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Synergy and Antagonism in Antimalarial Crude Extract Combinations

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Abstract

Background: Malaria accounts for around 4.8% of all recorded fatalities in Tanzania. Medicinal plants such as *Caesalpinia bonducella, Azadirachta indica,* and *Annickia kummeriae* have demonstrated promise in treating many diseases, including malaria. However, their combined activity against malaria has yet to be documented. Combination therapy using some medicinal plants with antimalarial activities may enhance safety and efficacy and reduce the evolution of parasite resistance**.**

Objectives: This study aimed to investigate antiplasmodium activities of different combinations of crude extracts from selected medicinal plants. *A. indica leaves*, *A. kummeriae* and *C. bonducella* roots were extracted using dichloromethane (DCM).

Methods: The *in vivo* antiplasmodial activity of individual and combined crude extracts was performed in mice inoculated with *Plasmodium berghei* (ANKA strain) using Peters's 4-day suppressive test.

Results: Individually, *C. bonducella* crude extracts exhibited the highest *in vivo* antiplasmodial efficacy (91% parasite suppression) than *A. kummeriae* (73% parasite suppression) and *A. indica* (60% parasite suppression) at 800 mg/kg/day. The *A. indica* and *A. kummeriae combinations and A. indica and C. bonducella demonstrated higher antiplasmodial activity (synergism-combination index 0.29 and 0.97,* respectively) than their constituents. However, combining *A. kummeriae* and *C. bonducella* produced the lowest antiplasmodial activity (antagonism- combination index 40.67) than its extracts. The high antiplasmodial potencies (ED_{50}) demonstrated by AiAk and AiCb are significant and critical results for traditional, complementary and alternative medicine.

Conclusion: These preliminary findings suggest that AiAk and AiCb are potential antiplasmodium herbal therapies. Further research should be undertaken to investigate the antiplasmodium effect of AiAk and AiCb in humans.

Keywords: Complementary and Alternative Medicine, Synergist- anti-plasmodium activities *Caesalpinia bonducella*, *Azadirachta indica*, *Annickia kummeriae*

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Introduction

Malaria is the most important health problem in Tanzania, where it causes 3.1% of all malaria cases and deaths and 4.1% of all deaths worldwide in 2021 (WHO, 2021). This means that preventing and controlling malaria should be a top priority. According to recent data, about 90% of the population in mainland Tanzania live in malaria-transmission areas. Strategies used for malaria control include malaria case management, malaria vector control using ITN, and malaria intermittent treatment in pregnant mothers (WHO, 2017). Malaria control in Tanzania through treatment faces challenges due to widespread antimalarial drug resistance (Schönfeld, 2007; Kumar *et al.,* 2015). Drug resistance to antimalarial drugs has become a significant hurdle in the successful treatment of the *P. falciparum* infection and has contributed significantly to global malaria-related mortality (WHO, 2017). *Plasmodium falciparum* has resisted nearly all current antimalarial drugs, which hinders malaria control strategies (Arrow *et al.,* 2004). Therefore, developing new effective and affordable anti-malarial drugs to combat this disease is essential.

The development of new antimalarials from highly active natural products is crucial in order to overcome the increasing resistance of *Plasmodium* to malarial drugs (Bero, 2009; Akin-Osanaiye *et al.,* 2013; Moustapha *et al.,* 2018). Historically, medicinal plants have served as sources of new pharmaceutical products like quinine and artemisinin (Newman *et al.,* 2000; Koehn, 2005) and inexpensive starting materials for synthesising many known drugs. Lemma *et al.,* 2017 identified 977 plant species with potential antiplasmodial activities. Accordingly, about 70 to 80% of people in developing countries rely on using herbal remedies for malaria treatment (WHO, 2015). *In vivo, antiplasmodial activity of some individual medicinal plants has* indicated relatively low parasitaemia suppression. Studies that assess the interaction between crude extracts from medicinal plants with antiplasmodial activity are scarce. Evidence suggests that the interaction of different crude extracts may be vital in enhancing therapeutic efficacy, optimizing dosage, increasing the level of target inhibition, reducing or delaying the development of drug resistance and simultaneous reduction of toxic effects (Williamson, 2001; Bero, 2009; Ginsburg & Deharo, 2011).

Several studies on antiplasmodial activity of *A. indica, A. kummeriae* and *C. bonducella* indicated antiplasmodial activity ranging from 30% to 70% (Moshi *et al.,* 2009; Nondo *et al.,* 2016; Akin-Osanaiye *et al.,* 2013; Malebo *et al.,* 2015). However, the nature of the interaction between the combinations of different crude extracts from these plants on plasmodium infection has not been studied. Therefore, this study intended to use a mice model to assess the *in vivo* antiplasmodial activity of individual crude extracts of selected medicinal plants and evaluate their interactional antiplasmodial effects. Specifically, we determined the antiplasmodial activity of *A. indica*, *A. kummeriae*, and *C. bonducella* in mice infected with *P. berghei*. It was postulated that the combined utilization of crude extracts would yield greater efficacy than their application in animal models. The findings of this study will be helpful in the quest for more effective herbal remedies for malaria and the development of novel anti-malarial medications based on complementary, alternative, and traditional medicine.

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Materials and methods

Collection and Authentication of Plant Materials

Three medicinal plants were harvested from different areas in Tanzania. *Azadirachta indica* leaves and *C. bonducella* roots were collected from Makuburi and Pugu, respectively, in Dar es Salaam, whereas the *A. kummeriae* roots were collected from Kisiwani Kisarawe, Pwani. The collected medicinal plants were identified in the Department of Botany, University of Dar es Salaam. The voucher specimens were deposited for future use.

Preparation and Extraction of Plant Materials

The harvested plant materials were air-dried for three weeks at room temperature and then pulverized into coarse granules ready for extraction. About 150 g of each of the selected plant materials (*A. indica, A. kummeriae* and *C. bonducella*) was extracted using the cold maceration method by soaking in 1 litre of Dichloromethane at room temperature for 24 hours and filtered through Whatman filter paper number 1 to remove debris. The procedure was repeated twice to ensure exhaustive extraction of plant materials. The extracts obtained were pooled together. The filtrates obtained were then concentrated in a rotary evaporator to remove solvent at 45°C under reduced pressure to prevent the thermal decomposition of labile compounds. The extracts were dried on air and kept in a freezer at -20°C until the day of use.

Phytochemical Analysis of Crude Extracts

The crude extracts were subjected to qualitative phytochemical analysis (based on chemical reactions) of secondary metabolites, including alkaloids, flavonoids, tannins, steroids, phenolics, saponins, cardiac glycosides and terpenoids (Mamta & Jyoti, 2012; Trease & Evans, 2002).

In vivo Antiplasmodial Activity Assay and Study Design

In vivo, antiplasmodial activity of individual and combined extracts was determined using the 4-day suppressive test described by Peters W 1975. An experimental study design was employed whereby 150 albino mice were used (Figure 1). The age and size of the animal were considered when making the choice. Individual crude extracts were tested at three nontoxic doses (200 mg/kg, 400 mg/kg, and 800 mg/kg) (Figure 1). The same procedure was followed when crude extract was used in combination.

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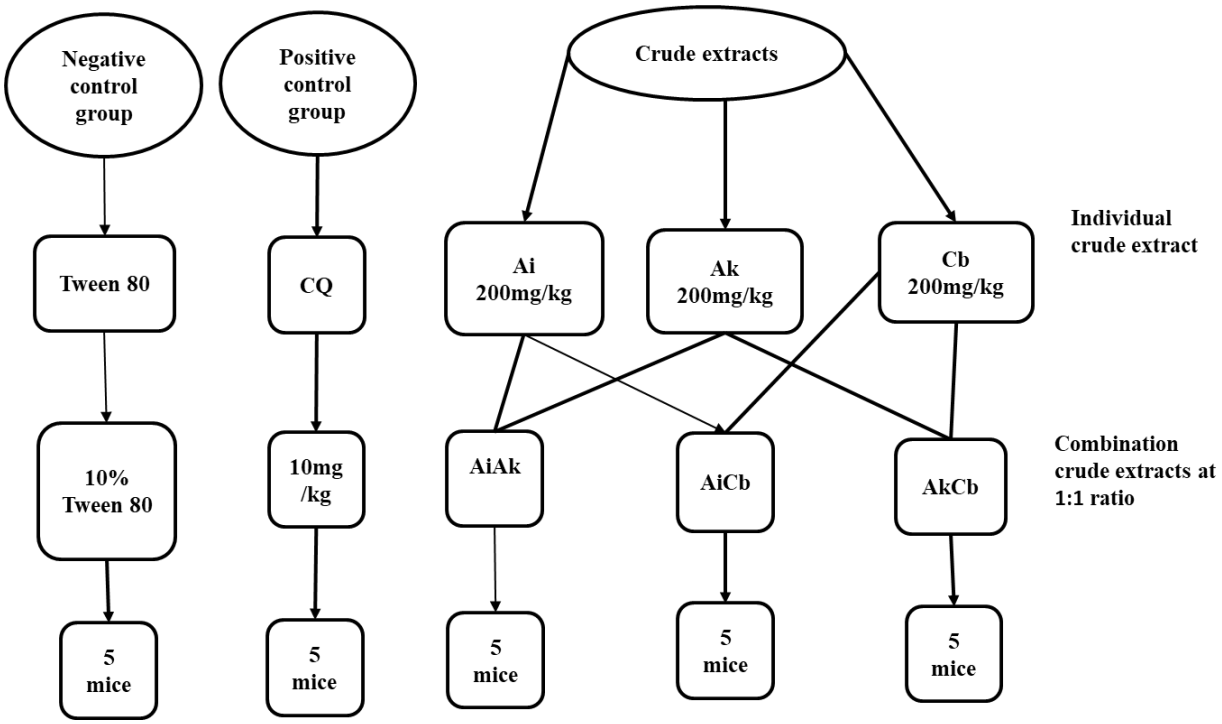

Figure 1: Study design to assess antiplasmodial activity for individual and combined crude extracts for all selected dosages.

Study Animals

Swiss albino mice weighing 20–30 g of either sex, raised at the University of Dar es Salaam, Department of Zoology and Wildlife Conservation were used in this study. Animals were acclimatized to the laboratory conditions and supplied with food and water *ad libitum* for two weeks before being used for the test. The animals were handled according to the National and International Guidelines for Handling of Laboratory Animals as well as per the Organization for Economic Cooperation Development (OECD) Guideline no. 425 and the study received ethical clearance from the University of Dar es Salaam and the National Institute for Medical Research (NIMR) in Tanzania.

Malaria Parasites and Preparation of Infected Red Blood Cells Suspension

Blood stage *P. berghei* ANKA parasites used in the study were kindly donated by Dr. Lindsay Stewart of the Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, United Kingdom to Muhimbili University.

Donor mice with high parasitaemia were anesthetized by diethyl ether; blood was collected through the sinus vein and diluted with sterile normal saline (0.9% w/v sodium chloride) to make a suspension of 1×10^8 infected red blood cells (iRBCs) per mL, which was used to infect test mice. Each mouse was inoculated with 2×10^7 iRBCs with *P. berghei* in 0.2 mL via tail vein and left for three hours before crude extracts administration.

Dosage Preparation and Administration of Extracts to *P. berghei* Infected Mice

Each crude extract was dissolved in 10% Tween 80 to make individual dosages of 200 mg/kg, 400 mg/kg and 800 mg/kg (Nondo *et al.*, 2016). Then dosages at 1:1 ratios for each combination were prepared. Before dose administration, the body weight of each infected mouse was assessed, and the dose administered crudely was calculated according to the body weight.

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Administration of the Extracts to *P. berghei* Infected Mice

After three hours post-infection, the mice were randomly allocated into groups of 5 mice each: The negative control group received 10% Tween 80 (5 ml/kg/day), the positive control group received chloroquine (10 mg/kg/day), and treatment groups received different doses of extracts (200, 400, or 800 mg/kg/day). Dose administration was done orally, once daily, starting on the day of infection and continued for four doses while parasitaemia was determined on day 5.

Malaria Parasitaemia Determination

On the fifth-day post P. berghei infection, thin blood smears were prepared from a drop of blood taken from the tail snip of each mouse. The smears were fixed with methanol and stained with 10% Giemsa solution. Malaria parasitaemia was determined under a microscope (×100 magnification). The number of parasitized erythrocytes was examined under three different fields on each slide and averaged to give the parasitaemia of individual animals. The percentage parasitaemia and suppression were calculated for all the doses of plant extracts using formulas.

The percentage of parasitaemia was then calculated using the formula:

$$\% \text{ Parasitaemia} = \frac{Number\ of\ infected\ RBCs}{Total\ number\ of\ RBCs} \times 100\%$$

The extract activities were also determined by calculating the percentage of parasitaemia suppression by using the formula:

$$\% \text{ Suppression} = \frac{Parasitemia\ of\ negative\ control - Parasitemia\ of\ test}{Parasitemia\ of\ negative\ contol} \times 100\%$$

Data Analysis

Determination of Antiplasmodial Activity of Individual Extracts in *P. berghei* Infected Mice

Data from all experimental animals were tested for conformity to normality (Kolmogorov–Smirnov's test) and variance homogeneity. The results were expressed as mean ± standard error of the mean (SEM). One-way ANOVA was used to establish differences between mean parasitaemia suppression between groups. Also, Tukey's comparison test was used to compare the antiplasmodial ability of crude extracts in lowering the number of parasitaemia in the control group. Significant differences were considered when $P<0.05$.

Determination of Interaction Effects between DCM **Crude Extracts in *P. berghei* Infected Mice**

A dose-response curve was generated for both individual and combination extracts to analyse the interaction effect between different extract combinations. The logarithm of their dose was plotted against the activity to obtain a nonlinear regression curve-fitting (Bell, 2005). The ED_{50}, a dose or amount of drug that produces a healing response or desired effect in 50% of the subjects taking it, was then determined from their nonlinear regression equations to determine the type of interaction. Synergy was considered when the effect of the combination was more significant (lower ED_{50}) than the one expressed from individual plant extract doses. At the same time, antagonism was considered when the combination effect was lower (higher ED_{50}) than individual plant extracts (Williamson EM, 2001). Furthermore, according to Gathirwa *et al.*, 2007 and Tarkang *et al.*, 2014 the interaction effects were evaluated by calculating the combination index (CI), a quantitative measure of drug combination effects and the obtained values were compared to the standards; whereby synergistic reaction was considered when CI < 1, additive 1 < CI < 2 and antagonistic when CI > 2.

The formula calculated the combination index:

https://dx.doi.org/10.4314/thrb.v25i2.17

$$\text{Combination index} = \frac{\text{ED50 Extract A in combination}}{\text{ED50 Extract A alone}} + \frac{\text{ED50 Extract B in combination}}{\text{ED50 Extract B alone}}$$

Results

Phytochemical Analysis

Phytochemical analyses of crude extracts from all plant species collected showed *C. bonducella* (roots) with more phytometabolites than *A. indica* (leaves) and *A. kummeriae* (roots) (**Table 1**)

Table 1: Phytochemical analysis of *Azadirachta indica, Annickia kummeriae* and *Caesalpinia bonducella* DCM crude extracts

SN	Phytochemical Test	Medicinal Plant		
		A. Indica	*A. kummeriae*	*C. bonducella*
1.	Alkaloids	+	+	+
2.	Flavonoids	-	-	+
3.	Tannins	-	-	-
4.	Steroids	-	-	-
5.	Phenolics	-	-	-
6.	Saponins	-	-	-
7.	Cardiac glycosides	-	-	+
8.	Terpenoids	-	-	+

+ = present; - = absent

Antiplasmodial Activity of *A. indica, A. kummeriae* and *C. bonducella* DCM crude extracts

Parasitaemia suppression in *P. berghei*-infected mice with *A. indica, A. kummeriae* DCM *C. bonducella* extract increased significantly in a dose-dependent manner, with a high dosage of 800 mg/kg exhibiting a percentage of parasite suppression approaching that of the positive control (**Table 2**). Of the three DCM crude extracts, *C. bonducella* had the highest antiplasmodial activity, followed by *A. kummeriae* and *A. indica*, which showed the lowest antiplasmodial activity.

Table 2: The summary of antiplasmodial activity of dichloromethane extract of *A. indica, A. kummeriae* and *C. bonducella* DCM crude extracts at different doses against *P. berghei* ANKA

Treatment Group	Mean percentage parasitaemia at day 5±SEM (n=5)			Mean percentage suppression of parasitaemia at day 5		
	A. indica	*A. kummeriae*	*C. bonducella*	*A. indica*	*A. kummeriae*	*C. bonducella*
Negative Control (10% Tween 80)	52.09 ± 2.04	50.55 ± 0.00	53.73 ± 2.41	0	0	0
200 mg/kg	40.15 ± 2.08	33.66± 1.01***	22.23 ± 1.43***	22.93	33.41	58.61
400 mg/kg	31.32 ± 2.39**	27.74 ± 4.10***	10.42 ± 5.44***	39.88	45.12	80.60
800 mg/kg	20.72± 6.32***	13.51 ± 1.82***	4.98 ± 7.54**	60.24	73.26	90.72

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Positive Control (CQ 10 mg/kg)	2.01 ± 0.83***	2.01 ± 0.83***	2.01 ± 0.83***	96.63	96.63	96.63

Note: *** means, the mean percentage parasitaemia of the extracts were extremely significantly different from that of negative control

Antiplasmodial Activity of Combined DCM Crude Extracts

On day five post-infection, the percentage parasitaemia was measured in *P. berghei*-infected mice receiving combinations of *A. indica* and *C. bonducella* (AiCb) and *A. indica* and *A. kummeriae* (AiAk) at three different doses (200, 400, and 800 mg/kg). Additionally, the percentage parasitaemia suppression was computed and recorded. The *in vivo* antiplasmodial activity of combined crude extracts also exhibited a dose-dependent suppression of parasite growth. The AiCb combination had higher antiplasmodial activity compared to *A. indica* and *C. bonducella* extracts when used individually. The same trend was observed in AiAk combination that also demonstrated higher antiplasmodial activity compared to its constituent extracts when used individually (Figure 2, 3 and 4). On the other hand, the combined crude extract of AkCb showed a lower percentage of parasitaemia suppression than the individual crude extracts, indicating antagonistic actions.

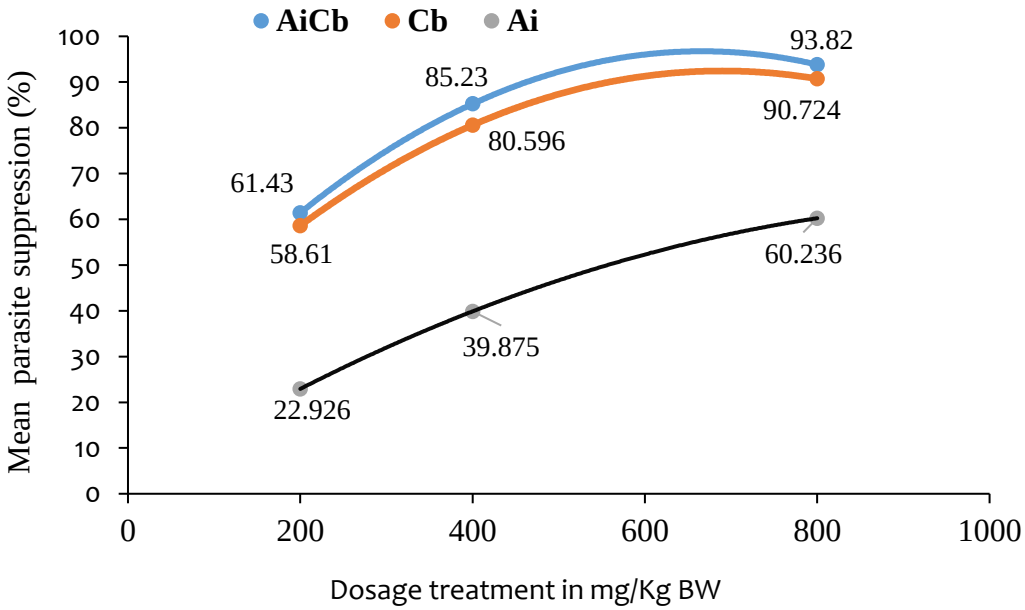

Figure 2: Percent parasitaemia suppression by AiCb combination in comparison to individual crude extracts in experimentally *P. berghei-infected* mice

Dose-Response Curves of AiAk, AiCb and AkCb crude extracts Combinations

The potency of separate and combined crude extracts was measured by determining the ED_{50}, the dose or amount of medication that generates a healing response or desire effect in 50% of participants. The ED_{50} for individual and combined crude extracts was determined on day 5 post infection (Figure 6, 7 and 8). Individually, the ED_{50} values were low in *C. bonducella* (126.63), moderate in *A. kummeriae* and higher in *A. indica* as shown in Table 3 and Figure 4. AiCb and AiAk combinations had lower ED_{50} values compared to individual extracts. However,

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the ED_{50} for the AkCb combination was much higher compared to its constituents, demonstrating antagonistic interaction (Figure 5, Table 3). Among the formed combinations, the effective dose (ED_{50}) value was lower in AiAk, followed by AiCb and higher in AkCb combination (Figure 5 and - Table 3).

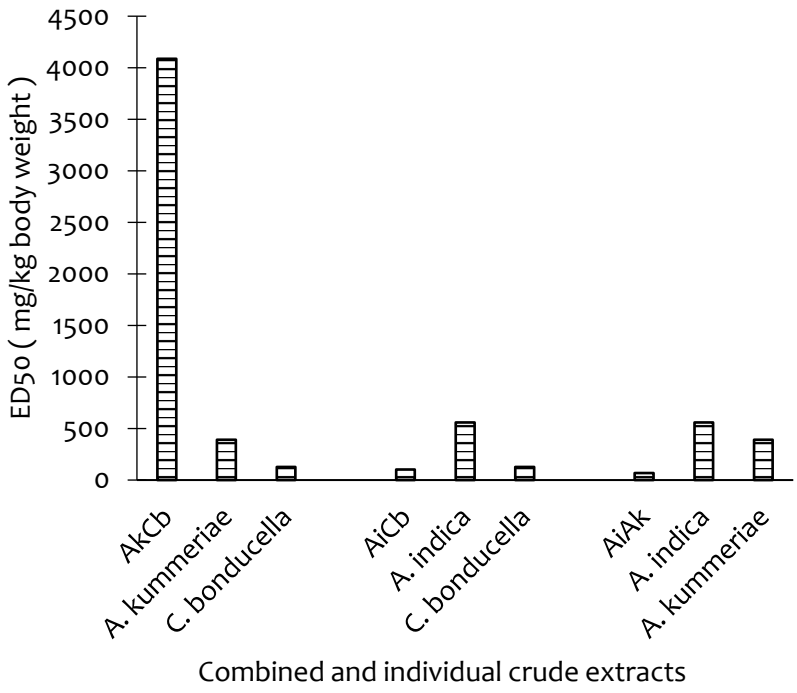

Figure 5: The ED_{50} for individual and combined DCM crude extracts of *A. indica*, *A. kummeriae* and *C. bonducella* in *P. berghei* infected mice

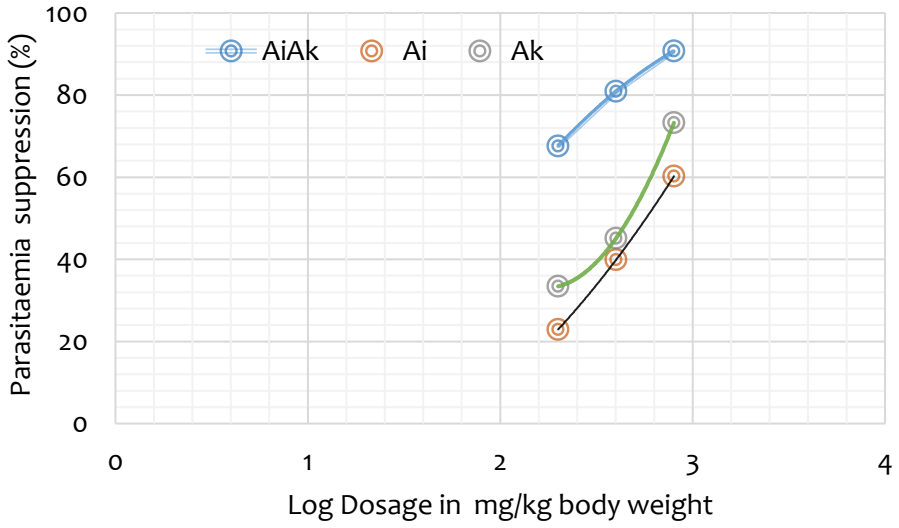

Figure 6: A dose response curves of AiAk DCM crude extracts combination

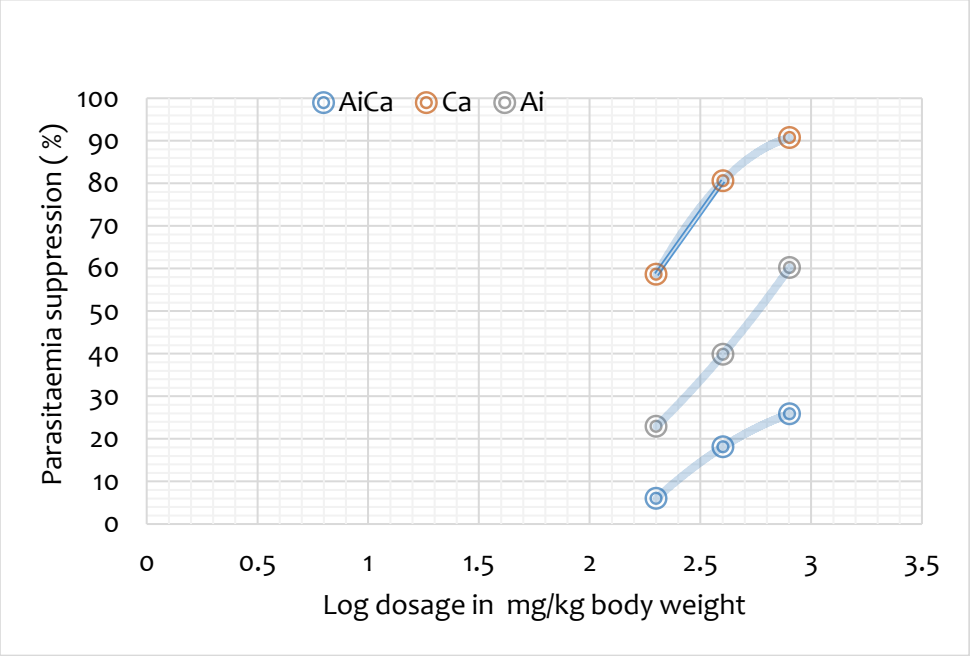

Figure 7: A dose response curves of AiCb DCM crude extracts combination

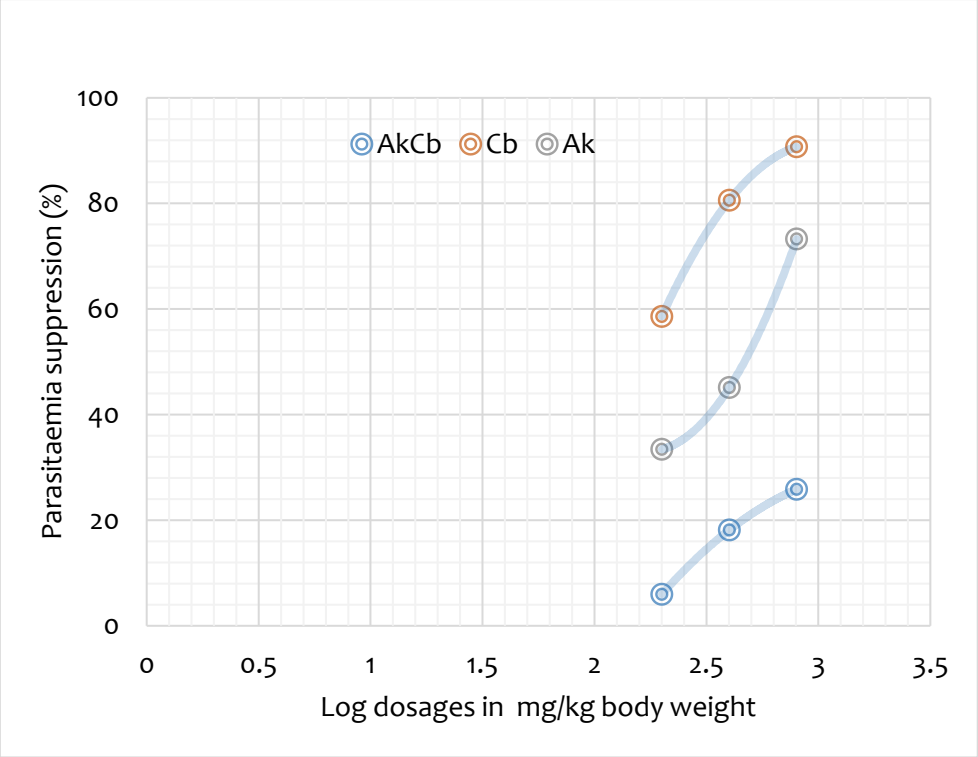

Figure 8: A dose response curves of AkCb DCM crude extracts combination

To investigate the nature of interaction, the combination index (CI), a quantitative measure of drug combination effects was calculated. AiAk and AiCb combinations had the combination index (CI) of 0.293 and 0.979, respectively, indicating synergism. However, AkCb combination had the CI of 40.67 indicating a strong antagonism (Table 4).

Table 3: Effective dose of individual and combined DCM crude extracts in mice infected *Plasmodium berghei* at 200, 400 and 800 mg/kg

Plants name or combination	Effective dose ED_{50} (mg/kg/day)	Retention factor (R^2)	95% confidence interval (mg/kg)	Regression equation
A. indica	558.45	0.9972	226.28 – 1378.25	y= 61.977x - 120.55
A. kummeriae	391.73	0.9464	203.44 – 754.27	y= 66.194x - 121.64
C. bonducella	126.63	0.9565	44.33 – 361.70	y= 53.346x - 62.162
AiAk	67.47	0.9925	15.73 – 289.37	y=38.455x - 20.338
AiCb	101.02	0.9520	15.73 – 648.74	y= 52.143x -54.516
AkCb	4085.95	0.9837	749.99 – 22260.26	y= 33.03x - 69.281

Table 4: Interaction between *A. indica, A. kummeriae* and *C. bonducella* DCM crude extracts against *Plasmodium berghei* at different combinations

	Ratios of extracts in mg/kg		Mean FED_{50} ± SEM		
Combinations	Extract A	Extract B	FED_{50} of Extract A	FED_{50} of Extract B	Combination Index (CI)/ Mean FED_{50}
AiAk	1	1	0.1208 (Ai)	0.1722 (Ak)	0.293 SYN
AiCb	1	1	0.3634 (Ai)	1.603 (Cb)	0.979 SYN
AkCb	1	1	10.42 (Ak)	32.25 (Cb)	40.67 MKD-ANT

Discussion

This investigation validates the antiplasmodium properties of crude extracts of *A. indica, A. kummeriae*, and *C. bonducella* that were obtained from locations other than Dar es Salaam (Malebo *et al.,* 2013; Akin-Osanaiye *et al.* 2013; Nondo *et al.* 2016). In the current study *C. bonducella* exhibited the highest *in vivo* antiplasmodial efficacy compared to that of *A. kummeriae* and *A. indica* at 800 mg/kg/day. *Caesalpinia bonducella* suppression rate was nearly to that of the standard antimalarial drug, CQ. The detected highest antiplasmodial efficacy of *C. bonducella* might be due to the presence of more than one class of phytochemicals, namely, alkaloids, terpenoids, flavonoids and cardiac glycosides. Evidence suggests that complex mixtures of phytochemicals tend to interact, either in potentiating the antimicrobial effect or interfering with each other activity (Lila & Raskin, 2005; Credo et al., 2018). The interaction between phytochemicals in *C. bonducella* extract may have potentiated the antiplasmodium activity observed in this study. In addition, *C. bonducella* had the lowest ED_{50} compared to *A. indica* and *A. kummeriae*. The lowest ED_{50} in *C. bonducella* further suggests highest potency against malaria parasite. This was more apparent from a dose-response curve, where the ED50 values of the extract in combination were lower than that of individual extracts (*A. indica* and *A. kummeriae*), suggesting higher potency.

The current study demonstrates for the first-time nature of the interaction between *A. indica, A. kummeriae* and *C. bonducella* crude extracts in *P. berghei-infected* mice. The AiAk and *AiCb* combination revealed a synergistic effect. This was more apparent from a dose-response curve, where the ED50 values of the extract in combination were found to be lower than that of individual extracts (*A. indica, A. kummeriae* and *C. bonducella*);

suggesting higher efficacy. Moreover, by comparing the combination index (CI) to the standards, the CI of AiAk and AiCb combination also suggest a strong synergism between the extracts against Plasmodium infection.

The current study is consistent with previous research that found synergistic interactions between many plant extracts, such as those which protect against *P. berghei* and include cryptolepine and artemisinins (Forkuo et al., 2016), artemisinin and triclosan (Mishra *et al.,* 2007), and *Uvaria acuminata* and *Premna chrysoclada* (Gathirwa *et al.,* 2010). It follows that given the current reliance on herbal therapy in traditional, complementary and alternative medicine (TCAM) in Sub-Saharan Africa (James et al., 2018), the high antiplasmodial potencies demonstrated by AiAk and AiCb are significant and key results for TCAM. Therefore, these findings, while preliminary, suggests that AiAk and AiCb are potential antiplasmodium therapies. Further research should be carried out to investigate safety of AiAk and AiCb in *P. falciparum* infection in human.

In contrast to AiAk and AiCb combinations, the ED50 and CI values of the AkCb exhibited characteristics consistent with an antagonistic reaction. The formed combination had the lowest antiplasmodial activity compared to that of *A. kummeriae* and *C. bonducella* when used individually. These findings suggest A. *kummeriae* and *C. bonducella* work best when used individually rather than in combination. Gathirwa *et al.* (2010) reported an antagonistic antiplasmodium reaction between *Grewia plagiophylla* and *Combretum illairii* crude extracts. However, it is beyond the scope of this study to examine the mechanism involved the antagonist activities observed from the A. *kummeriae* and *C. bonducella* combination against Plasmodium infection. Nevertheless, the parasitemia suppression rate that *A. kummeriae* demonstrated in this study, means that it can still be regarded as a potential antimalarial in line with earlier findings by Malebo et al. (2013), who discovered that compounds isolated from methanolic plant extracts showed high activities against multi-drug resistant *P.falciparum* K1 strain using *in vitro* model.

Overall, this study reports for the first time the existence of synergistic activity between *A. indica* and *A. kummeriae* as well as between *A. indica* and *C. bonducella* against *P. berghei* infection, suggesting that AiAk and AiCb can be considered as potential plant extracts-antimalarial combinations. This study also provides an exciting opportunity to advance our knowledge on exploring compounds responsible for the increased antiplasmodium efficacy in AiAk and AiCb formed extracts. Further studies need to be carried out in order to determine the optimal and safer dosing of these herbal extracts in human subjects and the biochemical mechanisms behind their antiplasmodial interactions.

Conclusions

The DCM crude extracts of *A. indica, A. kummeriae* and *C. bonducella* demonstrated a dose dependent antiplasmodial activity in *P. berghei* infected mice. The AiAk and AiCb combination exhibited synergistic anti-plasmodium activity. A*. indica, A. kummeriae* , and *C. bonducella* work best in AiAk and AiCb DCM combination rather than individually. The high antiplasmodial potencies demonstrated by AiAk and AiCb are significant and key results for TCAM.

Conflict of Interest

All authors declare that they have no conflicts of interest that could potentially influence the integrity, objectivity, or credibility of the research findings presented in this paper. All authors are committed to upholding the highest standards of ethical conduct in academic and scientific research.

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