International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.11 No.01, pp 64-74, 2018

The combination of antiretroviral and *Phyllanthus niruri* extract is more effective to increase CD4 cells count on HIV patients: a pilot study

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Abstract : Some in vitro researches reported that application of *Phyllanthus niruri* extract exhibited HIV reverse transcriptase enzyme inhibitor and immunostimulator effects. The immunostimulator activity, especially the effect on absolute CD4 cells count was determined in HIV patients from a teaching hospital in Surabaya, Indonesia and a teaching hospital in Sidoarjo, East Java, Indonesia. In a quasi-experimental trial with a pretest-posttest control group design, a combination of antiretroviral therapy (ART) and capsules of *Phyllanthus niruri* extract (contained 50 mg dried powdered extracts of *P. niruri*) were administered to 15 HIV patients in the intervention group, while ART only was administered to 13 HIV patients in the control group. After six months of therapy, results from paired t-test revealed the treatment group achieved higher absolute CD4 cell count than the control group ($p<0.001$). A multivariate analysis with the generalized linear models (GLMs) showed that being in the intervention group significantly increased the probability of having higher absolute CD4 cells count by 62.365 cells/μL after six months of therapy (95% CI, 35.732 - 88.998) compared to the absolute CD4 cells count of study participants in the control group, adjusted for the absolute CD4 cells count before therapy, age, stage of AIDS and adherence to therapy.
Keywords : HIV, *Phyllanthus niruri* extract, antiretroviral therapy, absolute CD4 cells count.

Introduction

HIV infection and AIDS are a global problem. Indonesia was one of five countries in the South-East Asia Region, which accounts for the majority of HIV burden [1]. The HIV-positive adult prevalence in Indonesia is stable at 0.3% regionally, but sub regional epidemic is still rising. From 1987 until the end of December 2015, there were approximately 191,073 reported HIV infection cases that were spreading over 407 (80%) of 507 districts/towns in Indonesia. Around 66.7% of HIV infection in Indonesia was transmitted by heterosexual contact, 11.4% by sharing contaminated needles, 2.9% by homosexual contact, 0.3% by perinatal infection, and the rest by other means of infection. Majority of HIV-positive individuals were males (55%), but the percentage

of HIV-positive housewives was increasing [2].

From 1987 to December 2015 there were around 77,112 reported AIDS cases in Indonesia. AIDS mostly affected those aged 20-29 years (31.8%), 30-39 years (29.9%), and 40-49 years (12.1%). The national AIDS case rate / 100,000 populations in Indonesia was 26.4, and the Case Fatality Rate (CFR) of AIDS decreased from 2.8% in 2013 to 0.95% in 2015. Five provinces with the highest AIDS case rate were Papua (416.7), West Papua (216.5), Bali (140.4), Jakarta Capital Region (65.2), and West Kalimantan (45.9) [2].

Up to the present time, the management of patients with HIV and AIDS in Indonesia is concentrated in clinical therapy by using a combination of antiretroviral therapy (ART) to optimize efficacy and reduce the likelihood of developing a drug resistance. Of 2015, the free ART coverage in Indonesia reached 81.2% of the eligible HIV-positive individuals, around half of them (52.3%) were still receiving ART while the rests were lost to follow up (20.5%), died (16.3%), moved to other health facilities (8.9%) or stopped taking ART (2.1%). Approximately 75.6% of those taking ART were in their original first line therapy, consisted of two nucleoside reverse-transcriptase inhibitors (NRTI) and one non-nucleoside reverse-transcriptase inhibitors (NNRTI). Around 21.2% were also in the first line therapy, but one antiretroviral had been substituted with other antiretroviral; and 3.3% were in the second line therapy [2].

Despite the beneficial effects of ART in reducing the mortality [3,4] and improving the quality of life of HIV and AIDS patients [5,6], the ART effectiveness could decrease due to poor adherence to therapy [7,8], the development of virus resistance [7,9,10], and high risk sexual behavior [11,12]. Further, a substantial percentage of HIV-positive patients reported that they refused or stopped taking ART related to its adverse reaction and toxicity [13–15]; local cultural frameworks, transport cost, logistical challenges and stigma [16,17].

A research conducted in 2010-2011 in five African countries revealed an average cost of care provided to HIV-positive patients receiving ART was $208 per patient-year (ppy) across Ethiopia, Malawi, Rwanda and Zambia, and $682 ppy in South Africa. The cost included medications, laboratory services, direct and indirect personnel, patient support, equipment and administrative services [18]. An amount of cost around $229 to $287 ppy was reported in Kenya [19].

There has not been any published research on ART cost carried out in Indonesia, however providing free ART during lifetime of each HIV-infected individual requires a huge amount of funding. In addition, large sections of population especially in developing countries still rely on a broader, safer and also cheaper repertoire of herbal medicine [20].

Since the ancient past, the Indonesian traditional herbal medicines, or *jamus*, have been known and practiced in the Indonesian community to maintain good health and to treat diseases [21]. However, the earliest known documentation of their use was dated centuries ago around A.D. 772 related to an archaeological finding from the Borobudur Temple in Central Java, which contained a carving of medicinal plants. Similar documentations had been found in reliefs from Prambanan, Penataran and Tegalwangi temples [22]. Written information on herbal medicines were also found in Lontar leaves (leaf from a kind of palm tree), as well as three books on the art of traditional healing, which are kept in the palace library of Yogyakarta and Surakarta. These are: *Usada* (Book of Healing), *Serat Kawruh bab Jampi-jampi* (A Treatise on All Manner of Cures), and *Serat Centhini* (Book of Centhini) [23].

The abundant rain forests of Java and outside Java provided a great range of were medicinal plants, including *Lengkuas*, the rhizome of *Languas galanga* (L) Merr. (for treating ringworm); *Kumiskucing*, the leaf of *Orthosiphon stamineus* Benth. (as a diuretic); *Tempuyung*, the leaf of Sonchus arvensis L. (for treating urolithiasis); *Karamunting*, the fruit and leaf of *Rhodomyrtus tomentosa* (Aiton) Hassk. (for treating diabetic ulcer); and Meniran, the leaf of *Phyllanthus niruri* (for treating kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B) [22,24,25].

Of many *Phyllanthus* species from Euphorbiaceae family that are widely distributed in most tropical countries, only a few have been studied and indicated to possess potential benefits in clinical practice [25]. The first preclinical study of *Phyllanthus niruri* as an immunostimulator in mice was carried on by Ma'at [26]. In his dissertation, Ma'at demonstrated that *Phyllanthus niruri* enhanced the activities of both humoral and cellular immunities. Such immunostimulator effect of the herb was further evaluated in several clinical trials involving patients from several hospitals in Indonesia with pulmonary tuberculosis [27,28]; vaginal candidiasis [29]; varicella [30];

and tonsilopharyngitis [31].

Scientific studies have showed that extracts and purified isolated compounds of *Phyllanthus niruri* possessed antiviral effects against herpes simplex virus (HSV), dengue virus (DENV), hepatitis B and HIV reverse transcriptase enzyme [32,33]. In addition, Phyllanthus niruri exhibited immunostimulator[34,35], and hepatoprotector [36–38] effects. Therefore, this study aimed to determine the effectiveness of a combination between the first line of ART and *Phyllanthus niruri* extract, compared to ART only in HIV patients.

Materials and Methods

This pilot study used a quasi-experimental design on 28 stage 2 and 3 treatment naïve HIV patients at Dr. Ramelan Naval Hospital, Surabaya and Sidoarjo General Hospital, Sidoarjo, East Java Province, Indonesia. The clinical stage of AIDS was based on WHO guideline [39,40]. The original sample size consisted of 32-treatment naïve HIV patients. The calculation was based on the sample size estimation for clinical trial with 10% estimation of drop out rate [41]. However, one of participants from stage 4 of AIDS in the intervention group and one in the control group had died in the first week of the study, before they were given any ART or *Phyllanthus niruri* extract. Two of study participants in the control group were lost to follow up in the first month of study, since they had moved to other cities and they could not be reached. Therefore, the analysis consisted of 15 participants in the intervention group and 13 participants in the control group. The study was carried out from September 2013 to March 2015.

The inclusion criteria for participants were: treatment naïve AIDS patients aged 17 years and over, willing to participate voluntarily in the study by signing informed consent, and eligible for the first-line antiretroviral therapy in accordance with the national guidelines for the clinical management of HIV infection and antiretroviral therapy in adults [40]. The exclusion criteria were: having symptoms of cardiac failure, symptoms of mental disorders or malignancy. Participants were dropped out from the study if ART was discontinued for more than a week due to any cause, died, could not be reached, or requested to withdraw from the study.

It was difficult to find any treatment naïve AIDS patients in the teaching hospitals that met the inclusion criteria. Majority of AIDS patients in the hospitals had already taken antiretroviral therapy. If there were any treatment naïve AIDS patients, usually they were on the late stage of AIDS related to their denial or refusal to be taken to the hospital. Furthermore, the patients came to the hospitals one by one for several months. Those who had been diagnosed with HIV should have clinical and laboratory examination immediately and then should be provided with a prompt therapy according to the guideline. Therefore, random assignment of participants was not possible to be conducted. The participants were assigned into intervention or control groups by consecutive sampling. The research ethics approvals were acquired from the Human Research Ethics Committee at Hang Tuah University (No. 04/M/DU/KEPUHT/IX/2013) and Dr. Ramelan Naval Hospital, Surabaya (No. 65/EC/KERS/2013).

The purpose, procedure and conditions of the study were explained beforehand to the patients who met the inclusion criteria. All study participants agreed not to take any vitamin or other herbal medicine during the study period. A written consent was obtained from each patient who agreed to participate voluntarily in the study.

All participants underwent clinical examinations, complete blood count, chest X-ray, sputum test, liver function test (SGOT and SGPT), renal function test, CD4 cell count, body weight and height measurement. The clinical examination was conducted by the internists and pulmonologists at the Care, Support and Treatment (CST) clinics of both teaching hospitals. The absolute CD4 cells count and CD4 percentage were measured with immunofluorescence analysis using flow-cytometry [42] using BD FACS Calibur™ (Fluorescence Activated Cell Structure) count. The liver function tests were carried out according to the International Federation of Clinical Chesmistry (IFCC) recommendation using Dimension® RxL® Max, Siemens. All laboratory tests were performed at the Clinical Pathology Laboratory, Dr. Soetomo General Hospital, Surabaya. The demographic characteristics and adherence to therapy were evaluated using questionnaire.

Co-trimoxazole was provided to all participants for two weeks prior to the start of ART to examine the patients' adherence to medication, and as the primary prophylaxis against toxoplasmosis and pneumocystis

jirovecii pneumonia, in accordance with the WHO recommendation [40,43].

The intervention group was provided with a combination of the first-line ART and capsules of *Phyllanthus niruri* extract for six months, while the control group was provided with the first-line ART for six months. The intervention group took two capsules of *Phyllanthus niruri* extract (each contained 50 mg extract of *Phyllanthus niruri*) three times a day, 15 – 30 minutes after meal, before taking ART. The capsules of *Phyllanthus niruri* extract were produced by "PJ. Tradimun", a small industry of traditional medicine in Gresik Regency, East Java Province, Indonesia, batch number 170613, the National Agency of Drug and Food Control (NA-DFC) registration number (POM TR) 063 358 851, expired date on June 2017, and were available as an over the counter medicine under the name "Phyllanthus". Each bottle of Phyllanthus consisted of 100 capsules of *Phyllanthus niruri* extract.

Figure 1. Phyllanthus niruri capsule

The control group took combination of the first-line ART and two capsules of placebo contained 50 mg of *Amylum manihot* three times a day. However, the placebo was given only for a week. The *Phyllanthus niruri* extract and placebo capsules were produced by the same company and provided in the same weight, shell color, and bottle.

The stage of AIDS was categorized into stage 2 and stage 3. The adherence to therapy was categorized into <95% and ≥95%. The key population was categorized into no, female sex worker (FSW), high risk men (HRM), man who have sex with man (MSM), and transvestites. The HRM, MSM and transvestites were all males. The education was categorized into elementary school, junior high school, senior high school, and university/college.

There were several different statistical analyses used in this study, depending on the results of the normality and homogeneity of variance tests. If the data were not met the criteria for parametric tests, then the data were analyzed using non-parametric tests.

The statistical analyses were performed using bivariate analyses (2-sided Fisher exact test, independent t-test and Mann-Whitney U test) and generalized linear models (GLMs) as a multivariate analysis with IBM-SPSS version 21.0 for Macintosh. Confidence interval of 95 percent and $p < 0.05$ were considered as significant.

Results

Table 1 – Demographic characteristics of participants in the intervention and control groups

Characteristics		**Intervention n = 15**	**Control n = 13**	**Test results (2-sided)**
		No (%)	No (%)	
Sex	Female	2 (13.3)	5 (38.5)	Fisher's Exact
	Male	13 (86.7)	8 (61.5)	test, p=0.198
Key population	No	0	1 (7.7)	
	FSW	2 (13.3)	4 (30.8)	
	HRM	4 (26.7)	4 (30.8)	

	MSM	7 (46.7)	2 (15.4)	
	Transverstite	2 (13.3)	2 (15.4)	
Education	Elementary sch	3 (20.0)	2 (15.4)	
	Junior high sch	2 (13.3)	5 (38.5)	
	Senior high sch	8 (53.3)	5 (38.5)	
	Univ/college	2 (13.3)	1 (7.7)	
		Mean ± SD	Mean ± SD	Indep t-test
Age		28.1 (7.3)	34.5 (8.1)	p = 0.039

Table 2 – Comparison of clinical and laboratory tests between intervention and control groups

Characteristics		**Intervention n = 15**	**Control n = 13**	**Test results (2-sided)**
		No (%)	No (%)	
Stage of AIDS	Stage 2	12 (80.0)	12 (92.3)	Fisher's Exact
	Stage 3	3 (20.0)	1 (7.7)	test, p=0.6000
Ever diagnosed	No	8 (53.3)	9 (69.2)	Fisher's Exact
with STI	Yes	7 (46.7)	4 (30.8)	test, p=0.460
Adherence	<95%	5 (33.3)	3 (23.1)	Fisher's Exact
to therapy	≥95%	10 (66.7)	10 (76.9)	test, p=0.686
		Mean (± SD)	Mean (± SD)	
Body weight	before treatment	61.4 (6.3)	58.8 (10.4)	Indep t-test, p = 0.432
	after 6 months	67.7 (6.7)	61.8 (10.9)	Indep t-test, p = 0.090
Hb	before treatment	14.4 (1.6)	13.8 (1.9)	Mann-Whitney U p = 0.419
	after 6 months	14.5 (1.4)	13.8 (1.8)	Mann-Whitney U p = 0.344
SGOT	before treatment	26.4 (6.5)	31.8 (11.6)	Mann-Whitney U p = 0.145
	after 6 months	27.7 (6.5)	61.3 (38.3)	Mann-Whitney U p = 0,000
SGPT	before treatment	31.1 (10.2)	36.4 (18.3)	Mann-Whitney U p = 0.611
	after 6 months	34.1 (12.6)	49.8 (19.4)	Mann-Whitney U p = 0.009
CD4%	before treatment	11.4 (4.1)	10.7 (2.4)	Mann-Whitney U p = 0.908
	after 6 months	19.5 (7.3)	12.7 (2.9)	Mann-Whitney U p = 0.002
Absolute CD4 cells count	before treatment	229.7 (66.9)	216.2 (57.7)	Indep t-test, p = 0.576
	after 6 months	315.9 (101.3)	241.7 (60.9)	Indep t-test, p = 0.030

Twenty-eight HIV patients were participated in this study. The mean of age in the control group was significantly older than that in the treatment group (2-sided independent t-test, p = 0.039). There was not any

other significant difference in demographic characteristics, clinical, adherence to therapy, and laboratory test results before treatment between intervention and control groups (Table 1 and Table 2).

Based on the Saphiro-Wilk test of normality, it was showed that absolute CD4 cells count before (p = 0.456) and after six months of therapy (p = 0.263), as well as body weight before (p = 0.690) and after six months of therapy (p = 0.538) were normally distributed. Based on the Levene's test for equality of variances, it was showed that absolute CD4 cells count before (p = 0.429) and after six months of therapy (p = 0.092), as well as body weight before (p = 0.296) and after six months of therapy (p = 0.238) had equal variances. Therefore, the parametric tests were used to analyzed these data.

However, the hemoglobin, liver function tests and CD4 percentage were not normally distributed. Therefore, these data were analyzed using non-parametric tests.

The 2-sided independent t-tests showed there were differences in SGOT (p < 0.001), SGPT (p = 0.009), CD4% (p = 0.002) and absolute CD4 cells count (p = 0.030) after six months of therapy between intervention and control groups. The intervention group significantly had higher mean of absolute CD4 cells count (315.9 cells/μL) compared to control group (241.7 cells/μL), and had higher CD4% (19.5%) compared to control group (12.7%) after six months of therapy. In contrast, the intervention group had lower liver function tests (SGOT and SGPT) compared to the control group after six months of therapy.

Table 3 – Parameter Estimates from the Generalized Linear Models (GLMs)

Parameter	β	SE	95% Wald CI		Hypothesis Test		
			Lower	Upper	Wald Chi-sq	df	Sig.
(Intercept)	-45.730	39.889	-123.911	32.451	1.314	1	.252
[Trial=1]	62.365	13.589	35.732	88.998	21.064	1	.000
[Trial=2]	0[a]	.	.	.	.	.	.
[AIDS=2]	23.449	20.683	-17.088	63.986	1.285	1	.257
[AIDS=3]	0[a]	.	.	.	.	.	.
[Adherence=0]	1.287	14.761	-27.644	30.218	.008	1	.931
[Adherence=1]	0[a]	.	.	.	.	.	.
Age	.238	.829	-1.387	1.862	.082		.774
CD40	1.190	.115	.965	1.415	107.279	1	.000
(Scale)	923.294[b]	246.761	546.823	1558.953			

[a]. Set to zero because this parameter is redundant.
[b]. Maximum likelihood estimate.

In the GLMs (Table 3), the absolute CD4 cells count after six months of therapy was treated as the dependent variable, while the absolute CD4 cells count before therapy and age were treated as a covariate. The trial group, stage of AIDS, and adherence to therapy were treated as the factors.

Results from the GLMs showed that being in the intervention group significantly increased the probability of having higher absolute CD4 cells count by 62.365 cells/μL after six months of therapy (95% CI, 35.732 - 88.998) compared to the absolute CD4 cells count of the study participants in the control group, adjusted for the absolute CD4 cells count before therapy, age, stage of AIDS and adherence to therapy. In addition, every one unit increase of the absolute CD4 cells count before therapy significantly increased the absolute CD4 cells count after six months of therapy by 1.190 cells/μL (95% CI, 0.965 - 1.415), adjusted for the absolute CD4 cells count before therapy, age, stage of AIDS and adherence to therapy.

Discussion

Phyllanthus niruri is known as *meniran* (Javanese) and widely used as a traditional herbal medicine by Indonesians to treat fever, inflammation, urinary tract infections, gastrointestinal infections, and impaired liver function [44]. Several clinical trials on patients with pulmonary tuberculosis [27,28]; vaginal candidiasis [29]; varicella [30]; and tonsilopharyngitis [31] conducted in Indonesia showed an immunostimulator effect of *Phyllanthus niruri*.

Based on the National Agency of Drug and Food Control regulation number HK.000.05.41.1384 in 2005, the preparation of herbal medicine were divided into three categories: jamu (the efficacy is based on the empirical data), standardized herbal medicine (the efficacy is based on preclinical data) and phytopharmaca (the efficacy is based on clinical data). The *Phyllanthus niruri* extract used in the present study was categorized as phytopharmaca, thereby the efficacy of the extract was based on clinical data.

The study participants in the control group took the first-line ART and two capsules of placebo contained 50 mg of *Amylum manihot* three times a day. However, the placebo was given only for a week, since two study participants complained of nausea and dizzy after taking the capsules.

This study revealed that study participants in the intervention group benefitted from the combination therapy by having higher absolute CD4 cells count after six months of therapy compared to the absolute CD4 cells count of the study participants in the control group, adjusted for the absolute CD4 cells count before therapy, stage of AIDS and adherence to therapy.

The increase of absolute CD4 cell counts in the intervention group was due to immunostimulator and reverse-transcriptase inhibitory effects of *Phyllanthus niruri*. Several active phytochemicals in *Phyllanthus niruri* were flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins. Coumarins and repandusinic acid (hydrolysable tannins) inhibited the reverse-transcriptase enzyme and HIV-1 replication [32,45].

Phenolic compounds were known to demonstrate inhibitory action in the HIV replication, from fusion, adsorption, reverse-transcription, integration and protein cleavage [46]. Terpenoid compounds were known to have anti-HIV activity [47], while flavonoids showed immunostimulator effect by increasing the phagocytosis activity of macrophage and stimulating activation of effector cells such as lymphocytes and macrophages to release cytokines, interleukins and tumor necrosis factor alpha (TNF-alpha) [48].

Several factors were recognized to influence the absolute CD4 cells count, including sex, age, race, diurnal variation, physical stress, pregnancy, drugs taken, tuberculosis, viral infection, anti lymphocyte autoantibody, splenectomy, and calibration [49]. The baseline data analyses showed that age was significantly different between intervention and control groups, therefore, age was included in the multivariate analysis. However, in the multivariate analysis age was not a significant predictor of the absolute CD4 cells count after six months of therapy. Sex was not significantly different between the intervention and the control groups. All study participants were Javanese, except one female participant was a Torajanese from South Sulawesi Province. There was not any pregnant female participant, participant who suffered from tuberculosis, participant who suffered from hepatitis, or participant who underwent a splenectomy in this study. All participants agreed not to take any vitamin or other herbal medicine during the study period, therefore, they only consumed prescribed drug from the CST clinics. All blood samples were collected between 09:00-11:00 AM to minimize diurnal variation of the absolute CD4 cells count [50]. The absolute CD4 cells count and CD4 percentage were measured with immunofluorescence analysis using flow-cytometry as recommended by WHO [42]. However, the researchers did not measure physical stress and anti lymphocyte autoantibody.

Although herbal medicinal products are widely considered to be of lower risk compared to synthetic drugs, they are not completely excluded from the possibility of having toxic or other adverse effects [51]. Deficiencies such as under-reporting of adverse reactions, lack of toxicological information on herbs, and the quality of the reported information present challenges when signals of safety concern arise [52]. An animal study on the acute toxicity of aqueous leaf extract of *Phylanthus niruri* reveled there was not any statistical differences between a single oral low dose of 2,000 mg/kg body weight and a single high dose of 5,000 mg/kg body weight of *Phyllanthus niruri* on urea, creatinine, total protein, albumin, globulin, direct bilirubin, indirect bilirubin, total bilirubin, γ-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. Therefore, the aquous leaf extract of *Phyllanthus niruri* could be considered non-toxic at the acute level and consequently, the LD_{50} of *Phyllanthus niruri* leaf extract was more than 5,000 mg/kg body weight [52].

The combination of ART and *Phyllanthus niruri* extract was administered for study participant in the intervention group in our present study. In a review, the concomitant use of conventional and herbal medicines could lead to clinically relevant herb-drug interactions [53]. However, during our study period, there was not any report of adverse event from the study participants. The ART was provided two weeks after the administration

of co-trimoxazole in accordance with WHO recommendation [40,43], and the combination of ART and *Phyllanthus niruri* was also provided two weeks after the administration of co-trimoxazole. This strategy could minimize the possibility of undetected adverse events with the development of unmasking immune reconstitution inflammatory syndrome (IRIS) that was often experienced by HIV patients who did not received therapy for their opportunistic infection, but immediately received ART [40,43].

The bivariate analyses in the present study also indicated a significant different in CD4 percentage and liver function tests after six months of therapy. The increased of liver function tests in the control group might be due to hepatotoxic effect of ART, especially evafirenz and nevirapine [40,54]. Several animal studies reported a hepatoprotective effect of Phyllanthus niruri extract through its antioxidant property [38,55,56]. Therefore, the use of Phyllanthus niruri extract as an adjuvant therapy in combination with ART in the intervention group showed promising beneficial effect against ART hepatotoxicity.

A limitation of the present study was the small sample size, although using the formula for calculating sample size for this quasi-experimental trial 14 HIV patients were enough for each group of this study. A further limitation of this study was the research design. Accordingly, we suggested a randomized clinical trial with bigger sample size to be carried out with the same purpose as this study.

Conclusion

The combination of the first line of ART and *Phyllanthus niruri* extract was more effective in increasing the absolute CD4 cells count compared to the administration of ART alone in HIV patients.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgements

This study was funded by the Health Professional Education Quality (HPEQ) Project, IBRD Loan No. 7737-ID, 2013. We express our sincere gratitude to *Phyllanthus niruri* research group; Prof. Soeprapto Ma'at; the director and staffs of Dr. Ramelan Naval Hospital Surabaya; Director and staffs of Sidoarjo General Hospital, Sidoarjo; NGO "Genta" Sidoarjo; the head and staffs of Clinical Pathology Laboratory, Dr. Soetomo General Hospital, Surabaya; the head and staffs of Puskesmas Perak Timur Surabaya, and all of our study participants for supporting this research.

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