# The Effect of Artemisin Combined with *Moringa oleifera* Leaf Extract on CD4+ and CD8+ Percentage of Mice Infected with *Plasmodium berghei*

Melda Fio Flora BR. Sijabat[1], Tinny Endang Hernowati[2, 3*], Loeki Enggar Fitri[4]

[1]Biomedical Science Magister Program, the Faculty of Medicine, Brawijaya University, Malang, Indonesia
[2]Departement of Clinical Pathology, dr. Saiful Anwar Public Hospital, Malang, Indonesia
[3]Departement of Clinical Pathology, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[4]Departement of Parasitology, Faculty of Medicine, Brawijaya University, Malang, Indonesia

**ABSTRACT**

The study aims to determine the effect of Artimisin and *Moringa oliefera* leaf extract combination on CD4+ and CD8+ T cells percentage of mice infected with *Plasmodium berghei*. Both of which have a role in the elimination of intracellular *Plasmodium* parasites that cause malaria. Artemisin, the effective anti-malaria, kills the *Plasmodium* parasite through its free radicals. However, free radicals can damage immune cells such as CD4+ and CD8+ T cells, and as the *M. oleifera* leaves contain the bioactive flavanoids quercetin and kaempferol, both being strong anti-oxidant and anti-inflamatory agents, it is hoped that they will reduce these negative effects. A post test group experimental design was employed on 6 groups of mice, 4 groups of mice infected with *P. berghei*, and then each administered 0.004mg/gBW Artemisin (A), a combination of Artemisin 0.004 mg/gmBW in 0.125 mg/gBW (DK1), 0.250 mg/gBW (DK2) and 0.500 mg/gBW (DK3) of *M. oleifera* leaf extract,  and 2 control groups of mice, one group of normal mice and another untreated group infected with *P. berghei*. Blood samples were collected randomly from each group on days 3 and 7, parasitemia levels were then calculated microscopically at 1000 × and the percentage of CD4+ and CD8+ T cells were obtained through flowcytometry. The results indicated that the combination of Artemisin and *M. oleifera* leaf extract over 7 days increased the percentage of CD4+ T cells for the DK2 group by p = 0.001 and the DK3 group by p = 0.000 and reduced parasitemia levels by p = 0.000 in DK1, DK2 and DK3 respectively, whereas, levels of CD8+ T cells did not rise. And the combination did have an effect on parasitemia levels in the treated groups (p = 0.000) and the percentage of CD4+ T cells (p = 0.000) but not on that for CD8+ T cells.

**Keywords**: *Parasitemia, CD4+ and CD8+ T cells, Moringa oleifera*

## INTRODUCTION

Malaria is a deadly intracellular disease caused by the parasite *Plasmodium* [1]. The five strains of plasmodium that most frequently infect humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malaria* and *Plasmodium knowlesi* and all these strains are carried by the Anopheles mosquito females who then bite and infect humans [2].

The main actors in the human immune response to malaria are CD4+ and CD8+ T cells. CD4+ T cells act to eliminate intraerythrocytes parasites, to regulate cellular and humoral immune activity and to induct CD8+ T cells through interferon and it these cells who play the major part in attacking these intrahepatic parasite [2,3]. *P. falciparum* and *P. vivax* infections induce significant falls in absolute T CD4+ T CD8+ cell and lymphocytes levels [3,4].

The standard anti malarial drug Artemisin works by killing malarial parasites through the free radicals it contains formed by the molecular interaction of its endoperoxide and $Fe^{2+}$-heme erythrocytes in infected cells (parasite killing) [5].

*Corresponding author:
Tinny Endang Hernowati
Departement of Clinical Pathology, Faculty of Medicine,
Brawijaya University
Jalan Veteran, Malang 65145, Indonesia
E-mail address: tinnyendang@yahoo.com

The *Moringa oleifera* leaves contain such things as amino acids, proteins, carbohydrates, vitamins A, C and E as well as calcium, magnesium and phosphorous. Its bioactive flavanoids, especially quercetin and kaempferol, its fenols such as (gallic and salicylic acids and protocatechnic) are all strongly antioxidant and anti inflammatory compounds [7, 8, 9, 10].

Thus the aim of this study was to determine the effect of Artemisin and *M. oleifera* leaf extract combination on CD4+ and CD8+ T cell percentage in mice infected with P.berghei in order to observe the pathogenesis of malaria in these animals that mimics that in humans.

## MATERIALS AND METHODS
### Research design
A post test control groups design was employed in this research.

### Sample collection and treatment
This study used healthy and active male Balb/c mice of between 6 and 8 weeks old weighing from 20 − 40 gms obtained from Vetenaria Farma, Surabaya. Prior to the commencement of the experiments, approval was received from Ethics Committee of Faculty of Medicine, Brawijaya University.

There were 36 subjects mice which were divided into 6 groups : (1) A negative control group of normal untreated mice (KN), (2) A positive (infected) control group but untreated (KP), and 4 groups infected with P.berghei and treated with: a) Artemisin only 0.004 mg/gBW (A), and combination doses of Artemisin 0.004 mg/gBW with 0.125 mg/gBW Moringa oleifera leaf extract (DK1), 0.250 mg/gBW leaf extract (DK2) and 0.0500 mg/gBW leaf extract (DK3). On days 3 and 7 of treatment, 3 mice were selected randomly from each group and had their parasitemia levels determined microscopically at 1000×, their CD4+ and CD8+ T cell percentage were calculated by using flowcytometry

### The inoculation with Plasmodium berghei
The *P. berghei* for inoculate was obtained from Biomedical Laboratory of Faculty of Medicine, Brawijaya University. 0.2 mL of mice blood containing erythrocytes infected with $1 \times 10^6$ P. berghei parasites were inoculated intraperitoneally [5]. The parasitemia levels per 1000 erythrocytes in infected mice were counted using a binocular microscope and a Giemsa dye [1, 5].

### The Moringa oleifera extraction process
Leaves from Nusa Tenggara Timur variety of *M. oleifera* were obtained from PT. Timor Mulia Sentosa. The extract was processed at the Pharmacology Laboratory, Brawijaya University in 3 stages, namely, drying, extraction and evaporation. First dry leaves were ground up, then 100 g were placed in a 1 L Erlenmeyer flask and dissolved in an 80% methanol solution until the volume became 900 mL and finally it's water content was evaporated.

### Measuring CD4+ and CD8+ T cells percentage
First 1 mL of blood was taken from the heart of each mice and their mononuclear cells were extracted. Then, the mononuclear cells were cleaned in a Phosphate Buffered Saline Solution (PBSS) and dyed with CD4 and CD8 antibodies for 3 minutes at 4°C, after that the mononuclear cells ($10^6$ cells/mL) were incubated with specific mouse CD4 and CD8 monoclonal antibodies. Analysis was done by flowcytometer employing a BD FACS caliber 3 colour flowcytometer per 10,000 leukocytes (4).

### Data analysis
Statistical analysis on the data for parasitemia levels, CD4+ and CD8+ T cells percentage were done by employing a *SPSS 17* programme for *Windows* software. In order to analyze different in parasitemia levels, CD4+ and CD8+ T cells percentage in each group between days 3 and 7, paired t- tests were used, whereas, to determine the differences between groups a one-way ANOVA test was utilized. The relationship between the various combinations of Artemisin and the Moringa oleifera leaf extract to parasitemia levels, CD4+ and CD8+ T cells percentage were obtained through a Pearson correlation test. The results were considered significant if they reached $p < 0.05$.

## RESULTS AND DISCUSSION
### The differences of parasitemia levels
An analysis of the results from the differing in parasitemia levels between the samples taken on Day 3 and those taken on Day 7 from each group (except for the negative control group) indicated a significant rise in the KP group (p = 0.031), and a significant in the Artemisin only group of p = 0.21, in the DK2 group the drop was p = 0.004 and for DK3 p = 0.000. The differences in the findings for parasitemia levels between Day-3 and Day-7 are shown in Figure 1.

The results of the Kruskal Wallis tests on the difference in parasitemia levels between groups on Day-3 used a post hoc Mann Whitney test indicated that there was a difference in parasitemi a levels between
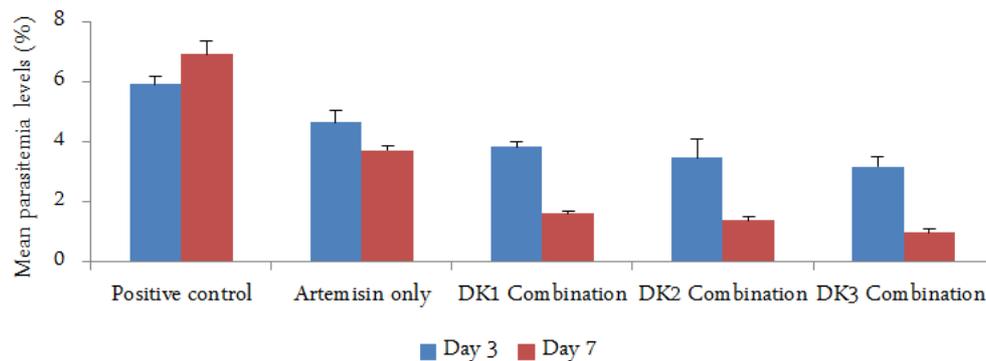
Figure 1. Differences in parasitemia levels between day-3 and day-7. In the positive control group (untreated infected mice) there was a significant increase in parasitemia levels (p = 0.031), whereas the group receiving Artemisin only recorded a significant decreased (p = 0.021), and this was also true for the DK2 (p= 0.004) and the DK3 groups (p = 0.000). However, in the DK1 group there was no significant difference (p = 0.050). A value of p < 0.05 was considered to be significant. The differences between the positive control group, the Artemisin only group and for the DK2 and DK3 groups were calculated via an unpaired t-test and the DK1 group a differential Mann Whitney test. All resuts were mean standard deviations.
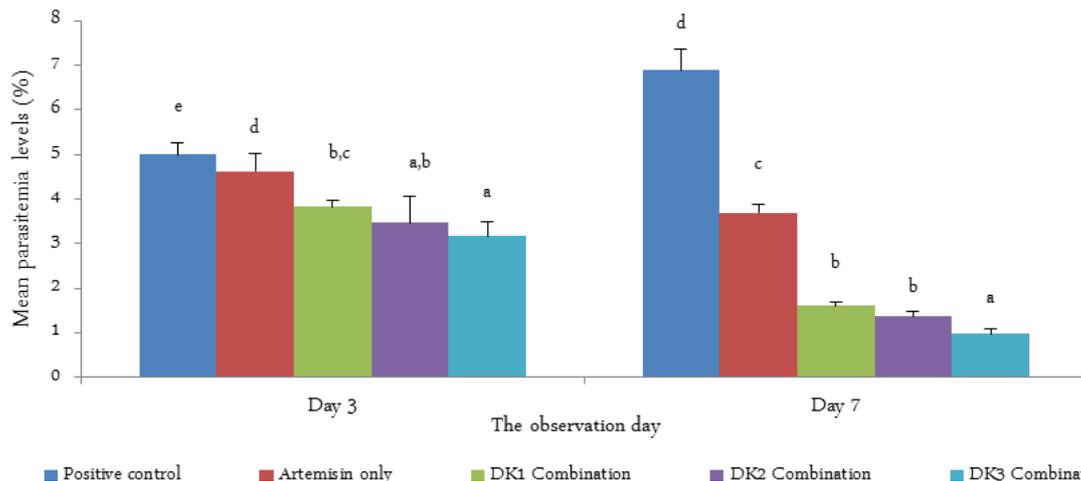


Figure 2. The difference in parasitemia levels between the groups on day-3 and day-7. The differences in the reading obtained on these days indicated a significant variation in each group's readings (a significance value of p < 0.05), whereas, readings of the same value demonstrated that there was no significant difference (p > 0.05) on day-3. Also the parasitemia levels of the Artemisin only group were lower than those of the Positive control group (p= 0.049) but higher than those of the DK1 (p = 0.049), DK2 (p = 0.049), and DK3 (p = 0.049) groups. Furthermore, on day-7, the parasitemia levels of Artemisin only group were lower than those of the Positive control group (p= 0.000), yet they were higher than those of the DK1 (p = 0.000), DK2 (p = 0.000), and DK3 (p = 0.000) combination groups. The lowest parasitemia levels on day-7 were found in the DK3 group (Artemisin 0.004 mg/gBW and 0.500 mg/gBW *M. oleifera* leaf extract).

groups, namely, that the level for the Artemisin only group was lower than that for positive control group (no treatment) with p= 0.049, however, it was higher than that of the groups treated with an Artemisin and M. oleifera leaf extract where for DK1, DK2 and DK3 p= 0.049 respectively. The results the One Way ANOVA Test on the differences in parasitemia levels between groups on Day-7 employed a post hoc Tukey analysis, which revealed that there was indeed a differ-

ence in parasitemia levels between groups, that the levels in the Artemisin only group where p= 0.000, although it was still higher than those respectively. The differences in parasitemia levels groups on Day-3 and Day-7 are laid out in Figure 2.

The results of this study demonstrate that the administration of Artemisin in doses of 0.004 mL/gBW/day for 7 days was able to reduce parasitemia levels [5, 14] with an inhibition concentration (IC50)

of 46%. Parasitemia levels in the groups given the combination treatment, i.e. DK1, DK2 and DK3, were lower than those in the Artemisin only group, namely, with an IC50 of 77%, 80% and 86% respectively. This was possibly due to the skizonticidal effect of the extract as proved by Patel et.al (2010) in their in vitro research [15]. Where Artemisin treatment has reached half life or where the therapy has not been completed, it is possible that some parasites still survive and could replicate resulting in its parasitemia levels being higher in the Artemisin only group than those in the groups receiving the combination therapy. This study also confirmed that there was a relationship between Artemisin with the *M. oleifera* extract combination and parasitemia levels. The Pearson correlation test results for the samples collected on Day-3 showed that there was a negative correlation with coefficient correlation value of −0.834 (p= 0.002), in addition, a Spearman correlation test result on Day-7 found a negative correlation with coefficient correlation value of -0.968 (p = 0.000). This negative correlation indicates that the higher the combination dose, the lower the parasitemia levels and visa versa. Furthermore, this research discovered that the lowest parasitemia level was obtained in the group that was administered the highest doses of the Artemisin and *M. oleifera* leaf extract combination, i.e., DK3, with doses of 0.004 mL/gBW Artemisin and 0.500 mg/gBW/day *M. oleifera* leaf extract.

### The differences of CD4+ T cells percentage

Analysis of the differences in CD4+ T cells percentage between day-3 and day-7 indicated a significant decrease in the KP (p= 0.017), but a significant increase in the Artemisin only group (p= 0.032) as well as in the DK1 (p= 0.032), and DK2 (p= 0.011) groups, whereas, in the DK3 there was no significant difference (p= 0.051). The differences CD4+ T cells percentage between day-3 and day-7 are shown in Figure 3.

The results of a one-way ANOVA test on CD4+ T cells percentage on day-3 showed that there was a significant difference between groups. The results of an analysis from a post hoc Tukey tests showed that the CD4+ T cells percentage from the Artemisin only group were not significantly different from those from the KP (p = 0.268) but lower compared to those from DK1 (p = 0.007), DK2 (p = 0.000), and DK3 (p = 0.000) groups. On day 7, CD4+ T cells percentage in the Artemisin only group were higher than those in the KP group (p= 0.002), whereas, they were still lower than those found in the DK2 (p = 0.001) and DK3 (p = 0.000) groups, yet, there was no significant difference

when compared to the DK1 group's percentage. These differences are laid out in Figure 4.

The low levels of CD4+ T cells percentage in the KP group compared to those in all the other groups both on day-3 and 7 are in agreement with the results of Malaguanera and Musumeci's (2002) results which found that immune suppression of CD4+ T cells percentage had occurred in mice infected with malaria. This immune suppression was caused by the accumulation of parasite hemozoine pigment in immune cells such as monocytes/ macrophages which went on to interfere with phagocyte function. In addition, hemozoin also inhibits cell immune response to IFN-γ and thus in CD4+ T cells activity. Furthermore, Dogruman et. al (2009) discovered that CD4+ T lymphocyte count in malaria infected mice were also lower [5].

The main flavonoids found in *M. oleifera* leaf extract are quercetin and kaempherol. Quercetin gives protection against oxidative stress and reduces its damage to lymphocytes improves immune cell function. Also, quercetin can reduce excess macrophage activity, lower the excretion of IL-1β and IL−6 both of which have roles in the production of pro inflammation cytokine [17, 18, 19]. Kaempherol can help by interfering with the formation of reactive oxygen species (ROS) such as super oxide anion, radical hydroxyl and peroxinitrite, by reducing intracellular ROS oxidative stress as well as the activity of ROS producing enzymes (such as xanthine oxidase) as well as NF−kB activities in expressing pro-inflammation cytokine [17, 18, 19]. It can also help Reactive Oxygen Species (ROS) such as superoxide anion, reduce intracellular ROS levels that induces oxidative stress and the activity of ROS producing enzymes (such as xanthine oxidase) as well as Nf-kb activities in expressing pro-inflammation cytokine (TNF-α, IL-1, IL-6, IL-8, COX-2, and iNOS) [20, 21, 22]. The 3 groups which received the Artemisin/ leaf extract combination (DK1, DK2, DK3) had higher antioxidant, anti-inflammation and CD4+ T cells counts than those from the Artemisin and KP groups. These findings agree with Gaikwad et. al (2011), which demonstrated that there had been a rise in the humoral and cellular immune systems through increases in humoral titer antibodies and in T and macrophage of M. oleifera leaf extract over 7 days [17]. In addition, Banji et al's (2012) research found that the flavonoid content in *M. oleifera* leaves activated lymphocytes [20, 21, 22]. In this study, the results of the Pearson correlation test between the Artemisin-*M. oleifera* leaf extract combinations and CD4+ T cells percentage found a positive correlation (the correlation coefficient value was 0.966
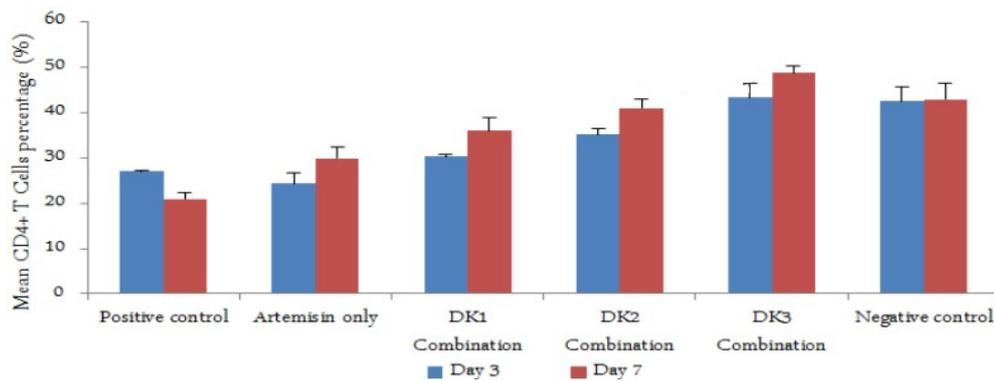
Figure 3. The differences in CD4+ T cells percentage between day-3 and day-7. In the Positive control group there was a significant fall in CD4+ T cells percentage (p= 0.017), whereas, in the Artemisin only group there was a significant rise (p= 0.032) as well as in the DK1 (p= 0.032) and DK2 (p= 0.011) groups. However, for the DK3 group there was no significant difference (p= 0.052). The significance value was p<0.05. All group results were analyzed by paired T-tests. The results were mean + standard deviation.
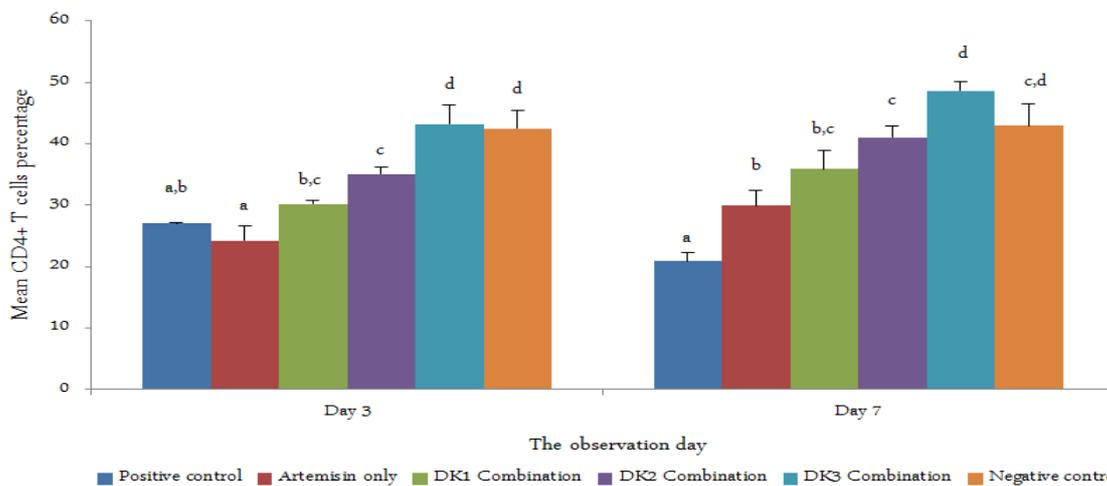


Figure 4. The differences in CD4+ T cells percentage between groups for days 3 and 7. The variations in the figures for the above days indicates there were significant differences in these cell percentages (p < 0.05 being a significant value), on the other hand, results with the same figures for individual counts show there were no significant differences (p > 0.05). On day-3, the CD4+ T cells levels in the Artemisin only group were not significantly different from those in the positive control group (p = 0.268), but they were lower than those of DK1 (p = 0.007), DK2 (p = 0.000) and DK3 (p = 0.000) groups. On day-7, CD4+ T cells percentage rise in the Artemisin only group and became higher than those from the positive control group (p= 0.002), yet they were lower than those from the DK2 (p= 0.001 and DK3 groups (p = 0.000). The highest CD4+ T cells percentage were from day-7 in the DK3 group, the group that received the highest doses of Artemisin and M. oleifera leaf extract combination.

with p=0.000 and in day 3 it was 0.961 and p=0.000) on day 7. This correlation indicates that the higher the doses of Artemisin-M. oleifera leaf extract combination, the higher the CD4+ T cells percentage and visa versa. In this research, the highest CD4+ T cells percentage were found in the group receiving the highest doses of the M. oleifera combination, namely, DK3 (Artemisin 0.004 mg/gBW and 0.500 mg/g/gBW/day M. oleifera leaf extract).

## The differences of CD8+ T cells percentage

Analyses of the differences in CD8+ T cell levels between those for Day-3 and Day-7 between each group indicate that there was a significant drop in the Artemisin only group (p = 0.022) and for the DK1 combination it was p = 0.047, whereas for the others (DK2 and DK3) there was no significant difference where p = 0.067, p = 0.746 and p = 0.807 respectively, as laid out in Figure 5.
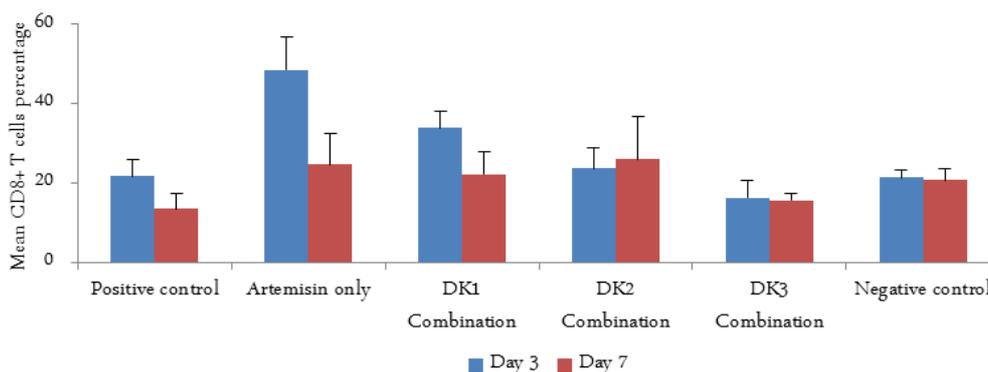
Figure 5. The differences in CD4+ T cells percentage between day-3 and day-7. In the Positive control group there was a significant fall in CD4+ T cells percentage (p = 0.017), whereas, in the Artemisin only group there was a significant rise (p = 0.032) as well as in the DK1 (p = 0.032) and DK2 (p = 0.011) groups. However, for the DK3 group there was no significant difference (p = 0.052). The significance value was p<0.05. All group results were analyzed by paired T-tests. The results were mean + standard deviation.
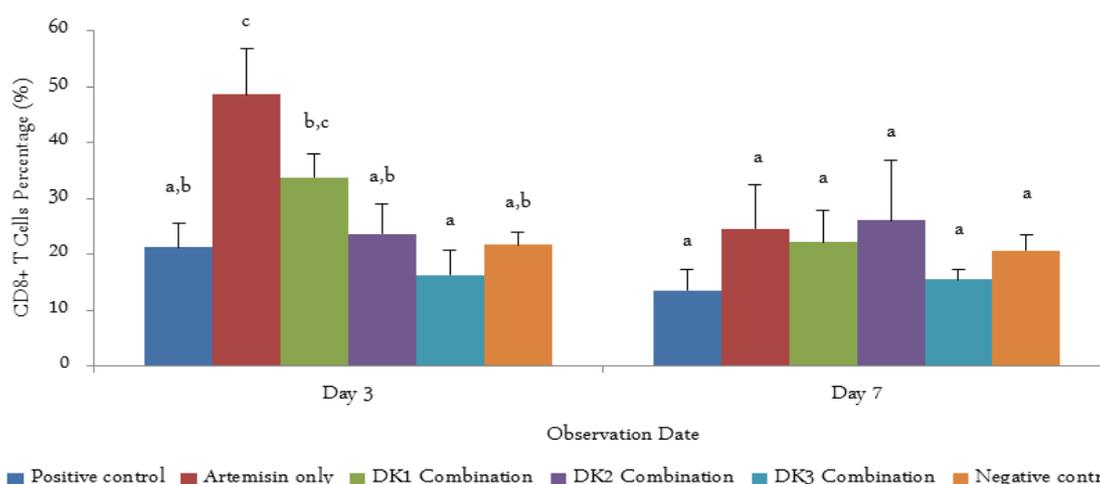


Figure 6. The differences in CD8+ T cells percentage between groups for days 3 and 7. The variations in the figures for the above days indicates there were significant differences in CD4+ T cells percentages (p < 0.05 being a significant value), on the other hand, results with the same figures for individual counts show there were no significant differences (p > 0.05). On day-3, the CD8+ T cells percentage in the Artemisin only group were higher than positive control group (p = 0.001), DK2 (p = 0.002), and DK3 (p = 0.000) as well as KN groups. On day-7, there were no significant differences CD8+ T cells percentage between groups (p > 0.05).

The results of the One-Way ANOVA test for CD8+ T cell percentage on Day-3 show that there was a significant difference in levels between groups. The results of the post hoc Tukey test revealed that the CD8+ T cell percentage for the Artemisin only group were higher than those of the positive control group (p = 0.001) and for DK2 it was p = 0.002, DK3 p = 0.000 and for the Negative control group p= 0.100. The results of the One-Way ANOVA test for CD8+ T cell percentage differences between groups on Day-7 showed that a significant difference of p = 0.147. Those differences are shown in Figure 6.

The Pearson correlation test analysis between the Artemisin with the *M. oleifera* leaf extract combination and CD8+ T cells percentage on Day-3 found a negative correlation with a correlation coefficient value of -0.920 (p = 0.000). This negative correlation indicates that the higher the doses of the Artemisin and Moringa oleifera leaf extract combination, the lower the percentage of CD8+ T cells and visa versa, whereas, the results of the Spearman correlation test on Day-7 showed that there was a significant relationship (p = 0.082). However, the highest percentage of CD8+ T cells on Day-3 were found in the Artemisin only group which is possibly due to CD8+ T cell immune activity in the early

stages on the *Plasmodium* infection, while parasitemia levels were still high [17, 21]. The high percentage of CD8+ T cells on Day-3 in the Artemisin only group compared to the that of the combination treatment groups could have been caused by a disturbance in the IFN-γ response when activating the CD8+ T cells to function as intrahepatocyte phagositosis [13, 16]. In addition, CD8+ T cells percentage in the Positive control group were the lowest of all the groups. This demonstrates that while the *Plasmodium* infection lasted, there was a drop in CD8+ T cell percentage [4], and where parasitemia levels from the Positive control group (infected no treatment).

Based on the above data, it is clear that the combination Artemisin with *M. oleifera* leaf extract by day-7 had produced lower levels of parasitemia and higher percentage of CD4+ T cells than those from the Artemisin only group. Furthermore, there was a negative correlation between the Artemisin with the *M. oleifera* leaf extract combination and parasitemia levels. A positive correlation between the Artemisin with the *M. oleifera* leaf extract combination on CD4+ T cells percentage. Whereas, for the CD8+ T cell percentage there was no significant difference between the Artemisin only group and the Artemisin and M. oleifera leaf extract combination groups.

## CONCLUSION

This research found that the combination of Artemisin and *M. oleifera* leaf extract over 7 days increased the percentage of CD4+ T cells for the DK2 group by p=0.001 and the DK3 group by p=0.000 and reduced parasitemia levels by p=0.000 in DK1, DK2 and DK3 respectively, whereas, levels of CD8+ T cells did not rise. And the combination did have an effect on parasitemia levels in the treated groups (p=0.000) and the percentage of CD4+ T cells (p=0.000) but not on that for CD8+ T cells.

## ACKNOWLEDGMENT

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