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Research article

A COMPARATIVE EVALUATION OF *IN VIVO* ANTIPLASMODIAL ACTIVITY OF AQUEOUS LEAF EXRACTS OF *Carica papaya, Azadirachta indica, Magnifera indica* AND THE COMBINATION THEREOF USING *PLASMODIUM* INFECTED BALB/C MICE.

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ABSTRACT: Combination of herbal plants may produce synergistic, additive or antagonistic effects. A concoction prepared from the aqueous leaf extracts of *Azadirachta indica* (ALEAI), *Magnifera indica* (ALEMI), *Carica papaya* (ALECP) has been used in the treatment of malaria in some parts of Ghana. In the present study, ALEAI, ALEMI, ALECP and their combination (AMC) were evaluated *in vivo* for antiplasmodial activities using *Plasmodium berghei* infected BALB/c mice. Freeze-dried extracts of the combined therapy as well as those of the individual plants were tested *in vivo* on *Plasmodium berghei* infected BALB/c mice at 50 mg/kg and 100 mg/kg dosages. Coartem at 20 mg/kg and water were used as positive and negative controls respectively. *Cryptolepsis sanquinolata* was also used as a positive control. The extracts showed varying antiplasmodial activities using Four day suppressive and Seven day repository tests. The antiplasmodial activities of the extracts were significant compared with the negative control ($P<0.05$). Exceptions were ALECP at 50 mg/kg (3.98%) and the combined therapy (AMC) at 100 mg/kg (4.36%) which were only significant in the repository test. Although the combined therapy at 50 mg/kg showed the highest chemo-suppression (54.07%) when compared with the individual test extracts (ALECP = 19.13%; ALEMI = 51.81% and ALEAI = 48.95%) at similar dose, the activity was neither a demonstration of synergistic nor additive effect. These results suggest that the active components in the various single plant extracts may overlap in their modes of action. At dose 100 mg/kg, the AMC showed the least chemo-suppression suggesting an increased amount of a possible antagonistic component. Phytochemical screening of the various aqueous extracts revealed the presence of bioactive compounds with reported antiplasmodial properties. The LD_{50} recorded for the various extracts was greater than 5000 mg/kg. In conclusion, ALEAI, ALEMI, ALECP and their combination (AMC) possess antiplasmodial properties and this supports their use in folkloric medical setting in treating malaria.

Keywords: Antiplasmodial assay, phytochemistry, combination therapy, synergy, antagonism

INTRODUCTION

Malaria is a major endemic disease with high mortality rate in many tropical and subtropical countries. The burden of this disease is on the rise partly due to the increasing resistance of *Plasmodium falciparum* against the widely available antimalarial drugs. According to the WHO, there is an estimated 225 million malaria cases, with 800,000 deaths among the 3 billion people at risk Worldwide. About 91% of total deaths occur in Africa with pregnant women and children under 5 years being the most affected group (WHO 2002; 2011). The resistance of *Plasmodium falciparum* to the commonly used antimalarial drugs including the newly introduced Artemisinin Combination Therapy (ACTs) has resulted in resurgence in treatment failures. Hence, new highly efficacious and affordable antimalarial agents are urgently needed. For hundreds of years, plants have constituted the basis of traditional medicine systems and natural products have been a good source for drug development. Discovery of potent anti-malarial agents from plants has therefore generated much interest amongst the scientific community. It is of great importance to constantly screen medicinal plants for potential antimalarial agents. Ethnomedicinal claims usually forms the basis for selection of medicinal plants for evaluation. In traditional medical practice, several plants are often used in combination instead of isolated compounds or single plant extracts. Several investigations have proven that crude single plant extracts often have greater *in vitro*/ and *in vivo* antiplasmodial activity than isolated constituents at an equivalent dose.

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There is also evidence for several different types of positive interactions between different components of a single plant crude extract used in the treatment of malaria. However, synergistic interaction or multi-factorial effects between compounds present in herbal mixtures has not been extensively exploited in conventional anti-malarial therapy.

Mangoes are a member of the genus *Magnifiers*, consisting of many varieties of tropical trees in the plant family *Anacardiaceae*. Mango trees (*Magnifera indica* L.) grow up to about 35–40 m (115–130 ft) in height, with a crown radius of 10 m (33 ft). Extracts from *M. indica* leaf and stem bark are reported to be pharmacologically active. The extracts have been found to possess antimalarial (Ayoola *et al*., 2008), antidiabetic, (Girón *et al.,* 2009), antioxidant, antifungal (Kanwal *et al*., 2010) antimicrobial, anti-inflammatory, antiviral (Garcia, *et al*., 2006) and anticancer activity (Williamson, 2002). *Azadirachta indica* (Neem) is one of the two species in the genus azadirachta and is native to India, Burma, Malaysia and others growing in tropical and semi- tropical regions. *A. indica* (Neem) is reported to contain active secondary metabolites that are proven to be antiemetic, antifeedant, antifungal, anti-inflammatory; antiseptic, antipyretic and antiviral. Several other reports also suggest antiplasmodial activity of *A. indica* extracts (Oseni and Akwetey, 2012; Aladesanmi *et al.,* 1988; Chatopadway, 1998). The papaya is a large tree-like plant, with a single stem growing from 5 to 10 metres (16 to 33 ft) tall with spirally arranged leaves confined to the top of the trunk. Extracts from *Carica papaya* have been reported to possess biological activity including antimalarial (Praveen and Surolia, 2001; Arise *et al*., 2012), antibacterial (Doughari *et al*.,2007) and anti-inflammatory (Imaga *et al*., 2010).

Leaf extracts of *Azadirachta indica, Mangifera indica*, *Carica papaya* have been used in the treatment of malaria in traditional practice amongst many tribes of Northern Ghana. An aqueous decoction is usually prepared from a combination of leaves from these plants and used in malaria treatment. Combining these herbs may produce either an additive, synergistic and or antagonistic effects. The present study seeks to evaluate and compare the antimalarial properties of aqueous extracts from the leaves of *A. indica, M. indica, and C. papaya* when used as single plant herbal remedies with the extract from the combination of all the leaves using Balb/C mice infected with *Plasmodium berghei.* The various preparations will be
screened for phytochemicals.

MATERIALS AND METHODS

MATERIALS

Reagents and Chemicals

All chemicals and reagents used were of analytical grade and were used without further purification

Plant Raw Materials and Herbal Standard

Plant Raw Materials and Herbal Standard

Azadirachta indica , Mangifera indica and Carica papaya old leaves were obtained from a piece of land at Mampong-Akuapem, Ghana and were then authenticated by Dr. Yaw Ameyaw, a botanist of the production Department at the Center for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem, Ghana.

Animals

Seven-week old female Balb/c mice (average mass of 30 g) were obtained from the Animal unit of the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem, in the Eastern Region of Ghana. The animals were fed on powdered feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were allowed free access to sterile distilled water.

Malaria Parasites

Plasmodium berghei NK65 strain from the University of Copenhagen Denmark and maintained for more than 20 years in liquid nitrogen with occasional passage in Balb/c mice in the Department of Immunology, Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, Legon, Accra, Ghana, was used for the experiment.

METHODS

Preparation of Herbal Extracts

To about 100 g portion of each milled single herbal species, was added 2 L of distilled water and then boiled for an hour at room temperature. Equal masses (100 g) of each powdered leaf were combined to a mass of 300g. About 3L of distilled water was then added and boiled for an hour under room temperature. The decoctions were then filtered through a funnel packed with cotton wool.

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The extracts obtained were concentrated and then freeze dried. They were labeled appropriately as ALEAI, ALEMI, ALECP and AMC representing A. indica, M. indica, C. papaya and their combinations respectively. These were reconstituted in distilled water before use.

Inoculumn Preparation

A stock of parasitized erythrocytes was obtained from infected Balb/c mice, with a minimum peripheral parasitaemia of 20% by cardiac puncture in heparin-coated tube. The cell concentration of the stock was determined and diluted with physiological saline such that 0.2 mL of final inoculumn contained 10^6 parasitized red blood cells (RBCs).

Treatment of Animals

Sixty mice were selected and divided into seven groups with five mice per group in groups 1 and 2 and ten mice per group in the other groups. Each mouse was inoculated intraperitoneally with the prepared parasite. Group 1 animals received distilled water (negative control at 0.2 ml) and group 2 animals received an ACT; Coartem as positive drug control at 20 mg/kg. Group 3 animals received Cryptolepis sanquinolenta as positive herbal control, group 4 animals received ALEAI, group 5 animals received ALEMI, group 6 received ALECP and group 7, the extract of the combined therapy AMC. Animals in groups 3 to 7 were further divided into two sub-groups within each group (each sub-group comprising five animals). Thus each group received two dosages (50 mg/kg/day and 100 mg/kg/day). Blood counting was done on the fourth (day 3) and seventh day (day 6) after drug administration according to Peter's 4-Day Suppressive test (Peters, 1967).

Monitoring of Parasitemia and Antimalarial Activity

On the fourth and seventh days after drug administration, thin blood smears were prepared using blood from the tail vein of each mouse. Each smear was air-dried, fixed in methanol, air–dried again and stained with Giemsa for 15 minutes, and examined under the microscope. The slides were observed under oil immersion. Each slide was observed under three different fields and the parasitized red blood cells (RBCs) and total number of red blood cells (RBCs) for each field was recorded.

$$Percentage\ parasitaemia = \frac{Total\ number\ of\ parasitized\ RBCs\ counted}{Total\ number\ of\ RBCs\ counted} \times 100$$

$$\%\ Chemo - suppression = \frac{Parasitaemia\ of\ control - Parasitaemia\ of\ test}{Parasitaemia\ of\ control} \times 100$$

In vivo Acute Toxicity Assay

48 Mice (Balb/c, 30 g ± 2 g) were used and were then kept in filter top cages and housed in environmentally controlled rooms. There were three groups of mice (4 mice per group) for each of the test drugs. The sets of groups were given doses 1250 mg/kg, 2500 mg/kg and 5000 mg/kg orally and then studied for a period of 14 days (Hodge and Sterner scale).

Phytochemical Screening of Extracts

The aqueous extracts of Azadirachta indica (ALEAI), Magnifera indica (ALEMI), Carica papaya (ALECP) prepared singly and the combined therapy (AMC) were screened for the presence of groups of phytochemicals such as saponins, reducing sugars, phenolics, cyanogenic glycosides, phytosterols, triterpenes, polyamides, flavonoids and alkaloids Reported procedure (Sofowora 1982; Harborne 1983) were adopted in the current study.

Statistics

Data obtained from this work are expressed as means ± SEM and were analyzed statistically using ANOVA (One-way) followed by student's t-test. Differences between means were considered significant at $p < 0.05$.

RESULTS

Phytochemical composition of extracts

Of the extracts screened, ALECP and ALEAI were found to contain most of the phytochemicals. Phytochemical screening of the extracts presents interesting results Table 1. Alkaloids and flavonoids which were found to be present in ALEAI were absent in the combination extract AMC. This suggests presence of possible antagonistic compounds in the other plants in the combination. Triterpenes, flavonoids, phenolics, phytosterols and alkaloids have been implicated as antimalarial agents in many plants (Milliken et al., 1997). The absence of alkaloids and flavonoids in the combination extract may have an effect on its antiplasmodial activity.

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Table 1: Results of phytochemical screening of the extracts.

PHYTOCHEMICALS	*C. papaya ALECP*	*M. Indica ALEMI*	*A. indica ALEAI*	*Combination AMC*
Alkaloids	-	-	+	-
Saponins	-	+	+	+
Reducing sugar	+	+	+	+
Flavonoids	-	-	+	-
Cyanogenic glycoside	-	-	-	-
Polyamides	+	-	-	+
Phenolics	+	+	+	+
Triterpenes	+	-	-	-
Phytosterol	+	-	-	-

- = phytochemical absent + = phytochemical present

In vivo toxicity study

In vivo toxicity test in mice showed no obvious toxic side effects and treated mice were found healthy and normal with no record of weight loss, hair loss, allergy or any other symptoms of discomfort. The LD_{50} recorded, was greater than 5000 mg/kg and thus may be classified as practically non-toxic and fall within the acceptable margin of safety (Hodge and Sterner Scale). Table 2.

Table 2: Acute Toxicity Test for the extracts

	ALEMI	ALECP	ALEAI	AMC
No. of animals	12	12	12	12
Sex	Females	Females	Females	Females
No. of groups	3 (N=4)	3 (N=4)	3 (N=4)	3 (N=4)
Administration	Oral	Oral	Oral	Oral
Formulation	Freeze dried aqueous extract	Freeze dried aqueous extract	Freeze dried aqueous extract	Freeze dried aqueous extract
Dose administered (mg/kg)	1250, 2500, 5000	1250, 2500, 5000	1250, 2500, 5000	1250, 2500, 5000
Period of observation	14 days	14 days	14 days	14 days
No. of deaths	0	0	0	0
Approximate lethal dose(LD_{50})	>5000 mg/kg	>5000 mg/kg	>5000 mg/kg	>5000 mg/kg

The LD_{50} of all test drugs were greater than 5000 mg/kg and thus, may be classified as practically non-toxic and fall within the acceptable margin of safety (Hodge and Sterner scale).

In vivo study on antimalarial activities of plant extracts.

Results of in vivo antiplasmodial test are presented in Table 3. The extracts show significant ($p < 0.05$) antiplasmodial activity at the various concentrations against the parasite.

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Table 3*: Table showing the results of average percentage parasitaemia and percent chemo-suppression of extracts and controls*

	DAY FOUR		DAY SIX	
Drug	**Parasitemia (%)**	**Chemo-suppression (%)**	**Parasitemia (%)**	**Chemo-suppression (%)**
Control-Water	4.92±0.42$^{\#}$		12.70±1.02$^{\#}$	
Artemether (Coartem)	0.00±0.00*	100.00±0.00	0.00±0.00*	100.00±0.00
MI 50 mg/kg	2.37±0.25$^{*\#}$	51.81±5.11$^{\#}$	9.03±0.51$^{*\#}$	28.88±4.04$^{\#}$
MI 100 mg/kg	2.71±0.67$^{*\#}$	44.88±13.70$^{\#}$	8.19±0.61$^{*\#}$	35.53±4.77$^{\#}$
CP 50 mg/kg	3.98±0.06$^{\#}$	19.13±1.30$^{\#}$	8.34±0.67$^{*\#}$	34.31±5.27$^{\#}$
CP 100 mg/kg	3.41±0.47$^{*\#}$	30.72±9.53$^{\#}$	7.86±0.35$^{*\#}$	38.08±2.74$^{\#}$
AI 50 mg/kg	2.51±0.86$^{*\#}$	48.95±17.58$^{\#}$	9.85±1.56$^{*\#}$	22.40±12.31$^{\#}$
AI 100 mg/kg	1.84±0.10$^{*\#}$	62.50±2.05$^{\#}$	8.21±0.47$^{*\#}$	35.36±3.69$^{\#}$
AMC 50 mg/kg	2.26±0.13$^{*\#}$	54.07±2.62$^{\#}$	8.18±0.14$^{*\#}$	35.60±1.13$^{\#}$
AMC 100 mg/kg	4.36±0.53$^{\#}$	11.30±10.81$^{\#}$	9.49±0.56$^{*\#}$	25.26±4.43$^{\#}$
Crypto 50 mg/kg	1.70±0.06$^{*\#}$	65.36±1.28$^{\#}$	6.91±0.39$^{*\#}$	45.60±12.38$^{\#}$
Crypto 100 mg/kg	1.67±0.18$^{*\#}$	66.11±3.64$^{\#}$	6.60±0.45$^{*\#}$	48.02±3.53$^{\#}$

Values are means ± SEM of n=5 *values significantly different ($p<0.05$) from control (water), $^{\#}$values significantly different ($p<0.05$) from Artemether.

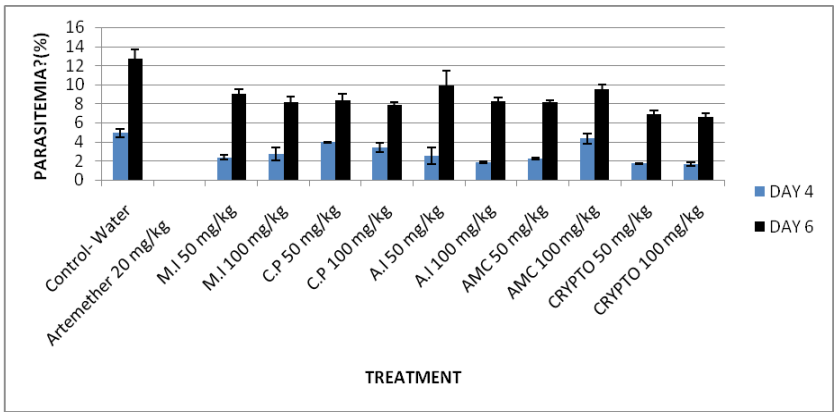

Figure 1: ***Graph showing the percent parasitaemia***

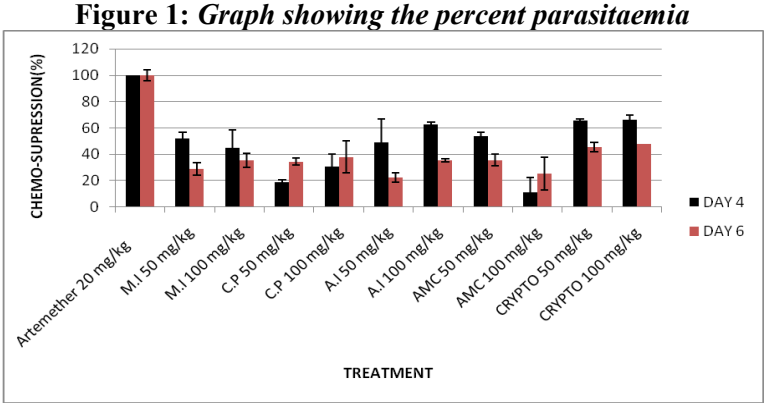

Figure 2: ***Graph showing the percent chemo-suppression***

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DISCUSSION

Triterpenes, flavonoids, phenolics, phytosterols and alkaloids which have been implicated as the antimalarial agents in many plants (Milliken *et al*., 1997) were present in some of the test drugs. The presence or absence of certain phytochemicals in a plant extract may be due to the mode of extraction or the season of harvest. The results showed significant differences between controls and test drugs in both average percent parasitaemia and percent chemo-suppressions on both test days. Exceptions were C. papaya (ALECP) at 50 mg/kg and the combined therapy (AMC) at 100 mg/kg. From Peter's four day antimalarial assay, *P. berghei* infected mice treated with 50 mg/kg of combined therapy (i.e. AMC) experienced a significant reduction in parasitaemia level (i.e. 54.07%) which was higher in efficacy than those treated with single plant extracts. The higher potency attained by the combined therapy at its minimum dose may be due to the presence of certain phytochemicals which were dominant in the entire single extracts. For instance, Table 1 shows the presence of phenols in all the single plant extracts as well as in the combined extract. A combination of all the single plant extracts could increase the concentration of phenols in the mixture, thus improving the activity. Phenols elevate red blood cell oxidation and inhibit the parasite's protein synthesis (Phillipson and Wright, 1990; Chandel and Bagai, 2010). This activity nullifies the oxidative damage induced by the malaria parasite Ayoola *et al*., (2008), Hilou *et al*., (2006). Thus the strong antimalarial activity observed for AMC may be due to the presence of numerous phenols. The strong activity of AMC at 50mg/kg may also be as a result of the presence of components with similar mode of actions, hence not leading to additive or synergistic effects.

AMC at 100 mg/kg gave the least chemo-suppression compared to those of the single plant extracts on Day 4. This might have resulted from some inactive components in the combined therapy suppressing the activity of the other components, thus leading to antagonistic effect. The LD_{50} for all extracts was found to be greater than 5000 mg/kg.

CONCLUSION

The aqueous leaf extracts of *C. papaya*, *M. indica* and *A. indica* showed significant antiplasmodial activity when compared with the negative control. Although the concoction obtained from the combination thereof demonstrated the strongest antiplasmodial activity when compared with the individual plant extracts, the activity of the combination was neither synergistic nor additive. In conclusion, these extracts possess antiplasmodial properties and our findings thus support the use of these concoctions in folkloric medical settings in the treatment of malaria.

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