ISSN 2089 - 2136

Tropical Medicine Journal

Volume 03, No. 1, 2013

TMJ	Volume 03	Number 01	Page 1 - 93	ISSN 2089 - 2136

Center for Tropical Medicine, Faculty of Medicine, Universitas Gadjah Mada
in collaboration with Indonesian Society of Tropical Medicine and Infectious Disease (PETRI)

Address : Tropical Medicine Journal, PAU Building, Jalan Teknika Utara Barek, Yogyakarta
Universitas Gadjah Mada, Yogyakarta 55281, Phone : +62-274-588483, E-mail: tropmedjournal@gmail.com

TROPICAL MEDICINE JOURNAL

ISSN : 2089-2136
Center for Tropical Medicine, Faculty of Medicine, Universitas Gadjah Mada in collaboration with Indonesian Society of Tropical Medicine and Infectious Disease (PETRI)

Volume 03, Number 01

CONTENTS

The Effect of *Pandanus conoideus* Lamk Extract to the Serum Level of TNF-α, IL-10 and Parasitemia of *Plasmodium berghei* Infected in Mice

[1]Zeth Robeth Felle*, [2]Mahardika Agus Wijayanti, [2]Supargiyono.
[1] Poltekes Kemenkes, Jayapura, Papua, Indonesia; [2]Department of Parasitology Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Corresponding author: zfelle@yahoo.com

ABSTRACT

Introduction: Study on the effects of red fruit (*Pandanus conoideus* Lamk) has been conducted with various result.
Objectives: In this study, the effect of red fruit extract on the level of cytokines TNF-α, IL-10 and the parasitemia of *Plasmodium berghei* infected Swiss mice were evaluated.
Methods: Quasi-experimental design with pre and post test only control group was applied. Sixty male Swiss mice of 8 weeks old and weighs 20-30 g, was simply randomized into four treatment groups. Group I (K1) was stimulated with the extract for 2 weeks before and 2 weeks after infection with *P.berghei*. K2 was stimulated with the extract for 2 weeks before infection, K3 was stimulated with the extract for 2 weeks after infection and K4, negative control, was given 0.6% tween 40. The dose of the extract was 7.8 mg/30g mice BW, intra gastric once a day. Serum level of TNF-α and IL-10 was measured by ELISA *Sandwich* methods and the number of parasitemia were examined microscopically. The difference level of TNF-α, IL-10 and parasitemia of each treatment group were analyzed by t-test, one way anova, honestly significant different (HSD) and multivariate analysis (manova).
Results: There were significance differences of parasitemia in K3 group compared to others. Parasitemia on day-3 was 18.464% and reduced to 1.054% on day-9. Parasitemia of K2 group was 13.204% on day-3 and 32.455% on day-9. Parasitemia of negative control group was 27.304% on day-3 and 78.506% on day-9. The TNF-α level of K3 group decreased along with the infection, it was 26.985 pg/mL on day-0 and 22.244 pg/mL on day-6. The IL-10 level increased at all groups and the highest level was on K3 group.
Conclusion: Effect of red fruit extract on *P. berghei* infected mice was reduced both parasitemia and TNF-α level but increased of IL-10 level.

Keywords : *Pandanus conoideus* Lamk, Malaria, Parasitemia, TNF-α, IL-10.

INTISARI

Pendahuluan: Penelitian tentang efek buah merah (*Pandanus conoideus* Lamk) sudah banyak dilakukan dengan hasil yang sangat bervariasi.
Tujuan: Dalam penelitian ini, akan dikaji pengaruh pemberian ekstrak buah merah terhadap kadar sitokin TNF-α, IL-10 dan angka parasitemia pada mencit swiss yang diinfeksi *Plasmodium berghei*.
Metode: Penelitian ini menggunakan desain *quasi eksperimental* rancangan *pre and post test only control group design*. Enam puluh ekor mencit swiss jantan berumur 8 minggu dengan bobot 20-30 gr, diacak sederhana ke dalam 4 kelompok perlakuan. Kelompok I (K1) diberikan ekstrak selama 2 minggu sebelum dan 2 minggu setelah diinfeksi *P. berghei*, K2 diberikan ekstrak sebelum infeksi, K3 diberikan EBM setelah infeksi dan K4 kontrol negatif. Dosis ekstrak adalah 7, 8 mg/30gr BB mencit/po/hari. Pemeriksaan kadar TNF-α dan IL-10 dari serum menggunakan metode ELISA *Sandwich*. Angka

parasitemia diperiksa secara mikroskopis dengan teknik apusan darah tipis. Perbandingan kadar TNF-α, IL-10 dan angka parasitemia dari setiap kelompok perlakuan dianalisis dengan uji-t, Anova satu arah, dilanjutkan dengan uji *honestly significant different* (HSD) dan juga analisis multitvarians (manova).
Hasil: Terdapat perbedaan angka parasitemia yang nyata pada kelompok K3 dibandingkan kelompok lainnya. Parasitemia pada hari-3 adalah 18,464% dan menurun menjadi 1,045% pada hari ke-9 pasca infeksi. Parasitemia pada kelompok K2 adalah 13,204% pada hari ke-3 dan 32,445% pada hari ke-9. Parasitemia kelompok kontrol negative juga meningkat dari 27,304% pada hari ke-3 menjadi 78,506% pada hari ke-9. Kadar TNF-α pada kelompok K3 menurun selama infeksi, 26,985 pg/mL pada hari ke-0 menjadi 22,244 pg/mL pada hari ke-6 pasca infeksi. Kadar IL-10 meningkat pada semua kelompok dan peningkatan paling nyata pada kelompok K3.
Simpulan: Ekstrak buah merah dapat menurunkan angka parasitemi dan kadar TNF-α serta meningkatkan kadar sitokin IL-10..

Kata Kunci: *Pandanus conoideus* Lamk , Malaria, Parasitemia, TNF-α, IL-10,

INTRODUCTION

Red fruit (*Pandanus conoideus* Lamk) is a Papuans traditional food that well-known of its efficacy and benefits. Empirically, the red fruit oil has been used to treat various diseases such as cancer, stroke, hypertension, hepatitis, liver cirrhosis, diabetes mellitus, sinusitis, ovarian cysts and epilepsy[1]. In case of HIV/AIDS, after being administered the red fruit oil jointly consuming 80% of animal protein each day, it can increase the number of $CD4^+$ T cells[2]. Content of red fruit dominated by unsaturated fatty acids such as, palmitoleic acid, oleic acid, linoleic acid, linolenic acid and some compounds of tocopherol (vitamin E), β-carotene (pro vitamin A), omega, omega 3, omega 6 and omega 9[1,3], is a complex compound of high anti-oxidant that also has a immunostimulant with an increased number of components of immunity, both cellular and humoral immunity, such as increase cell proliferation of lymphocytes, T helper cell activity and antibody production[3,4,5]. However, there are also a number of studies that actually contradicts with studies results and empirical evidence above, including the effect of red fruit extract in improving human immune response. Until now the effect of red fruit on cell culture experimental animal and human immune response still controversial.

In order to solve those problems basic research at the level of experimental animals using an infectious agent capable of inducing complex immune responses is essential. Malaria parasites is an appropriate agent for its ability to stimulate innate and adaptive immune response[6,7,8]. *Plasmodium berghei* infection in Swiss mice is one of animal malaria model that has properties resemble of *P. falciparum* in human. This animal model of infection will be used to study the effect of red fruit extract on cellular as well as humoral immune response during *P. berghei* infection[7,9]. Increased cellular immune response of the body that mediated Th1 ($CD4^+$), will be accompanied by pro-inflammatory mediator release by macrophages and Th1 such as, cells cytokine interferon gamma (IFN-γ), interleuin-1 (IL-1), IL-2, IL-12 and tumor necrosis factor alpha (TNF-α) [9,10,11].

Cytokine products of Th1 cells and macrophages tend to work together to stimulate immune response to suppress parasite[8,11], however excessive response may result certain pathological conditions, which can be fatal such as severe malaria, cerebral malaria[7,12]. As a protective mechanism, Th2 cell produce a number of anti-inflammatory mediators such as IL-4, IL-5, IL-9, IL-10 and IL-13,[7,11]. IL-10 can inhibit production of TNF-α in patient with falciparum malaria which may or reduce the pathological effect

of Th1 cell pathology, probably by inhibiting secretion of IL-12, IFN-γ and TNF-α[8,11]. IL-10 has also been reported to damages of the brain tissue caused by cerebral malaria[9,12,13].

The role of TNF-α and IL-10 in immunity is like a double-edged sword, that makes this concept is interesting to be studied. Similarly, a number of bioactive substances in red fruits which have several compound, is predicted to contribute in preventing pathological conditions during malaria infections[14].

In this study, *P.berghei* infected Swiss mice were used to evaluate the effect of red fruit extracts on changes of the level of parasitemia, TNF-α and IL-10 level in the serum during infection.

MATERIALS AND METHODS

Quasi-experimental designs with *pre and post test only control group design* was used in this study.

Experimental Animals

A total of 60 male Swiss mice of 8 weeks old and weighs 20-30 grams were selected after a period of acclimatization for 2 weeks, randomized into 4 groups of 15 mice/ group. Group-1 (K1) was administered the extract 2 weeks before and after *P. berghei* infection. Group-2 (K2) was administered the extract 2 weeks before infection. Group-3 (K3) was administered the extract 2 weeks after infection and Group-4 (K4), negative control, was administered with the solvent 0.6% of Tween 40.

Preparation of *Pandanus conoideus* Lamk Extract

The preparation of *Pandanus conoideus* Lamk (red fruit: ogi or mbarugum ; Wamena) with 1.3 m length per pieces, 11.5 cm diameter, and 6.4 kg weight without the fruit stalk was extracted by maceration using hot distilled water with a ratio of 100-150 ml / 250 grams of fruit meat[15]. The extract dose was adjusted from human to mouse. Human dose is 2 g / kg day/70 BB^2. The dose conversion factor to 20g mouse is 0.0026, therefor 5.2 mg/20 g BW or 7.8 mg/30 g BW mice. 0.6% of Tween 40 solution was used as a solvent in preparing the extract. The experimental mice were administered once daily intragastric according to the group.

Parasite

Plasmodium Berghei ANKA strain was used in this study[16]. The infection dose was 10^7 parasites / 0.2 ml. Parasitemia was calculated based on thin blood smear[17].

Serum isolation and ELISA tests

Serum was collected from cardiac puncture blood on D0 (before infection), D_3, D_6 and D_9 after infection. Serum TNF-α and IL-10 was measured by ELISA Sandwich method (Bendermed System)[18].

Data Analysis

Parasitemia, TNF-α and IL-10 level of experimental groups were analyzed using one-way ANOVA by significance of $p < 0.05$ and followed by a test of *honestly significant different* (HSD). T-test was also conducted to determine the differences among treatment groups (t-independent), and differences of every day examination in each treatment group (t-dependent). To avoid bias due to a separated test on the Anova test above, others statistical tests will be done well statistical Manova or multivariance analysis[19,20].

RESULTS AND DISCUSSION

The parasitemia, serum TNF-α and IL-10 level of each experimental group can be seen in the following tables and figures.

Table 1. The effect of *Pandanus conoideus* Lamk extract on parasitemia, serum TNF-α and IL-10 level of *P.berghei* infected mice

Experimental Group	Day	Parasitemia (%)		TNF-α (pg/ml)		IL-10 (pg/ml)	
		Mean	SD	Mean	SD	Mean	SD
K 1	0	0,497	0,201	34,859	22,469	10,472	3,327
	3	33,193	13,992	23,322*	8,819	24,214*	20,684
	6	-	-	133,697	47,018	71,211	26,062
	9	-	-	-	-	-	-
K 2	0	0,463	0,210	77,815*	29,964	10,938	0,624
	3	13,208	3,158	83,373	20,827	40,236*	22,974
	6	13,141*	6,883	107,341	2,610	92,802	63,771
	9	32,455*	-	108,722*	-	116,360	-
K 3	0	0,299	0,057	26,984	1,779	29,644	19,282
	3	18,364	18,571	23,269*	8,832	22,857*	19,023
	6	13,735*	1,714	22,244*	5,897	69,883	27,588
	9	1,054*	1,826	59,179	30,727	252,892	207,852
K 4	0	0,533	0,251	27,194	1,713	42,334	12,942
	3	27,304	15,506	69,983	15,993	120,332	44,103
	6	58,180	25,777	94,679	0,774	249,874	194,430
	9	76,506	6,406	44,064	15,405	181,030	61,360

Note :

K1: Administration of the extract 2 weeks before and after infection
K2: Administration of the extract 2 weeks before infection
K3: Administration of the extract 2 weeks after infection
K4: Negative control : 0.6% of Tween 40

* = There are significant differences in K4 at p <0.05
\- = Average (Mean) can not be counted (dead mice)

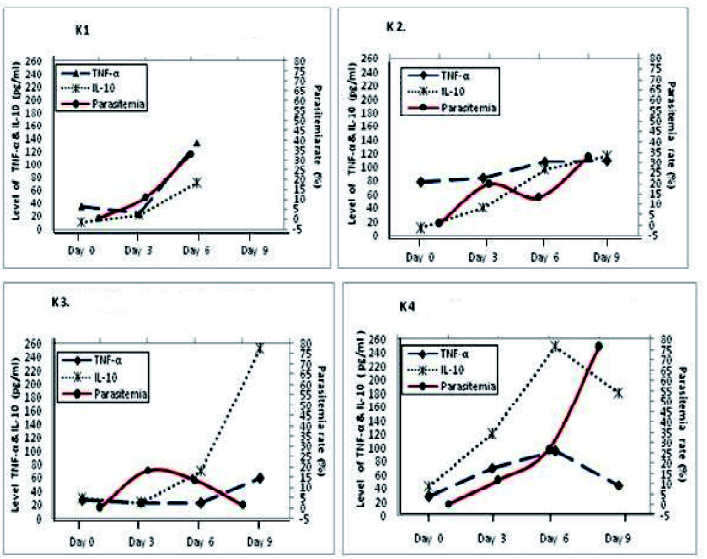

Figure 1. The effect of *Pandanus conoideus* Lamk extract on parasitemia, serum TNF-α and IL-10 level of *P.berghei* infected mice

A one-way Anova test of parasitemia on day 0 (D0), D3, D6 and D9, indicate the significant difference among experimental groups, $p<0.05$. The Tunkey HSD test showed that the difference was exist on D3, D7 and D9. Independent t-test between K3 and K4, has a significant value of $p = 0.004$, and p value between K2 and K4 was 0.019, However p value of K1 and K4 was 0.127.

Level of serum TNF-α was significantly difference among experimental groups on D0, D3, D6 and D9, $p <0.05$. The difference was pronounced on day 3 and 6 especially on K3.

Multivariate analysis, it showed that K1 has a significance value of parasitemia, TNF-α and IL-10 level, $p = 0.07$, 0.008 and 0.019 respectively. Serum TNF-α level was higher than IL-10, it seems directly proportional to the increase of parasitemia. Parasitemia, TNF-α and IL-10 of K3 was significantly difference to other groups, $p = 0.107$; 0.065 and 0.0005 respectively. The high level of serum IL-10 may contribute to the reduction of parasitemia and prevent mortality of *P.berghei* infected mice.

Pandanus conoideus Lamk extract was given for 28 days, i.e. 14 days prior- and post-infection (K1), resulting in higher serum TNF-α level and cannot be regulated by existed anti-inflammatory mediators, IL-10. Although the initial infection can slightly decreased TNF-α level as the indication of normal regulatory mechanisms of the immune system against higher TNF-α level due to the extract stimulation (Table 1). Similarly, under physiological conditions, when the body is exposed to an infectious agent of malaria parasites, the immune response is activated with the cellular immune response. Immune cells, especially macrophages will release pro-inflammatory mediators and anti-inflammatory simultaneously[13].

The serum IL-10 did not seem to increase significantly, even lower than that of TNF-α level in a physiological state as inversely conditions (See Figure 1; K1 and K4). This indicates that anti-inflammatory mediators including IL-10, are not able to reduce the TNF-α level as stimulated by two different factors at the same time, agent of infection (*P. berghei*) and *Pandanus conoideus* Lamk extract. In contrast, IL-10 and anti-inflammatory mediators could only suppress a slight increase in pro-inflammatory mediators, including TNF-α level, these conditions cannot suppress the parasite proliferation. TNF-α did not effect to the parasite directly but contribute to pathophysiological mechanisms that lead to the early death of mice at day 4 after infection. This is consistent with previous statements, that pro-inflammatory cytokine product of Th1 and macrophages that has important role in pathophysiological mechanisms of severe malaria is TNF-α [9.11]. Thus the provision of *Pandanus conoideus* Lamk extract in the long term, especially before and after infection, suppressed cellular immune response that resulting in increased parasitemia and pro-inflammatory mediators that resulted directly in pathophysiological mechanism.

Pandanus conoideus Lamk extract was given for 14 days before infection (K2), did not show significant immunostimulatory effect. At the beginning of the infection, serum TNF-α level was already quite high due to extract stimulation, as shown in Figure 1 and 2 above. This condition has been inferred by previous researchers, the red fruit can improve macrophage phagocytic activity, lymphocyte proliferation and activating the cellular immune response[21,22,23]. Despite the increased activity of cellular immune response with the release of pro-inflammatory mediators, including TNF-α, but it not completely suppress the proliferation of parasites (see Figure 1), on the contrary, lead to pathological conditions due to the death of mice that began D6 after infection with *P. berghei*. Although the death of mice also caused by pathogenic factors of *P. berghei*, which is lethal on D6 to D8 mice after infection [24]. Discontinuance of *Pandanus conoideus* Lamk extract will optimize the protection mechanisms on excessive

cellular immune response and pathological impact. A few days after *P.berghei* infection, or prior to reaching the highest peak of parasitemia, TNF-α level could be reduced by anti-inflammatory mediators, including cytokines IL-10 as product of macrophages, Th 2 and Th3 will increase, as shown in Figure 1 above. Serum level of IL-10 was higher than TNF-α on D9. Although the process is very slow that affecting pathophysiological mechanisms and death of mice on the previous day, but result is in line with Bratawidjaja's statement, that cytokines will be active at a very low level 10^{-10}-10^{-15} mol / l in order to stimulate the targeted cell[13]. Elevated IL-10 level were statistically significant in protective effect, but not, apparently also in line with previous statements, since the effect of cytokine antagonists has no tangible results because the compensation of other cytokines [13.32.]

A different and unique condition was showed on K3. The results showed that serum IL-10 level elevate significantly. This condition was consistent with that serum of patients with minor or uncomplicated malaria will contain the elevated IL-10 level and decreased TNF-α level[12]. In addition, the serum TNF-α level from K3 is lower compared to K4 (negative control group) (See Figure 1: K1 and K4) as noted in previous studies, the administration of red fruit oil shown to reduce cellular immune responses[5].

Elevated IL-10 level in K3 has significant reductions in parasitemia as illustrated in Figure 1, from D0 to D3. The results also showed that death of 1 mouse occurred on D8. After D3 infection, parasitemia decreased significantly but inversely proportional to serum IL-10 level, while TNF-α level tends to be more stable. Parasitemia of K3 decreased significantly on D9, reaching 1.054%, from the previous 18.365% (D3) and 13, 735% (D6). This condition indicate the effect of administration of *Pandanus conoideus* Lamk extract after malaria infection can increase serum IL-10 level, accompanied by significant decrease of parasitemia.

Adequacy of both cellular and humoral immunity comes with administration of *Pandanus conoideus* Lamk extract infection with *P. berghei*, mainly initiated by the changing role of β-carotene compound in *Pandanus conoideus* Lamk that is from pro-oxidant to anti-oxidant compounds[21]. In malaria parasite-infected conditions, a decline in the volume and capacity of oxygen (O_2) due to phagocytosis of erythrocytes parasites by the spleen lymphocytes and macrophages can cause anemia[8,9,28], besides the *Plasmodium* also invade erythrocytes of all ages[7,29]. The situation is certainly steadily reduced availability of oxygen level in the circulation and lowering partial pressure of oxygen (PO_2). Decreased PO_2 will spur changes in chemical structure and function of â-carotene from pro-oxidant to antioxidant compounds, as stated previously that β-carotene is an oxygen binder and as an potential anti-oxidant, but it is effective as a free radical binding when only 2-20% oxygen available and low PO2[21.30].

On the other hand, the content of tocopherols was previously functioning as phenolic hydrogen donors to neutralize β-carotene in pro-oxidant conditions, if the oxygen availability and PO2 is adequate, in pre-infection or early infection condition. However, after infection with the parasite, many pro-oxidant compounds or oxyradical can be generated either by immune component, and the consequences of such parasitic activity, ROS and ROI class, therefore, the availability of anti-oxidant compounds of tocopherols are not fulfilled. Instead these conditions, it further enhance the ability of anti-free radical of tocopherol and β-carotene, because the chemical structure of β-carotene change to pro-oxidant and anti-oxidant compounds due to the parasitic infection that led to the decreased PO2 pressure. In addition, a number of unsaturated fatty acids in *Pandanus conoideus* Lamk has function as an anti-free radical because the structure are susceptible to oxidation in the double bond, making the complex compounds as a powerful anti-free

radical[21,30]. Biochemical reaction mechanism is similar to the statement of deMan, that the rate of oxidation of fatty acids is influenced by the amount of oxygen, the degree of unsaturated lipid and the presence of antioxidants.

Provision of anti-oxidants contained in *Pandanus conoideus* Lamk extract, resulting in a number of free radicals substances ROI and RNI of effector immune cells, especially macrophages, will neutralize the non radical compounds. Neutralized free radicals substances of ROI and RNI group products on the immune effector cells, apparently weakens the protective functions of macrophages and Th1, in turn, it will decrease the production of TNF-α, IL-1, IL-2, IL-6, IL-8, IL-12 and IL-18. Instead these conditions facilitate the activation and differentiation of CD4 + T cells by a subset of Th2 cells is initiated by cytokines autoregulation or anti-inflammatory that released by macrophages, such as IL-4, IL-5, IL-10 and IL-13, in addition to the interaction of β-carotene (retinol and retinoic acid) and immune effector cells through the binding with Retinol Binding Protein Celular (CRBP), including macrophages, B cells, plasma cells, Th2, Th3 (Treg), CTL and NK, which facilitates the proliferation and differentiation. Given vitamin A plays an important role in the regulation of immune system both specific and non-specific, as well as it play a role in the process of Th2 cell differentiation, the growth and differentiation of B cells into plasma cells and antibody production by antigen specific configuration and maintain normal antibodies in the circulation under influence Th2 cells[13,30,32].

IL-10 is also the product of Th2 and Th3 (T reg), that will increase the regulator of immune response and the sensitivity and specificity of effector cells. A number of pro-inflammatory cytokines Th1 and macrophage products that released at the beginning of the infection, it will still continue to circulate the blood for a few days to several months, and Th1 cytokine macrophage products include TNF-α, may increase the activity of effector cells in the immune response order. Specifically, with the mediation of antibody, including increased activity of Plasmodicidal effector cells[9,10,11].

CONCLUSION

Based on those results, *Pandanus conoideus* Lamk extract showed effect on (1) reduction of serum TNF-α level; (2) increasing serum IL-10 level; (3) reduction of parasitemia. Those effects were more obvious if the extract administered after malaria infection.

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Tropical Medicine Journal is published in June and December by the Faculty of Medicine, Universitas Gadjah Mada.

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Sample References

Scientific Journal

1. *Standard journal article*

   You CH, Lee KY, Chey RY, Menguy R. Electro-gastro-graphic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980; 79(2):311-14.

   Goate AM, Haynes AR, Owen MJ, Farral M, James LA, Lai LY, et al. Predisposing locus for Alzheimer's disease on chromosome 21. Lancet 1989;1:352-55.

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   Coffee drinking and cancer of the pancreas [editorial]. BMJ 1981;283-628.

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   Massone L, Borghi S, Pestarino A, Piccini R, Gambini C. Localisations palmaires purpuriques de la dermatite herpetiforme. Ann Dermatol Venereol 1987;114:1545-47.

5. *Volume with supplement*

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    Spargo PM, Manners JM, DDAVP and open heart surgery [letter]. Anaesthesia 1989;44: 363-64.
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13. *Article containing retraction*
    Shishido A. Retraction notice: Effect of platinum compounds on murine lymphocyte mitogenesis [Retraction of Alsabti EA, Ghalib ON, Salem MH. In: Jpn J Med Sci Biol 1979; 32:53-65). Jpn J Med Sci Biol 1980;33:235-37.
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    Hemodynamics III: the ups and downs of hemodynamics [computer program]. Version 2.2. Orlando (FL): Computerized Educational System; 1993.

We thank to the reviewers of this edition:

dr. Abu Tholib, M.Sc., Ph.D., Sp.MK

dr. Ahmad Hamim Sadewa,Ph.D

dr. Arta Farmawati, Ph.D

dr. Elizabeth Henny Herningtyas, M.Si, Ph.D

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