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Annona muricata Linn and *Khaya grandifoliola* C.DC. Reduce Oxidative Stress *In Vitro* and Ameliorate *Plasmodium berghei*-Induced Parasitemia and Cytokines in BALB/c Mice

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Abstract

Background. Annona muricata and *Khaya grandifoliola* are ethnomedicinally used for the treatment of malaria and have been experimentally shown to have an anti-plasmodial effect, but the mechanisms involved are not fully understood. This study investigated the effect of the ethanol extracts of their leaves on parasitemia, radical scavenging and cytokines in *Plasmodium berghei* ANKA-infected BALB/c mice. *Methods. BALB/c* mice were infected with P. *berghei* and treated with chloroquine, *A. muricata* or *K. grandifoliola* extract for 4 days. The percentage of parasitemia and the level of cytokine expression were determined after treatment. Trace element, phytochemical and nitric oxide (NO) scavenging activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging properties assays were done to study the antioxidant effects of AN and KG *in vitro. Results. P. berghei* consistently increased parasitemia in *BALB/c* mice. The tested doses (100-, 200-, and 400 mg/kg) of *A. muricata* and *K. grandifoliola* attenuated the *P. berghei*-induced elevation of parasitemia and cytokines (TNF-α, IL-5, and IL-6) *in vivo* during the experimental period, though not as much as chloroquine. Moreover, both extracts scavenged the DPPH and NO radicals, though *A. muricata* had more anti-oxidant effect than *K. grandifoliola in-vitro. Conclusion.* The ethanol extracts of *A. muricata* and *K. grandifoliola* reduce parasitemia in *P. berghei*-treated mice BALB/c by scavenging free radicals and reducing cytokines, though the extracts were not as effective as chloroquine.

Keywords

Annona muricata, *Khaya grandifoliola*, Plasmodium berghei, BALB/c mice, cytokine inhibition, cerebral malaria

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Introduction

Malaria is caused in humans by the six (6) Plasmodium species (among over 100 species) including *P. falciparum*, *P. vivax*, *P. ovale, P. malariae, P. simiu* and *P. knowlesi*.[1] *P. falciparum* and *P. vivax* are the 2 species that mostly cause infection with severe anemia, though the most severe form of the disease (cerebral malaria, CM) is caused by *P. falciparum.*[2] Malaria is a tropical disease with a serious public health burden worldwide and a significant impact on Sub-Saharan Africa, as it is the major cause of mortality in middle-, low-income and developing countries. Globally, the WHO reported 200 million new cases of Plasmodium infections and 435,000 deaths in 2018.[3] About 106 countries are considered endemic to malaria infection, with a 3.3 billion population at risk.[4] In Uganda, 95% of the population is at risk of malaria infection,[5] which kills 70,000 and 100,000 children and pregnant women respectively each year.[6] The highest proportion of the malaria burden in Uganda affects children and pregnant women living in resource-limited settings such as hard-to-reach rural settlements, and poor people with limited access to health care services coupled with a lack of education.[7]

The CM is an encephalopathy with severe neurological impediment,[8] and often, less than 20% of cases survive with neurological disabilities[9] including cognitive and speech deficits, motor alterations and cortical blindness.[10] There are several pathophysiological processes involved in CM syndrome. For instance, investigations have shown that disproportionate signals in the pro- and anti-inflammatory immune response, a disruption of the blood-brain barrier and an activation of the endothelial cell system are fundamental processes in the pathophysiological development of CM.[11] Specifically, the overproductions of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), gamma interferon (IFN-γ), interleukins, macrophage colony-stimulating factor (M-CSF), and lymphotoxin[12] are pieces of evidence of the pathogenesis of CM.

Bioactive components from plant derivatives have been a pivotal source for the development of antimalarial therapeutics. For examples, chloroquine/quinine (*Cinchona)*[13] and artemisinin (herbal tree *Artemisia annua)*[14] are derived from plant constituents. Since the resistance against chloroquine was reported in 1957 in Thailand[15] and in 1976 in India,[16] malaria-endemic and the development of resistance to the current treatment spread globally, which has made malaria to be of great public health concern. A resistant strain of artemisinin and its derivatives,[17] which are presently the first line of antimalarial drugs, was reported in the Thai-Cambodia region in 2009.[18] Therefore, the need to search for new active antimalarial agents is important.

Evidence-based academic reports recognize *Annona muricata* Linn ethnomedicinal application in tropical regions for the management and treatment of diverse ailments, including fever,[19] pain, respiratory and skin illness, internal and external parasitic infections, bacterial infections,[19] inflammation,[20] diabetes[21] and cancer.[22] Apart from the local use of *A. muricata* in Uganda,[23] Togo, Cameroon and Vietnam[24] to treat malaria, studies have reported the antimalarial activity of *A. muricata* in *P. berghei* ANKA[25] and *P. falciparum*[26] infection models. Similarly, *Khaya grandifoliola* C.DC. has shown significant pharmacological potential as a hepatoprotective, antiviral and anti-inflammatory agent.[27] Apart from the folk medicinal use of *K. grandifoliola* in Uganda[28] and Nigeria to treat malaria and its symptoms like fever, its anti-malarial effect has been demonstrated against *P. berghei* and *P. falciparum* models of malaria.[29] However, the mechanisms of their antimalarial effect need to be investigated.

Even though they can't perfectly mimic the full human syndrome of the disease, malaria models have been developed in mice, monkey, and rats and they faithfully represent certain aspects of human malaria. The similarities between the human and murine antigens and immune response pathway have made the mouse a model of choice in malaria studies. Moreover, *P. berghei* is the most widely used parasite model of severe malaria infection in rodents because it sequesters within the microcirculation, which is the major characteristic of severe cerebral malaria.[30] In this study, the antimalarial effect and the mechanism of action of the *A. muricata* and *K. grandifoliola* leaf extracts in *P. berghei*-infected BALB/c mice were investigated.

Methods

Plant Collection Authentication and Extractions

A. muricata and *K. grandifoliola* were harvested from the Rubirizi district, Southwest of Uganda. The specimen was authenticated by a taxonomist, and the identification number (HOPE-PHA-2019/01, 2019/02) was left in the School of Pharmacy herbarium, Kampala International University, Uganda, for future citation/references.

Fresh plant leaves were crammed into sterile polythene bags and transported to the Department of Pharmacology Laboratory. The samples were washed and air-dried at room temperature for approximately 1 month, crushed into powder using a clean mortar and pestle, and stored in the desiccator until ready for use. *A. muricata* and *K. grandifoliola* ethanolic extracts were obtained by applying the method described by.[31] Briefly, 150 g of the plant powder was macerated on a separate jar in 900 ml of absolute ethanol and agitated every 24 hrs for 3 days. The solvent was decanted and filtered with Whatman No. 1 filter paper, followed by evaporation in a water bath at a temperature of 400°C and kept in the refrigerator until ready to use.

Trace Element Analysis

An atomic absorption spectrophotometer (AAS 969 Unicam Solar 32) was used to analyze the essential mineral content of the *A. muricata* and *K. grandifoliola* ethanolic extracts following the protocols of AOAC 2000, while Na and K were evaluated by flame photometry (JENWAY PF7).

Phytochemical Analysis

The presence of phytochemicals such as alkaloids, tannins, saponins, flavonoids, steroids, cardioglycosides, anthraquinones and triterpenoids was determined by standard methods reported by Trease and Evans.[32]

Induction of Parasitemia and Experimental Treatment Regimens

The chloroquine-sensitive *P. berghei* strain was sourced from the Uganda Medical Research Institute, Kampala. Mice were infected intraperitoneally (i.p.) with 0.2 10^7 ml parasitized red blood cells (RBCs). Before the commencement of the study, the number of parasitemia was observed and counted with a hemocytometer, while the parasites were adjusted to 0.5×10^6 in phosphate-buffered saline (PBS) sterile solution. Aside from the 4 mice that were used for the adjustment of parasitemia and another 15 mice used for the toxicity study, a total of 54 mice were divided into 9 groups (n = 6 per group) following the method of Charan and Kantharia[33] for the determination of animal sample size. The treatment groups were intraperitoneally injected with 200 µl inoculum of 0.5×10^6 parasites (named Day 0), and treatment started on day 1 up to day 4.

Group 1—Control group: parasitemia (day 0, i.p.) + distill water (placebo)
Group 2—Treatment group: parasitemia (day 0, i.p.) + *A. muticata* (100 mg/kg, p.o., day 1-4)
Group 3—Treatment group: parasitemia (day 0, i.p.) + *A. muticata* (200 mg/kg, p.o., day 1-4)
Group 4—Treatment group: parasitemia (day 0, i.p.) + *A. muticata* (400 mg/kg, p.o., day 1-4)
Group 5—Treatment group: parasitemia (day 0, i.p.) + *K. grandifoliola* (100 mg/kg, p.o., day 1-4)
Group 6—Treatment group: parasitemia (day 0, i.p.) + *K. grandifoliola* (200 mg/kg, p.o., day 1-4)
Group 7—Treatment group: parasitemia (day 0, i.p.) + *K. grandifoliola* (400 mg/kg, p.o., day 1-4)
Group 8—Drug control group: parasitemia (day 0, i.p.) + Chloroquine (5mg/kg, p.o., day 1-4)
Group 9—Normal group: distilled water (day 0-4).

Before treatments, indicator and physical signs of illness[30] such as piloerection, lethargy, reduction in locomotor, and passage of dark urine were checked in the parasite-infected mice and control on days 1 and 2. It was observed to be moderate in the groups on day 2, which became severe in the untreated mice on day 4 and mild in the other groups.

Antimalarial Activity of the Extracts

Thin blood smears were prepared from tail blood for days 0-4 for the percentage parasitemia level and suppressive test. The smear was fixed with methanol, allowed to dry for 15 min and stained with 10% Giemsa stain at pH 7.2 for 15 min and acridine orange. Afterward, the stained slides were washed and allowed to dry at room temperature. The slides for each mouse were examined under a UV illumination microscope (Olympus) with an oil immersion adjusted objective of 100× magnification. Each slide in different fields was examined, and the number of parasitemia counts was expressed as shown below.

% Parasitemia = (Number of parasitized RBCs / Total number of RBCs counted) × 100
% suppression = mean parasitemia of untreated group / Mean parasitemia of treated group X 100

Antioxidant Activity of the Extracts

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the ethanolic extracts. The scavenging ability of the extracts for free radicals was measured by assaying the DPPH radical scavenging activity as reported by Braca et al[34] An aliquot of 2 ml of 0.04 % DPPH solution in ethanol was mixed with 1.0 ml of each ethanolic extract/ascorbic acid of different concentrations (20, 40, 60, 80, 100 mg/ml) and vortexed and then allowed to attain a steady-state for 30 minutes at room temperature in a dark chamber. The inhibitory activity of DPPH by the extracts was determined by measuring the absorbance at 517 nm against a control (1 ml ethanol plus 2 ml of DPPH). The percentage inhibition of DPPH radical scavenging ability of the extract was expressed as follows:

% Inhibition = [(Ac-As)/ Ac] * 100, where Ac is absorbance of control; As is defined as the absorbance of extracts/ascorbic acid.

Nitric oxide scavenging activity of the ethanolic extracts. The nitric oxide (NO) scavenging activity of the ethanolic extracts was measured following the protocol of Braca et al, (2001).[34] A 0.5 ml aliquot of the different concentrations (20, 40, 60, 80 and 100 µ/ml) was mixed with 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4). The assortment was incubated at room temperature for 2 hours and 30 minutes. Afterward, 1 ml of incubated solution was added to 1 ml of Nedd reagent and incubated at room temperature for 30 minutes. Absorbance was measured using a spectrophotometer at 540 nm. Ascorbic acid was used as a standard. Blank was 1 ml of water, 2 ml of sodium nitroprusside and 1 ml of Nedd reagent. The control was 2 ml of sodium nitroprusside, 0.5 ml of phosphate buffer, 1 ml of Nedd reagent and 0.5 ml of methanol. The number of nitric oxide radicals scavenged was determined using the formula shown below:

% Inhibition = [(Ac-As)/ Ac] * 100

where Ac = Absorbance of the control and As = Absorbance of the plant extract.

Acute Toxicity Test

The standard protocol of CDER (1996) and WHO (1992) to investigate the acute toxicity of the extracts was adopted as described before[35] while the LD_{50} values for the 2 extracts were calculated according to Miller and Tainter.[36] A total of 25 mice was used for the toxicity study.

Cytokine Assays

Blood was collected from all groups by inserting a needle into the vein starting at the tip of the tail[37,38] days 1, 3 and 6. The blood was centrifuged at 1,000 g for 10 min to obtain serum. Cytokine ELISA kits (R&D System, USA) for tumor necrosis factor-alpha (TNF-α) and interleukin (IL-5 and IL-6) were assayed according to the manufacturer's instructions.

Statistical Analysis

Data were blindly analyzed with SPSS version 16.0 and expressed as the mean ± standard error of the mean. The percentage inhibition of DPPH and NO and the levels of cytokines were compared by 2-way analysis of variance (ANOVA), followed by the posthoc Bonferroni test for multiple comparisons. To control for the differences in the baseline value (which is a co-variate) of the parasitemic rats, analysis

Table 1. Phytochemical Screening of the Ethanol Extracts of *A. muricata* and *K. grandifoliola.*

Phytochemicals	*A. muricata* and	*K. grandifoliola*
Flavonoid	+	+
Saponins	+	+
Tannins	+	+
Steroids	−	−
Alkaloids	+	+
Anthraquinones	−	+
Triterpenoids	+	+
Reducing Sugar	+	+
Cardiac glycoside	+	+

+, Present; −, Absent.

Table 2. Trace Elements in the Ethanol Extracts of *A. muricata* and *K. grandifoliola.*

Elements (ppm)	*A. muricata*	*K. grandifoliola*
Sodium	39	30.443
Potassium	56.121	15.621
Magnesium	34.508	10.411
Iron	5.008	3.067
Lead	0.332	0
Copper	0.424	1.016
Manganese	0.201	0.121
Zinc	2.899	0.100
Nickel	0.022	0

of covariance (ANCOVA) was used to compare the parasitemia in all the groups, followed by a posthoc Bonferroni test. $P < 0.005$ was considered statistically significant.

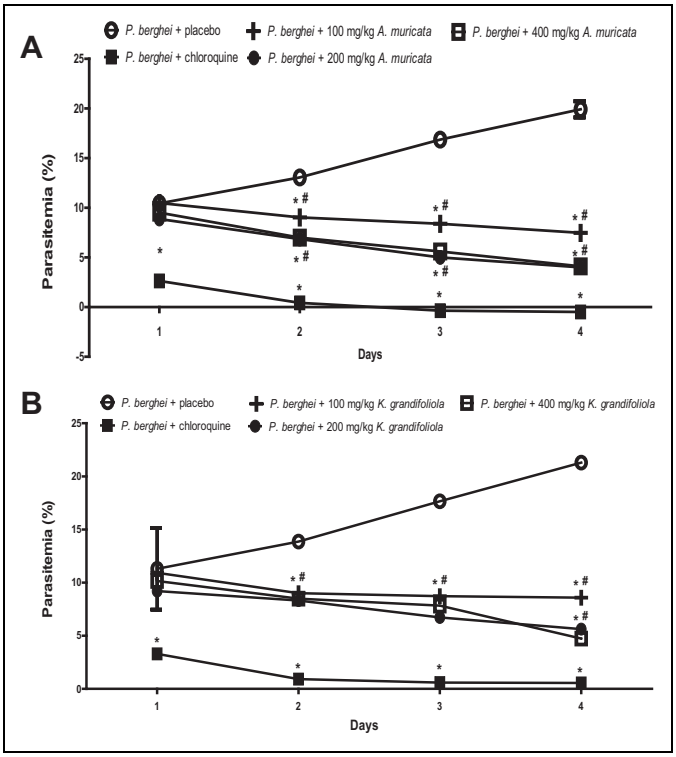

Figure 1. *A. muricata* (A) and *K. grandifoliola* (B) extracts reduced parasitemia in *P. berghei*-infected BALB/c mice. *$P < 0.05$ vs. *P. berghei* + placebo of the same day; #$p < 0.05$ vs. *P. berghei* + chloroquine of the same day.

Results

Phytochemical and Trace Elements in the AM and K. grandifoliola Extracts

Both *A. muricata* and *K. grandifoliola* contain flavonoids, saponnins, tannins, alkaloids, triterpenoids, reducing sugars and cardiac glycosides but not steroids (Table 1). Moreover, the *A. muricata* and *K. grandifoliola* had 9 and 7 essential elements respectively, with sodium (39 mg/100 g and 30.443 mg/100 g) and potassium (56.121 mg/100 g and 15.621 mg/100 g) respectively dominating both extracts (Table 2).

Acute Toxicity Test

There were no behavioral changes observed after the administration of the different doses of both extracts, and no death was recorded up to 7 days of the observation period. There was also a reduced aggressiveness and movement among the extract-treated groups compared to the control group during the first 4 hours. These indicate that the extracts are relatively safe at 2500 mg/kg with a greater LD_{50}. Therefore, the extracts are considered safe at the tested doses and practically nontoxic using Cotonat J (1996)[39] classification of a range of LD_{50}.

A. muricata and K. grandifoliola Reduced Parasitemia in P. berghei-Infected BALB/c Mice

Parasitemia was increased in *P. berghei*-infected BALB/c mice treated with placebo but reduced in *P. berghei*-infected BALB/c mice treated with *A. muricata*, *K. grandifoliola*, and chloroquine. In order to determine the impacts of *A. muricata* and *K. grandifoliola* on the daily parasitemia level, the adjusted means in rats that received *A. muricata* or *K. grandifoliola* was compared to those that received placebo and chloroquine. It was observed that none of the 3 doses of *A. muricata* and *K. grandifoliola* reduced parasitemia as much as chloroquine (Figure 1).

On day 1, only 200 mg/kg but not 100 mg/kg and 400 mg/kg of *A. muricata* significantly reduced the parasitemia in *P. berghei*-infected BALB/c mice when compared to the infected mice treated with placebo. Furthermore, all doses of *A. muricata* significantly reduced the parasitemia at days 2-4 when compared to the corresponding level in the infected mice treated with placebo (Figure 1A).

Similarly, on day 1, only 200 mg/kg but not 100 mg/kg and 400 mg/kg of *K. grandifoliola* significantly reduced the parasitemia in *P. berghei*-infected BALB/c mice when compared to the infected mice treated with placebo. Also, all doses of

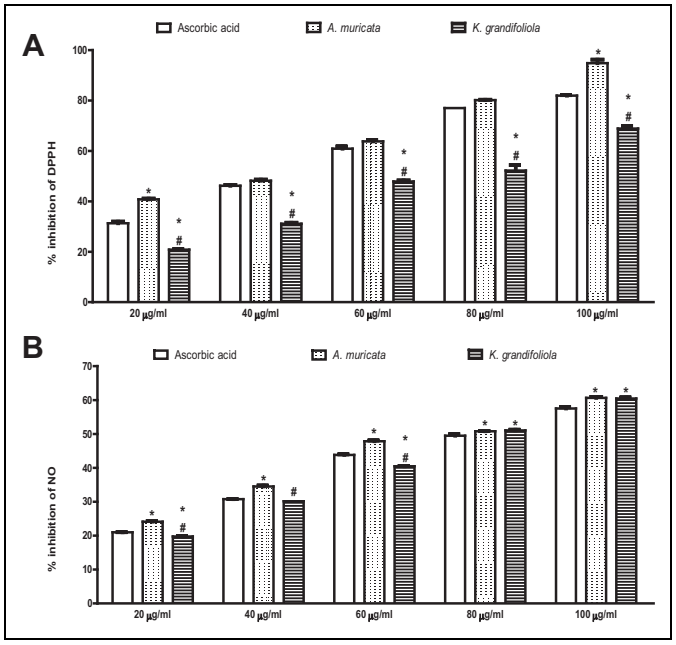

Figure 2. *A. muricata* and *K. grandifoliola* extracts scavenge DPPH (A) & NO (B) radicals *in vitro*. Each bar represents the mean ± S.E.M of triplicate experiments. DPPH, 2,2-diphenyl-1-picrylhydrazyl; NO, Nitric oxide; $^{*}p < 0.05$ vs. ascorbic acid of the same dose; $^{\#}p < 0.05$ vs. *A. muricata* L of the same dose.

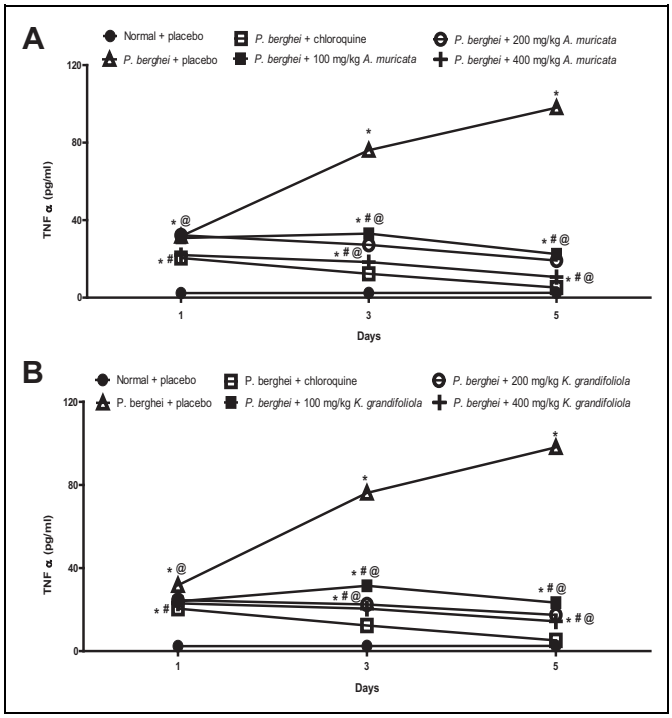

Figure 3. *A. muricata* (A) and *K. grandifoliola* (B) extracts reduced TNF-α in *P. berghei*-infected BALB/c mice. $^{*}p < 0.05$ vs. normal + placebo of the same day; $^{\#}p < 0.05$ vs. *P. berghei* + placebo of the same day; $^{@}p < 0.05$ vs. *P. berghei* + chloroquine of the same day.

K. grandifoliola significantly reduced the parasitemia at days 2-4 when compared to the corresponding level in the infected mice treated with placebo (Figure 1B).

The percentage suppression of parasitemia by the extracts showed the same pattern as the parasitemia data, and is therefore not shown in the figures.

In-Vitro Antioxidant Activity of the Ethanolic Extracts of A. muricata and K. grandifoliola

The *A. muricata* caused higher percentage inhibition of DPPH radical at 20 µg/ml and 100 µg/ml but the same percentage inhibition of DPPH radical as ascorbic acid at 40 µg/ml, 60 µg/ml and 80 µg/ml. On the contrary, all the doses of *K. grandifoliola* caused lower percentage inhibition of DPPH radical than ascorbic acid. Comparatively, *K. grandifoliola* caused lower percentage inhibition of DPPH radical than *A. muricata* across all the doses, even though the percentage inhibition of both extracts increased in a dose-dependent manner (Figure 2A).

Similarly, *A. muricata* caused higher percentage inhibition of NO radical than ascorbic acid at all the tested doses. On the contrary, the percentage inhibition of NO radical by *K. grandifoliola* was lower at the doses of 20 µg/ml and 60 µg/ml but higher at the doses of 80 µg/ml and 100 µg/ml and similar at the dose of 40 µg/ml when compared to ascorbic acid. Comparatively, *K. grandifoliola* caused lower percentage inhibition of NO radical than *A. muricata* at the doses of 20 µg/ml, 40 µg/ml, and 60 µg/ml but the same percentage inhibition as *A. muricata* at the doses of 80 µg/ml and 100 µg/ml, even though the percentage inhibition of both extracts increased in a dose-dependent manner (Figure 2B).

A. muricata and K. grandifoliola Reduced TNF- α in P. berghei-Infected BALB/c Mice

The TNF-α was increased in *P. berghei*-infected BALB/c mice treated with placebo but reduced in *P. berghei*-infected BALB/c mice treated with *A. muricata*, *K. grandifoliola*, and chloroquine while it does not change in non-infected mice that received placebo. To determine the impacts of *A. muricata* and *K. grandifoliola* on the daily TNF-α level, the means in rats that received *A. muricata* or *K. grandifoliola* was compared to those that received placebo and chloroquine. It was observed that none of the 3 doses of *A. muricata* and *K. grandifoliola* reduced TNF-α level as much as chloroquine (Figure 3).

The TNF-α levels in infected mice treated with all doses of *A. muricata* were higher than the levels in infected mice treated with chloroquine but lower than the level in infected mice treated with placebo throughout the experimental period (Figure 3A).

Similarly, the TNF-α levels in infected mice treated with all doses of *K. grandifoliola* were higher than the levels in infected mice treated with chloroquine but lower than the level in infected mice treated with placebo throughout the experimental period (Figure 3B).

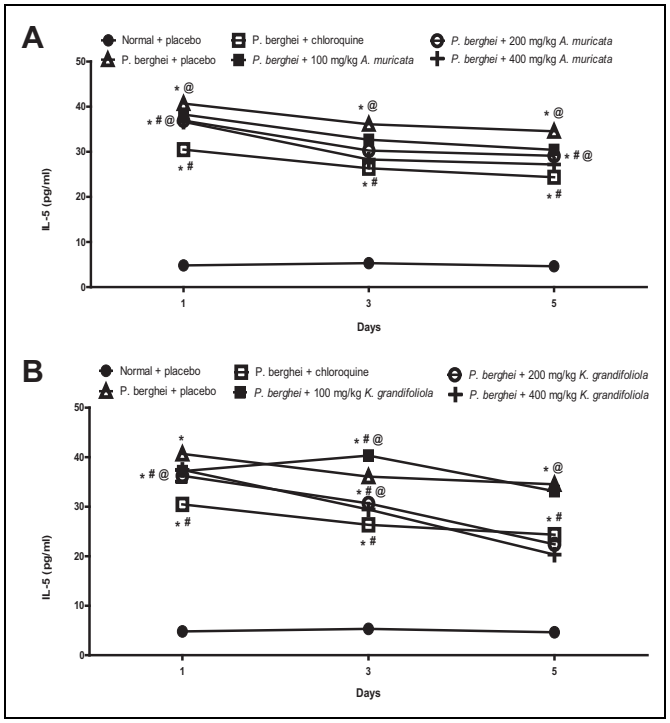

Figure 4. *A. muricata* (A) and *K. grandifoliola* (B) extracts reduced IL-5 in *P. berghei*-infected BALB/c mice. $^{*}p < 0.05$ vs. normal + placebo of the same day; $^{\#}p < 0.05$ vs. *P. berghei* + placebo of the same day; $^{@}p < 0.05$ vs. *P. berghei* + chloroquine of the same day.

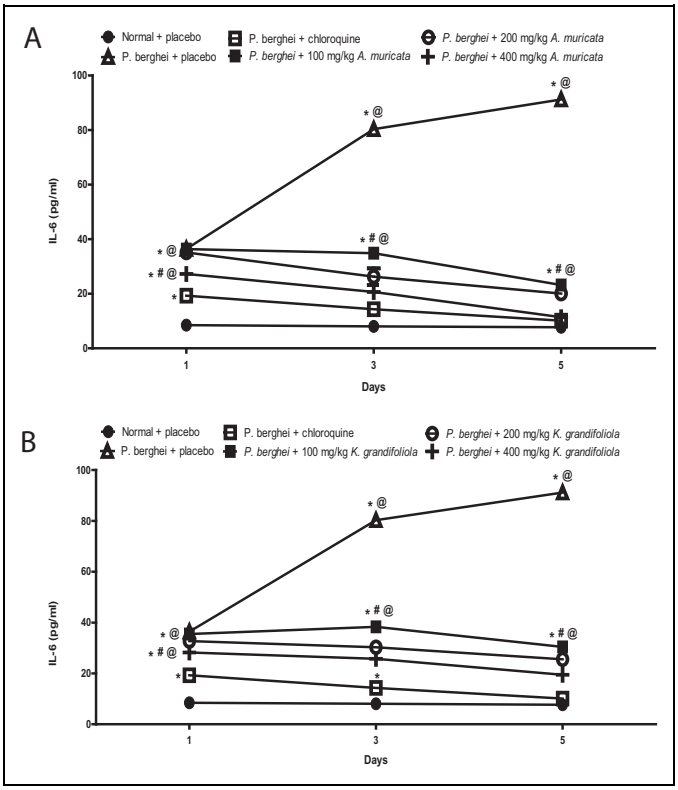

Figure 5. *A. muricata* (A) and *K. grandifoliola* (B) extracts reduced IL-6 in *P. berghei*-infected BALB/c mice. $^{*}p < 0.05$ vs. normal + placebo of the same day; $^{\#}p < 0.05$ vs. *P. berghei* + placebo of the same day; $^{@}p < 0.05$ vs. *P. berghei* + chloroquine of the same day.

A. muricata and K. grandifoliola Reduced IL-5 in P. berghei-Infected BALB/c Mice

The IL-5 was reduced in *P. berghei*-infected BALB/c mice treated with placebo, *A. muricata*, *K. grandifoliola*, and chloroquine while it did not change in non-infected mice that received placebo.

The IL-5 levels in infected mice treated with all doses of *A. muricata* were higher than the levels in infected mice treated with chloroquine but lower than the level in infected mice treated with placebo at throughout the observation period (Figure 4A). Similarly, the IL-5 levels in infected mice treated with all doses of *K. grandifoliola* were higher than the levels in infected mice treated with chloroquine (except those treated with 200 mg/kg and 400 mg/kg for 5 days) but lower than the level in infected mice treated with placebo (Figure 4B).

A. muricata and K. grandifoliola Reduced IL-6 in P. berghei-Infected BALB/c Mice

The IL-6 was increased in *P. berghei*-infected BALB/c mice treated with placebo but reduced in *P. berghei*-infected BALB/c mice treated with *A. muricata*, *K. grandifoliola*, and chloroquine while it does not change in non-infected mice that received placebo. It was observed that none of the 3 doses of *A. muricata* and *K. grandifoliola* reduced TNF-α level as much as chloroquine.

The IL-6 levels in infected mice treated with all doses of *A. muricata* were higher than the levels in infected mice treated with chloroquine but lower than the level in infected mice treated with placebo (Figure 5A). Similarly, the IL-6 levels in infected mice treated with all doses of *K. grandifoliola* were higher than the levels in infected mice treated with chloroquine but lower than the level in infected mice treated with placebo (Figure 5B).

Discussions

The extracts of *A. muricata* and *K. grandifoliola* are widely used as folk medicine in Africa due to their ethnomedicinal importance. The present study examined their antiparasitemic effects and explored their cytokine and free radical inhibitory potentials as possible mechanisms of their herbal treatment of CM induced by *P. berghei*. It was observed that both extracts attenuated the increases in parasitemia and cytokines induced *in-vivo* by *P. berghei*. It was also observed that both extracts scavenged the DPPH and NO radicals *in-vitro*. However, neither *A. muricata* nor *K. grandifoliola* caused anti-parasitemia as much as chloroquine, a known antimalarial drug. This is consistent with a previous report that methanol extract of *A. muricata* inhibits plasmodium parasite *in-vitro*[40] and *in-vivo*.[25]

Is the parasitemia induced by *P. berghei* related to the generation of free radical and enhancement of inflammatory cytokines? The regulatory role of the CNS on the release of TNF-α and other cytokines by the immune system is well known. Some CNS disorders, including cerebral malaria induced by *P. falciparum* and *P. berghei*, are known to modify the release of cytokines in humans and animals. Some clinical features of the CM have been associated with the accumulation of parasitized RBCs in the microvasculature of the brain due to interactions between the over-expressed adhesion molecules (caused by an increase in TNF-α) and parasite proteins. The increased TNF-α release from the host cells to the brain and plasma following exposure to various malarial antigens causes upregulation of adhesion molecule-1 and TNF-α receptors.[41] Studies have also shown that TNF-α production is phase-dependent, with the initial CM phase relating to a reduction in parasitic load while the late phase relates to disease severity.[42] The dual role of TNF-α led us to estimate its levels on different days of the experimental period. As expected, the TNF-α consistently increased in *P. berghei*-induced parasitemic mice when compared to the baseline and also to normal mice throughout the experimental period. This supports the contention that *P. berghei*-induced malaria infection in mice is associated with an increase in TNF-α as reviewed by some authors.[43]

Are interleukins 5 and 6 affected by *P. berghei*-induced malaria infection? The IL-6 is a pleiotropic cytokine that can be produced by many cell types, including T cells, monocytes and endothelial cells, all of which are key to the lesion of CM. Since the synthesis and release of IL-6 are induced by the TNF-α, its trend during the experimental period was investigated. Furthermore, thrombocytopenia is a known complication of malaria[44] and the role of IL-6 in the production of platelets has been well-documented. For instance, patients with reactive thrombocytosis have been reported to show an increase in IL-6,[45] while administration of IL-6 also increases circulating platelets count.[46] Though the present study is limited by the non-availability of data on platelets level, our observation that the IL-6 level increased in *P. berghei*-induced malaria infection is consistent with the report of Raza et al[44] which also observed an increase, albeit insignificant, in IL-6 level in *P. vivax*-associated thrombocytopenic Souther Pakistani population. It was speculated that the increase in IL-6 observed in this study might be associated with the increased TNF-α throughout the experimental period. Our association of increased IL-6 to malaria infection is similar to the previous report of Grau et al[47] who showed that IL-6 is produced in considerable amount during blood-stage infection by the malaria parasite, which is involved in hypergammaglobulinemia rather than in the pathogenesis of cerebral complications. Our data are also consistent with previous studies that reported an increase in IL-5 and IL-6 in various kinds of infectious diseases, including malaria.[48,49] Thus, the elevation of pro- and anti-inflammatory cytokines is consistent with the previous study of Basir et al[30] in *P. berghei*-treated ICR mice.

Plasmodia digest hemoglobin, which leads to the liberation of heme that will eventually trigger the production of ROS, thus implicating free radical in the pathophysiology of malaria.[50] The malaria-induced free radical generation has been reported to cause anemia[51] and apoptosis.[52] Moreover, the formation of free radical intermediates is the mechanism explored by some antimalarial drugs e.g artemisinin for the destruction of plasmodium parasites.[53] The DPPH assay is a commonly employed marker in redox studies of plant extracts or certain compounds within a short period as it provides information on the reactivity of extracts with a stable free radical. On the other hand, a combine USA/Tanzania study has shown that the suppression of plasma NO concentration is involved in the pathogenesis of cerebral malaria, as Tanzanian children with cerebral malaria had decreased plasma and urine nitrogen oxide concentration. Other studies have also shown an inverse relationship between NO and cerebral malaria.[54] Is the anti-parasitemia induced by *A. muricata* and *H. grandifoliola* associated with their anti-oxidant property?

Malarial infection and its consequences have been substantially ameliorated by plants and compounds that have anti-oxidant property. In fact, animal studies have demonstrated that the development of cerebral complications from malaria can be prevented by anti-oxidants. The anti-oxidant property of a polyherbal anti-malarial product was also reported.[55] In our *in-vitro* study, the free radical-scavenging effect of *A. muricata* and *K. grandifoliola* to that of vitamin C, a widely-known anti-oxidant, were compared. It was observed that the *A. muricata* caused higher percentage inhibition of DPPH radical at 20 μg/ml and 100 μg/ml but the same percentage inhibition of DPPH radical as ascorbic acid at 40 μg/ml, 60 μg/ml and 80 μg/ml, while all the doses caused more inhibition of NO than ascorbic acid. On the contrary, the percentage inhibition of DPPH radical was lower in all the doses of *K. grandifoliola* while the inhibition of NO was lower in only 20 μg/ml and 60 μg/ml but higher in 80 μg/ml and 100 μg/ml *K. grandifoliola* when compared to ascorbic acid. Comparatively, *K. grandifoliola* caused lower percentage inhibition of DPPH and NO radicals than *A. muricata*. Thus, our data provide *in-vitro* pieces of evidence that the anti-parasitemic effect of these 2 extracts are mediated by the anti-oxidant mechanism. The comparatively lower anti-oxidant effect of the 2 extracts, when compared to vitamin C, is also consistent with a report that *A. muricata*'s anti-oxidant property was 1000 times less active than the commercial butylated hydroxytoluene.[56]

Is the anti-parasitemic effect of *A. murucata* and *K grandifoliola* mediated by cytokines? Many natural products, including *A. murucata,* have been shown to have an immunomodulatory effect with promising anti-inflammatory effect. Having established the fact that *P. berghei*-induced parasitemia is associated with elevation of cytokines and oxidative stress, it was further investigated if the anti-parasitemic effect of the extracts is associated with cytokines depletion. The plasma levels of TNF-α, IL-5, and IL-6 were all reduced in *P. berghei*-infected mice treated with the extracts compared to the infected mice treated

with placebo, even though none of the extracts was as effective as chloroquine. Our study is consistent with the previous anti-inflammatory report of others where the ethanol extract of *A. muricata* reduced TNF-α, IL-1β and IL-6.[57,58] This is in addition to the modulation of CXCL10 (a strong independent marker of CM) by *A. muricata*.[59]

The extract of *A. muricata* has been reported to contain about 212 identified and isolated secondary metabolites like flavonoids, tannins, saponins, alkaloids, polyphenols, kaempferol, diterpenoids, essential oils, acetogenin compounds and megastigmanes,[60] some of which are known to possess anti-plasmodial and antimalarial effects.[61] More than 120 Acetogenins, which are the most predominant bioactive compounds, have been reported from the leaves, seeds, stems, fruit peel and pulp of *A. muricata,* out of which 46 have been identified from the leaves.[60] Also, around 22 alkaloids[62] and 34 phenolics[63] compounds have been identified in the *A. muricata* leaves. The total phenolic content of *A. muricata* has been positively correlated with its anti-oxidant potential.[64] In this study, it was confirmed that both *A. muricata* and *K. grandifoliola* contain flavonoids, saponins, tannins, alkaloids, triterpenoids, reducing sugars, cardiac glycosides and some essential elements including sodium and potassium. Speculatively, the phytochemicals and elements present in both plant extract could have synergistically contributed to the antimalarial, anti-oxidant and anti-inflammatory effects reported herein.

Conclusion

This study shows that the ethanol extracts of *A. muricata* and *K. grandifoliola* reduce parasitemia in *P. berghei*-treated mice BALB/c b scavenging free radicals and reducing cytokines, though the extracts were not as effective as chloroquine.

Authors' Note

Hope Onohuean conceived, designed, and carried out the study. Hope Onohuean and Abdullateef I. Alagbonsi analyzed and interpreted the data, drafted the manuscript. All authors revised the manuscript, read and make the final corrections. All the authors have read and agreed to the publication of the finding as contained in the manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The experimental protocol was approved by the KIU-Ethics Committee and carried out in accordance with the guidelines given by the Uganda Council for Higher Education (UCHE).

Declaration of Conflicting Interests

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