glacial acetic acid (7.5: 1.5: 0.8: 0.2) respectively, then was examined by UV_{254} and UV_{366}.

Statistical analysis

The values of non-specific parameters extract are expressed as percentages. Blood glucose level, Blood Urea Nitrogen and creatinine are expressed as mean±SD and were analyzed using one way anova followed by LSD test with p.0.05 significance.

RESULT

The Effect of Lowering Blood Glucose Levels

Measurement of blood glucose levels was done on days 0, 5, 7, 9, 13, and 19, after being induced by alloxan 150 mg/kgBW. Blood glucose measurement results are shown in Table 1.

Table 1 indicates that the induction of alloxan 150 mg/kgBW intraperitoneal could increase in blood glucose levels on day 5. Table 3 illustrates that administration of a combination of Blimbing wuluh fruit and tapak dara extract could lower blood glucose levels starting on day 2 until day 15 after treatment of extract.

The statistical analysis by one-way ANOVA showed that the positive control (glibenclamide 0.126 mg/200 gBW) were able to lower blood glucose levels, a result significantly different from the negative control both on the second day until after 15 days of treatment, which would indicate that method of test is valid. After two days of extract, the ANOVA test showed that the second and third doses of the combination decreased blood glucose levels significantly, being 4 days to 15 days after administration of the extract either a combination

Table 1. Mean and standard deviation of blood glucose levels before and after treatment (n=5)

Groups	Blood Glucose (mg/dl)					
	Day 0	Day 5	Day 7	Day 9	Day 13	Day 19
Positive control	84.25 ±8.0	199 ±24.5	180.5 ±49.4	158.3 ±35.6	124.8 ±26.3	139.5 ±14.2
Negative control	79 ±3.2	181.7±55.3	203.75±23.8	237.75±32.4	189.25±27.4	221.25±21.5
Combination I	87 ±10.7	221 ±45.3	193.2 ±72.3	134.6 ±61.9	111.6 ±48.7	77.6 ±10.9
Combination II	97.2 ±11.9	262.2±35	194.8 ±74.2	186.6 ±79.6	153.8 ±67.9	67.2 ±13.4
Combination III	77.6 ±21.1	278.6±10.6	212.2 ±74.5	204.8 ±70	151.2 ±66.0	162.8 ±39.2

Positive control: glibenclamide 0.126 mg/200 gBW

Negative control: CMCNa 2 ml/200 gBW

Combination I: Rats treated by combination Blimbing wuluh 40 mg/200 gBW and tapak dara 40 mg/200 gBW

Combination II: Rats treated by combination Blimbing wuluh 40 mg/200 gBW and tapak dara 80 mg/200 gBW

Combination III: Rats treated by combination Blimbing wuluh 80 mg/200 gBW and tapak dara 40 mg/200 gBW

Table 2. Mean and standard deviation of Blood Urea Nitrogen (BUN) levels before and after treatment (n=5)

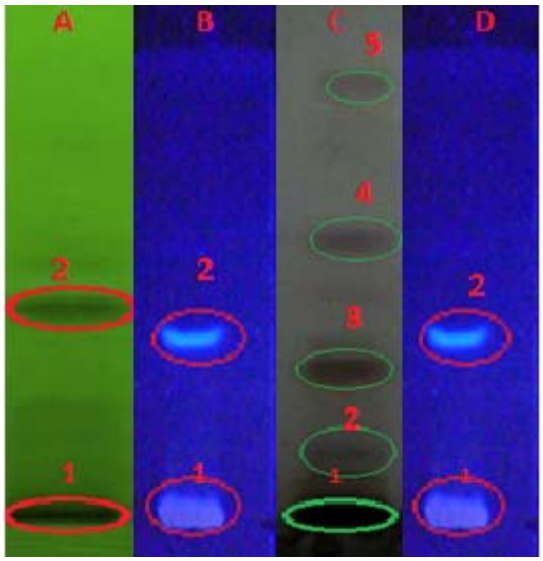
Groups	BUN (mg/dl)	
	Day 0	Day 19
Positive control	21.13±1.3	37.94 ±7.6
Negative control	22.88±2.4	32.57±5.5
Combination I	21.16±2.0	38.94±14.5
Combination II	22.51±2.2	28.30±12.2
Combination III	24.00±3.3	47.54±13.9

Table 3. Mean and standard deviation of serum creatinine levels before and after treatment (n=5)

Groups	Serum Creatinine (mg/dl)	
	Day 0	Day 19
Positive control	0.60±0.08	2.75±0.6
Negative control	0.63±0.05	2.90±0.6
Combination I	0.46±0.09	3.20±0.6
Combination II	0.50±0.01	3.00±0.5
Combination III	0.58±0.08	3.30±0.3

of I, II and III could lower blood glucose levels significantly ($p < 0.05$). A single dose of alloxan was able to increase the levels of BUN and creatinine, indicating that alloxan is nephrotoxic . BUN and creatinine data are shown in Table 2 and 3.

Tables 2 and 3 illustrate that an increase in BUN and creatinine were significant. At day 19 compared to BUN and creatinine baseline on day 0, they had increased approximately 5 times. In the ANOVA test, it was found that there were no significant differences in BUN and creatinine levels on day 19 among groups ($p > 0.05$). This means that both glibenclamide and combination extract I, II, and III were not able to prevent kidney damage caused by alloxan administration.



(A) The appearance of under UV_{254}dan (B) appearance under UV_{366} (C) derivatization with vanilin H_2SO_4 (D) derivatization with sitroborat

Fig. 1. TLC profile of ethanol extract of the Blimbing wuluh fruit (Averrhoa bilimbi L.)

Thin Layer Chromatography Profiles

On examination of the chemical constituents, test data includes the following results:

Ethanolic extracts of *A. bilimbi* L.

On examination TLC with silica gel GF_{254} plate and mobile phase toluene: ethyl acetate: methanol: glacial acetic acid (7.5 : 1.5 : 0.8 : 0.2) obtained the following results:

From Figure 1, several compounds may be identified and are listed in Table 4.

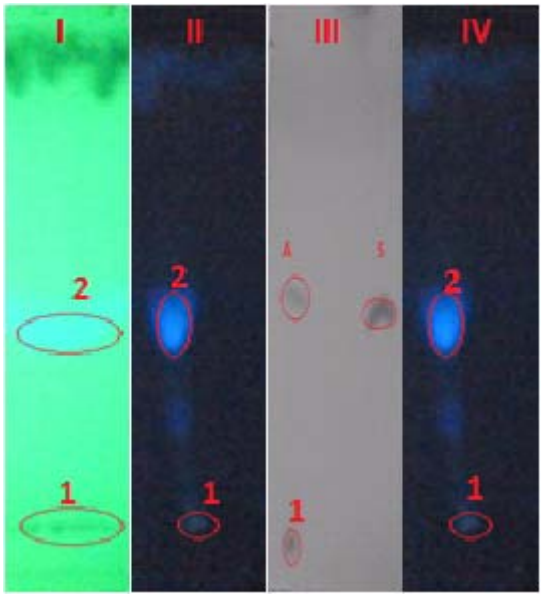
Ethanolic extract of *C. roseus* G

TLC examination results with silica gel GF_{254} plate and chloroform mobile phase: methanol (9.5 : 0.5), (I) appearance under UV_{254} and (II) under UV_{366} appearances (III) derivatization with vanilin H_2SO_4 (IV) derivatization with sitroborat obtained the results shown in Figure 2.

From Figure 2, several compounds may be identified and are listed in Table 5.

Table 4. Separation of ethanolic extract of Averrhoa bilimbi L fruit

Detection	No	hRf	Description of colour	Chemical compound
UV_{254}	1	0	Strong quenching	
	2	50	Strong quenching	
UV_{366}	1	0	Yellow fluorescence	Flavonoid
	2	47.5	Yellow fluorescence	Flavonoid
Vanilin H_2SO_4	1	0	Purple black	Terpenoid
	2	20	Purple black	Terpenoid
	3	45	Purple black	Terpenoid
	4	70	Purple black	Terpenoid
	5	95	Purple black	
Sitroborat		0	Yellow fluorescence	Flavonoid
		47.5	Yellow fluorescence	Flavonoid



A= *Catharanthus roseus* G sample, S = *ursolic acid standart* (hRf= 55)

Fig. 2. TLC profile of ethanolic extract *Catharanthus roseus* G leaves

DISCUSSION

Indonesia is unusually rich in medicinal plants. Among these medicinal plants, *A. bilimbi* L. fruit and *C. roseus* G. are often used for traditional medicinal purposes. In this study, the combination *A. bilimbi* L and *C. roseus* G dose IV (40:80 mg/200 g BW) and group V (80:40 mg/200 g BW) had the effect of lowering blood glucose, but were unable to prevent increasing BUN and creatinine levels in male rats induced by alloxan.

In previous studies, ethanol extract of Blimbing wuluh have had a hypoglycemic effect, anti-lipid peroxidative, antiatherogenic, and antihypertriglyceridemia in rats induced by streptozotosin. [32,33] The data indicates that ethanol extract dosage of 125 mg/kgBW was able to decrease blood glucose and triglyceride levels by 30-50%, and increases HDL by 60% compared to aquadest control.

Table 5. Separation of ethanolic extract C. roseus G

Detection	No	hRf	Description of colour	Chemical compound
UV 254	1	0	Weak quenching	
	2	50	Blue	
UV366	1	0	Blue fluorescence	Flavonoid
	2	50	Blue fluorescence	Flavonoid
Vanilin H^2SO^4	1	0	Purple black	Terpenoid
	2	55	Purple	Ursolic acid
Sitroborat	1	0	Yellow fluorescence	Flavonoid
	2	50	Yellow fluorescence	Flavonoid

The effect of lowering blood glucose levels in this study is consistent with the previous study. Treatment with the water fraction of ethanol extract dosage of 125 mg/kgBW significantly decreased blood glucose and trigliserida in rats induced by streptozotosin with a high fat diet. [34] Treatment by hydroalcoholic extract of leaves of *Averrhoa carambola* L. could lower blood glucose level on male Wistar rats by p 0.05.[35]

The addition of C. roseus G. can decrease blood glucose levels more than dosages without tapak dara. The combination A. *bilimbi* L. fruit 40 mg/200 gBW and C. roseus G. leaves 80 mg/200 gBW proved more effective in lowering blood glucose than the others. This research is consistent with research Som Nath Singh *et al*. performed, which stated that the dichloromethane extract of ethanol (1:1) 500 mg/kgBW dose is capable of lowering blood glucose in rats induced streptozotosin for 7 and 15 days with a 48.6% and 57.6% reduction.[36]

The study was also consistent with studies from Benjamin *et al*. (2004), stating that the water extract of dried leaves Tapak Dara was able to lower blood glucose levels by 60% .[37] The extract of dichloromethane and methanol (1:1) extracts (500 mg/body weight) for 20 days of leaf Catharanthus roseus decreased blood glucose levels on alloxan induced diabetic rats.[38]

In this study, ethanolic extract of A.*bilimbi* L. fruit contained flavonoids and terpenoids. This is consistent with other researchers who stated that A.*bilimbi* L. fruit extract contains flavonoids, saponins and terpenoids.[38,39] The chemical content of A.*bilimbi* L. reported amino acids, citric acid, cyanidin-3-O-â-D-glucoside, phenolics, potassium, and vitamin A. [40] The constituents of C. roseus G. leaves from this research are flavonoid, terpenoid, and ursolic acid. The study by Fereres at al. reported that besides alkaloids, other compounds of C. roseus are flavonoids and anthocyanins. Fifteen flavonol glycosides were identified from seeds, stems, leaves and flowers of C. roseus.[41]The study by Inte *et al*. (1998) notes that the chloroform extract of dried leaves of catharantus roseus contains ursolic acid, vindoline, oleanolic acid, and squalene.[42]

CONCLUSION

The combination of ethanolic extract of belimbing wuluh (*A. bilimbi* L.) fruit and Tapak dara (*C. roseus* G) leaves with dosages of 40 : 80 mg/ 200 gBW and 80 : 40 mg/200 gBW are effective for lowering blood glucose levels on day 7. On days 9, 13, and 19, combining dosages of 40:40 mg/200 gBW; 40:80 mg/200 gBW; and 80:40 mg/200 gBW are effective in lowering blood glucose ($p < 0.05$). All rats, BUN and creatinine levels increased on day 19. There is no significance difference in BUN and creatinine levels among treatment groups. Combinations of extract were not able to prevent kidney damage which was induced by alloxan ($p > 0.05$).

The chemical composition of 70% ethanolic extract Blimbing wuluh (*A. bilimbi* L.) are flavonoids and terpenoids. The chemical composition of 70% ethanolic extract of leaves of Tapak dara (*C. roseus* G) are ursolic acid, flavonoids, and terpenoids.

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