Research Article

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IN VIVO ANTI-HYPERCHOLESTEROLEMIA EFFECT OF INDONESIAN JAMU FORMULA

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ABSTRACT

Indonesia has many Jamu formulas to threat some diseases including for hypercholesterolemia. Aim of this research were to standardize and analyze the effectiveness of Jamu Formula as anti-hypercholesterolemia and see its toxicity to liver function. Jamu Formula in this research consisted of *Syzygium polyanthum*, *Casia alata*, and *Centella asiatica* leaves which were extracted by ethanol 30%, and *Curcuma xanthorrhiza* rhizome which were extracted by ethanol 70%. This Jamu fomula can inhibit *HMGCoA reductase* activity in previous study. This study was conducted in animals laboratory of Tropical Biopharmaca Research Center, Bogor, Indonesia. *In vivo* analysis using *Sprague dawley* rats fed a high-fat diet for 4 weeks, then treated with Jamu Formula for 2 weeks. Blood collection performed before and after treatment. The results showed that Jamu Formula have a good quality for plant raw material and safe to be used. It had LD_{50} higher than 5000 mg/kg BW. The Jamu formula dose 360 mg/kg BW can decreased cholesterol level and body weight of rats about1,5 and 2,5 times relative to negative control group. Total cholesterol and LDL level of Jamu formula dose 360 mg/kg BW can decrease to 92.4 ± 5.0 and 25.8 ± 8.6 mg/dL which was not significantly different with normal and simvastatin group. In conclusion, Jamu formula dose 360 mg/kg BW have anti-hypercholesterolemia effect as good as simvastatin group.

Keywords: Hypercholesterolemia, Jamu formula, Toxicity, *in vivo* analysis

INTRODUCTION

Since 20 years, Indonesia has undergone a transition state of health and nutrition. Increased nutritional conditions resulted in increased risk of hypercholesterolemia[1]. Hypercholesterolemia is a risk factor for heart disease. Coronary heart disease (CHD) is the leading cause of death in comparison to other diseases[2].Hypercholesterolemia and obesity are some of risk factors for cardiovascular disease (CVD)[3]. People with obesity have an accumulation of macrophage in adipose tissue, leading to systemic inflammation, and hypercholesterolemia can cause an endothelial dysfunction of artery wall. All of that conditions are the early stages of atherosclerosis and responsible for the pathophysiological changes of cardiovascular disease[4].

Indonesia has a lot of medicinal plants used traditionally to prevent and cure some disease in the community. A mixture of several medicinal plants that are traditionally consumed in Indonesia is called Jamu. Just as India that has Ayuverda therapy, Indonesia has Jamu as a therapy that is believed since ancient times. Indonesian people among all age and social group have a positive attitudes and perceptions on Jamu[5]. Nowadays, there is a methods that can give a good understanding about relationship between plants, Jamu, and their efficacy[6]. One of Jamu formula is often used to prevent and cure hypercholesterolemia.

This research used the mixture of *Syzygium polyanthum*, *Cassia alata*, and *Centella asiatica* leaves with *Curcuma xanthorhiza* rhizome. Those plants are the popular medicinal plants used in Indonesia.

Based on previous *in vitro* study, the combination of this Jamu Formula gives synergistic effect as an anti-hypercholesterolemia by inhibiting *HMG-CoA reductase* activity about 25% at concentration of 500 ppm[7]. *HMG-CoA reductase* is an enzyme which related to the mevalonic pathway to produce cholesterol and the isoprenoids compounds[8]. By inhibiting the activity of this enzyme, the cholesterol production will be decrease. The Jamu Formula used is prepared for oral formulation.

In vivo assay is needed to determine the efficacy of Jamu formula. The triglycerides, cholesterol, HDL, and LDL levels in serum of animal tested is determine to find the effect of consuming the formula. Besides that, the ALT and AST level in the serum is also determined to find the effect related to the liver function. Aim of this research were to standardize the plants raw material used in Jamu formula, and find the effectiveness of Jamu formula as anti-hypercholesterolemia by *in vivo* analysis.

MATERIALS AND METHODS

Plant Materials and Chemicals

The materials used in this research was *Syzygium polyanthum* leaves, *Cassia alata* leaves, *Centella asiatica* leaves, and *Curcuma xanthoriza* rhizome which were collected from conservation and cultivation Unit of Tropical Biopharmaca

Research Center, Bogor Agricultural University, West Java, Indonesia. The experiment was conducted from April to June 2015. Voucher specimens are deposited in the Herbarium of Tropical Biopharmaca Research Center. The other materials were ethanol (Sigma) for extraction, and ELISA kit for cholesterol, triglycerides, HDL, LDL, AST and ALT test (BioLab).

Formulation and Extraction

Composition of medicinal plants and the doses used in this *in vivo* assay are selected based on the most optimum result from the previous *in vitro*study. This composition was being tested as Jamu formula with two doses (360 and 720 mg/kgBW).All samples were washed in water, cut into small pieces and dried for ± 5 days(moisture < 10%), then ground into powder (size, 100 mesh). The powdered plant materials were extracted with ethanol 30% (for *Syzygium polyanthum*, *Cassia alata* and *Centella asiatica* leaves) and 70% (for *Curcuma xanthoriza* rhizome). The extracts were filtered using filter paper and concentrated in vacuum using a rotary evaporator. Determination of moisture, ash, heavy metals and microbe contaminant of all plant samples were using AOAC methods[9]. Extracts that have been standardized, then tested with *in vivo* assay for toxicity and efficacy.

Phytochemical analysis

Total phenolic content was determined according to the Folin-Ciocalteu method[10], using gallic acid (Wako Pure Chemical Industries, Ltd., Japan) as the standard. The sample were dissolved in 50% (v/v) methanol/water. 500 µL of sample solution was mixed with 500 µL of 1N Folin-Ciocalteu reagent (Wako Pure Chemical Industries, Ltd., Japan).The mixture was allowed to stand for 5 min, then followed by the addition of 1 ml of 20% Na_2CO_3 (Nacalai Tesque Japan). After 10 min of incubation at ambient temperature, the mixture was centrifuge for 8 min at 12000 g, and the absorbance of the supernatant was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligram per gram sample.

Tannin content of samples was determined by a protein precipitation method using Bovine Serum Albumin (BSA) (Wako Pure Chemical Industries, Ltd., Japan). This method was adapted from Kawamoto *et al*.[11] by analyzing the BSA content of the supernatant liquid rather than the BSA content of precipitate. Samples were diluted in 50% (v/v) ethanol/water to get a concentration of 2 mg/ml. 200 µL volume of sample solution was added to 200 µL of BSA solution (10 mg/ml, dissolved with 0,1 M acetate buffer, pH 5). After reacting at room temperature for 1 hr, the solution was centrifuged at 13000 g for 2 min. The remaining BSA in the supernatant was determined by HPLC with a reserved phase Develosil 300 C4-HG-5 column (4.6 mm i.d. x 150 mm, Nomura Chemical Co Ltd. Japan) monitored at 280 nm. The solvent system used was as follows: a linear gradient elution for 20 min from 80 to 20% solvent A (0,01% TFA in water) in solvent B (90% (v/v) CH_3CN/water containing 0,01% TFA) at flow rate 1 ml/min. The column temperature was 35°C.

Experimental Animals

This study has been approved by animal ethic committee of Tropical Biopharmaca Research Center, Bogor Agricultural University. Male *Sprague dawley* rats age 8-12 weeks (200 – 250 gram/rat) were placed on the room with temperature of 22°C (±3°C) with humidity of 30-70% and 12 hours light and 12 hours in dark. The rats were acclimatized for one week before the start of the experiment. The rats were treated humanely throughout the study period and were kept in a well-controlled area according to the guideline for use and care of animals[12].

Rats were randomly divided into five groups (n = 5), consist of control and treatment group. Rats in Normal group received only standard diet until the end of the treatment, while other rats received a high-fat diet in 4 weeks. Negative control group is hypercholesterolemia rats with no therapy. Positive Control group get simvastatin orally. Treatment groups were given daily extract of Jamu Formula dose 360 mg/kg BW (dose 1) and dose 720 mg/kg BW (dose 2), orally for 2 weeks. The rats body weight were measured every 5 days. Blood serum were collected and lipid profile (total cholesterol, LDL, HDL, triglycerides) of the rats were measured at the end of treatment.

Collection of Blood Samples

Blood samples were withdrawn from retro-orbital plexus on 0^{th} and 14^{th} day. At the end of the experimental periods, the rats were fasted for 8 hours, and then injected with ketamine and xylazine. After the rats anesthetized, blood samples were collected by cardiac puncture. The collected blood samples were centrifuged at 3500 rpm for 10 minutes and the serum collected for biochemical studies. The rats sacrificed by a blow to the head.

Evaluation of Acute Toxicity

Acute toxicity test was performed according to the Organization of Economic Co-operation and Development (OECD) guideline[13] for testing of chemicals 420 and the World Health Organization (WHO) guideline[14]. The extract was prepared at concentration 2500 mg/ml in distilled water. On the limit test, two rats (one rat per sex) were administrated a single oral dose of 5000 mg/kg BW. Body weight, signs of toxicity and mortality were observed after the administration at the first, second, fourth and sixth hour and once daily for 14 days. If there are rats dead after 24-48 hours from administrated, the treatment continue to the main protocol with reducing the dose. If no rats dead, the same dose is given to the rest rats (4 rats per sex).

Statistical Analysis

The data obtained were analyzed with completely randomized design (CRD) and analysis of variance (ANOVA) at the confidence level of 90%. *P* values < 0,05 indicates that there is a significant difference to the measured response. Further test used Duncan test. All data were analyzed with SAS program.

RESULTS AND DISCUSSION

Content of Raw Materials

Determination of compound extract were to control the quality of raw materials in accordance Indonesian Herbal Pharmacopoeia[15], which is important to standardize the Formula. Based on the standardization result (Table 1) all plant raw materials were safe to be used for Jamu Formula.

Table 1: The quality of plant raw materials

Parameter		*Cassia alata*	*Curcuma xanthoriza*	*Syzygium polyanthum*	*Centella asiatica*
Moisture content	%	11.76	17.44	10.53	8.8
Ash content	%	10.58	5.37	4.65	2.5
Acid unsoluble ash content	%	0.46	0.78	0.34	1.5
Pb	Ppm	Nd	Nd	Nd	Nd
Cd	Ppm	Nd	Nd	Nd	Nd
As	Ppm	Nd	Nd	Nd	Nd
TPC	CFU/g	$1.4x10^4$	$2.6x10^4$	$2.1x10^3$	$2.1x10^2$
Coliform	CFU/g	Negative	$1.6x10^3$	$1.4x10^2$	$1.7x10^3$
Kapang/Khamir	CFU/g	Negative	$2.3x10^3$	$1.4x10^2$	$1.7x10^3$

TPC – Total Plate Count; Ppm - part per million; Nd - Not detected; CFU – Colony Forming Unit

Content of Jamu Formula

All extracts used in this Jamu Formula are consisted of flavonoid, tannin, quinone, steroid, and triterpenoid qualitatively. Based on this information, the flavonoid, tannin, and fiber content are determined in the formula (Table 2).

Table 2: The Standard of Jamu Formula

Standard	Levels in Jamu Formula (%)
Moisture content	6.1
Ash content	2.2
Flavonoid content	1.2
Tannin content	22.8
Fiber content	4.31

Acute Toxicity

Both female and male rats fed with the extract at a dose of 5000 mg/kg BW did not show any signs of toxicity in the entire period of 14 days of observation. Neither body weight nor internal organ weight of treated rats was significantly changed relative to that of the control group. The internal organs of treated rats such as heart, lung, liver, spleen, kidney, pancreas, brain and sex organ showed no pathological abnormality relative to those organs of the Normal group (data not shown).The Jamu formula is not toxic since it has LD_{50} higher than 5000 mg/kg BW. It means the Jamu formula is save to use.

***In Vivo* Analysis**

Rat's body weight in all groups were measured every five days in three weeks. Cholesterol level were measured before and after treatment. The decrease of cholesterol level and body weight in rats were compared to the negative control group (Figure 1). Although statistical analysis show that there is no significant differences between all group, but descriptively the results show that Jamu Fomula dose 1 has better results than dose 2, with cholesterol decreasing 1,5 times relative to negative control group, as good as simvastatin group. Body weight decreasing of Jamu formula dose 1 was 2,5 times relative to negative control.

Analysis of lipid profile in the end of the study showed that total cholesterol in treatment groups were in normal range (40-113 mg/dL). Statistical analysis showed that at the end of treatment, Jamu Formula Dose 1 has Total cholesterol, LDL, and HDL value that is not significantly different from the normal and simvastatin group (Table 3).

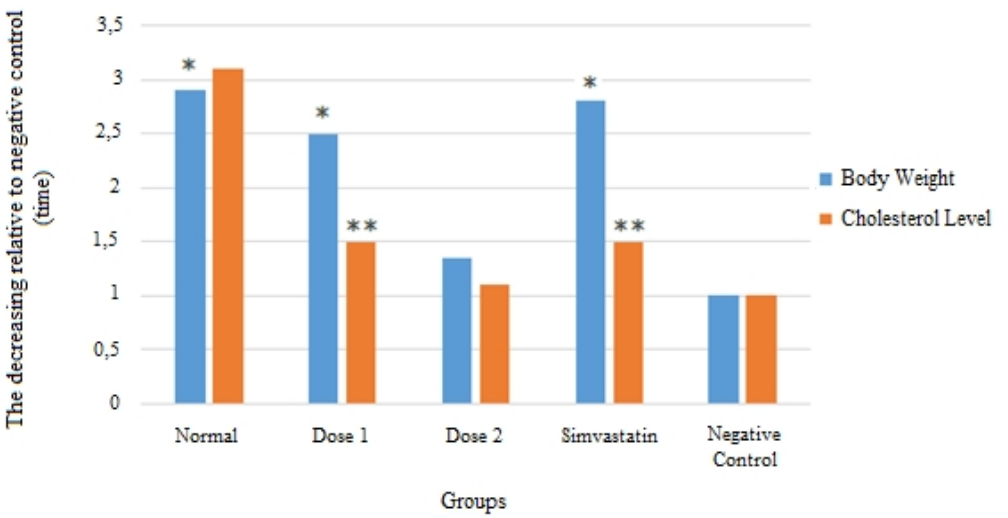

Figure 1: The decreasing of body weight and cholesterol level of all groups compared with negative control. The decrease of body weight in Jamu Formula dose 1 have lower value than Simvastatin group but higher than dose 2 group (*). The decrease of cholesterol level in Jamu Formula dose 1 have a similar value with Simvastatin group (**).

Table 3: Triglycerides, cholesterol, HDL, and LDL level in all groups after treatment

Groups	Triglycerides level (mg/dL)	Total cholesterol level (mg/dL)	HDL level (mg/dL)	LDL level (mg/dL)
Normal	90.8 ± 1.9 [a]	87.4 ± 8.4[a]	47.7 ± 1.6[a]	21.6 ± 4.5 [a]
Dose 1	117.9 ± 37.7 [ab]	92.4 ± 5.0[a]	43.0 ± 6.7[a]	25.8 ± 8.6 [a]
Dose 2	136.4 ± 58.2 [b]	103.8 ± 9.7 [b]	46.0 ± 7.8 [a]	28.9 ± 8.8 [ab]
Simvastatin	105.1 ± 28.4 [ab]	83.6 ± 6.2 [a]	40.7 ± 4.5[a]	21.4 ± 7.4 [a]

Values are expressed as mean ± S.E.M., n = 5. Values in the same column followed by the same letter are not significantly different at the test level 5%

Table 4: ALT and AST level in all groups

Groups	ALT (U/L)	AST (U/L)
Normal	37.0 ± 7.7	58.1 ± 12.0
Dose 1	30.7 ± 4.7	60.9 ± 20.0
Dose 2	33.8 ± 4.9	36.4 ± 21.0
Simvastatin	28.1 ± 3.7	49.7 ± 11.0
Negative Control	32.2 ± 7.5	69.1 ± 15.0

Values are expressed as mean ± S.E.M., n = 5

Effect of Jamu consumption in rats liver function studied by looking the activity of enzyme related to liver function. Based on the result of ALT and AST level in the end of treatment (Table 4) showed that Jamu Formula dose 1 and 2 did not cause disturbances in liver function. All group have normal liver enzyme level (ALT= 17.5 – 40.2 U/L; AST = 45.7 – 80.8 U/L).

DISCUSSION

The leaves of *Syzygium polyanthum,* or a synonym *Eugenia polyantha,* commonly known as 'Daun Salam' in Indonesia, and widely used as spice. In traditional medicine, this plant is used to cure diarrhoea, gastritis, rheumatism and intoxicated due to alcohol[16]. Ethanolic extract of this leaves also have an effect as antioxidant[17,18], antidiabetic[17] and antihypercholesterolemia[19]. *Cassia alata* commonly known as 'Ketepeng' in Indonesia, is traditionally used to cure skin lesion like tinea and ringworm, constipation, and stomatitis[16]. Ethanolic extract of *Cassia alata* leaf has an antipyretic effect which is higher than paracetamol[20]. *Centella asiatica,* known as 'Pegagan', is a herb that has been used for healing wound and leprosy in the folk medicine in Asia. It is also mention in Indonesian Herbal Pharmacopoeia as a treatment for icterus, diarrhea, haemorrhagic disease, and for blood circulation[15]. Many studies have shown that this herb have some effect such as immuno-modulation, antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, anti-fertility, neuro-protective, anti-anxiety, anti-depressant, and antiepileptic activity[21]. *Curcuma xanthorhiza,* also known as Javanese Turmeric or 'Temulawak', is a medicinal plant used for curing hepatitis, inflammation, indigestion, and to increased appetite[16]. This plant was used widely as antioxidant, , not only in Asia but also in Europa[22]. Several studies have shown that *Curcuma* hasan effects as antihyperglycemic,anti-inflammatory[23], antioxidant, hepato-gastroprotector[24,25], andpotential as a phytotherapeutic agent under atherosclerosis and cardiovascular disease[26].

Standardization in herbal medicines is the process of making a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, safety, efficacy and reproducibility[27]. Raw materials forming the anti-cholesterol formula is standardized according to the rules of Indonesia Health Ministry[15] and WHO[14]. The specification of WHO for total aerobic microorganisms and yeasts/moldsare not more than 10^7 CFU/gand 10^4 CFU/g for the plant material for use as teas and infusions and at most 10^5 CFU/g and 10^3CFU/g for internal use. Fungi with a high counts area risk because of the possibility to produce mycotoxin, such as aflatoxin, which is a carcinogenic[28].Limit values for heavy metal contaminant are ≤ 10 ppm for Pb, ≤ 0.3 ppm for Cd, and ≤ 5 ppm for As.

Based on the results, showing that the raw materials for Jamu formula are safe to be used because it does not contain heavy metals Pb, Cd and As. It also has a moisture and ash content within normal level and the microbial contaminants are still below the limits.

Quality of Jamu Formula is determined by the levels of flavonoids, tannins and crude fiber[29]. The group of flavonoid compounds have long been used to treat various diseases, especially as an antioxidant, which is one approach to overcoming the problem of hypercholesterolemia[30]. Tannins are a group of compounds that have the potential as a hypolipidemic[31]. Meanwhile, crude fiber has long been known to decrease the absorption of fat in the intestine[32].

In the end of treatment, both of Jamu dose 360 and 720 mg/kg BW showed the decrease of total cholesterol, but the best result was in Jamu formula dose 360 mg/kg BW. The results descriptively shown that the reduction in cholesterol levels of Jamu Formula dose 360 mg/kg BW have the same values with the positive control group (simvastatin), which is 1.5 times relative to the negative control group. Total cholesterol and LDL level of Jamu formula dose 360 mg/kg BW can decrease to 92.4 ± 5.0 and 25.8 ± 8.6 mg/dL which was not significantly different with normal and simvastatin group. It means this Jamu has anti-hypercholesterolemia effect as good as simvastatin group.

In acute toxicity study, after female and male rats were treated with the Jamu formula at dose of 5000 mg/kg BW, the results did not show any toxicity signs. There were no significant differences in body weight, internal organ weight, and general behaviors. These results indicate no toxicity effect of the substance due to no changes in such parameters. Furthermore, blood chemical examination was performed in order to evaluate any toxic effects on liver. In this study, the levels of these blood chemical values were minor changes and remained within thenormal range[33].The results suggest that the Jamu formula is not toxic after an acute exposure in rats.

CONCLUSION

The Jamu Formula is not toxic (LD_{50} higher than 5000 mg/kg BW) and do not cause disturbances in liver function due to the levels of AST/ALT are still in the normal range. Jamu Formula dose 360 mg/kg BW can decrease cholesterol level and the body weight of rats about 1,5 and 2,5 times relative to negative control group. It has anti-hypercholesterolemia effect as good as simvastatin group.

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