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Artemisia annua L.- *Vernonia amygdalina* Del: A Potential Herbal Artemisinin Combination Treatment against Malaria

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Authors' contributions

This work was carried out in collaboration between all authors. Author NC developed the concept, designed the protocol and carried out data collection. Author OPE designed the protocol, analyzed the data and wrote the manuscript. Author OB designed the protocol. Author AN managed the statistical analysis. Author ME designed the protocol and collected the data. All authors read and approved the final manuscript

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Original Research Article

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ABSTRACT

Aim of Study: The aim of the study was to determine the potential of *Vernonia amygdalina* - *Artemisia annua* combination as a possible herbal artemisinin combination treatment of malaria.
Place and Duration of Study: Study was conducted at Natural Chemotherapeutics Research Institute, Ministry of Health, Kampala Uganda and at the Centre for Traditional Medicine and Drug

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Research, Kenya Medical Research Institute, Nairobi Kenya, from March 2013 to January 2014.
Methodology: After authentication of the plants, mature healthy *Artemisia annua* and *Vernonia amygdalina* leaves were collected and shade dried over 2 weeks. The dry materials were then each separately extracted by maceration using Petroleum ether and methanol. The filtrates obtained were first concentrated by fanning and then oven dried at 50℃ to constant weight of dry extract. Eight groups of *Plasmodium berghei* ANKA infected mice were treated once daily for 4 days from the day of inoculation as follows; group I, 200 mg/kg bwt of *A. annua* methanol extract; group II, 125 mg/kg of *V. amygdalina* methanol extract; group III, 200 mg/kg of *A. annua* pet ether extract; group IV, 125 mg/kg of *V. amgydalina* pet ether extract; group V, 125 mg/kg of *V. amygdalina* + 200 mg/kg of *A. annua* methanol extracts group; VI, 125 mg/kg of *V. amygdalina* + 200 mg/kg of *A. annua* pet ether extracts; group VII (positive control), 15 mg/kg of artemisinin+ 3 mg/kg of naphthoquine and group VIII (negative control), 0.2 ml 10% Tween 80.
Parasitaemia in each mice was determined by microscopy on day 5 while survival times were recorded over 30 day period. The means of percent parasitaemia and survival times for each treatment group were determined and differences tested for significances using One-way analysis of variance followed by Student's t-test at P=0.05 using STATA version 13.0 statistical program.
Results: The petroleum ether extracts combination produced 100% parasite clearance by day 5 just like the Artemisinin-Napthoquine. The survival times for the herbal combination were however poor and significantly less i.e 10.67±1.09 days compared to more than 30.0 ± 0.0 days for the ACT, P=0.000.
Conclusion: The *V. amygdalina-A. annua* petroleum ether extract combination shows promise for use as a herbal artemisinin combination against malaria however the survival times need improvement to match that of the ACT.

Keywords: Artemisia annua; Vernonia amygadalina; herbal-combination; malaria.

1. INTRODUCTION

Malaria remains a leading cause of morbidity and mortality especially in children despite adoption of Artemisinin-Combination Treatments (ACTs) [1]. Artemisinin derivatives, such as artesunate and artemether were adopted as components of ACTs in the hope that effective treatment would reverse the apparently increasing death rates amongst especially African children [2]. However, ACTs remain inaccessible to all in need due to rampant poverty in Africa leaving malaria still rampaging many lives especially children under 5 years of age. On the other hand, the World Health Organization (WHO) estimates that 80% of the population in Africa uses traditional/herbal medicine (TM) of some sort [3]. This is because they are easily accessible compared to modern medicines. Medicinal plants have been used as cures for malaria in the Traditional Medicine practices of various communities. Two important antimalarials in clinical use today that is artemisinin from *Artemisia annua* L. and quinine from *Cinchona officinalis* were isolated from medicinal plants based on TM use point of view [4,5]. Other medicinal plants used in TM have been shown to have potent antiplasmodial effects. These include *Momordica foetida* [6], *Aspilia Africana* [7], *Maytenus senegalensis* [8], *Vernonia amygdalina* [9] and several others. In this paper *V. amygdalina* which is widely available in Uganda and used as a herbal antimalarial was studied in combination with *A. annua* using rodent malaria. Although *A. annua* was used for malaria treatment in Ancient China and also still used in some communities in Africa for both cure and prevention of malaria [10,11], recent studies have demonstrated that *A.annua* preparation's major drawback is the occurrence of parasite recrudescence and the fear of widespread artemisinin resistance occurring when used as single plant [12]. It is therefore thought that a combination of *A. annua* with another antimalarial plant may be able to overcome the challenge of recrudescence and provide an option to the inaccessible ACTs. In this paper, we report the outcome of *A. annua* extract combination with *V. amygdalina* extract against rodent malaria, a model often used in the study of antimalarial drugs.

2. METHODOLOGY

2.1 Study Design

This was an *in-vivo* experimental study in mice infected with rodent malaria parasites.

2.2 Collection and Extraction of Study Plant Materials

Healthy mature leaves of *V. amygdalina* were collected in March (2013) from a cultivated garden located in Nalugala village, Katabi sub-county, Wakiso district (0.088; 32.528), while aerial parts of *A. annua*, were collected in March (2013) from cultivated specimens obtained in Kampala district (0.361; 32.597) and both plant specimens were identified by A taxonomist Ms Wanyana Olivia at the Department of Botany Herbarium Makerere University. Voucher specimens NC-51 (*A. annua*) and NC-52 (*V. amygdalina*) were deposited at the Makerere University Herbarium, Kampala, Uganda.

The plant parts (leaves and aerial parts) were dried under shade at room temperature over 14 days. After drying each of the samples was pulverized using a grinder (Kemri miller no. JTK 0040). The respective powders were then subjected to maceration extraction procedures with redistilled petroleum ether and methanol as solvents. Powder of each plant 300 g was weighed, extracted by soaking them separately in 1,100 ml of pet ether for *A. annua* and in 800ml of pet ether for *V. amygadlina* over 48 hours. Another two sets of 300 g of each of the plant powders was extracted in 1,000 ml of methanol over 48 hours. The respective filtrates were concentrated by fan for 24 h followed by oven drying at temperature of 55ºC to a constant weight of dry extract and the yields computed. The extracts were then kept in umber coloured tightly closed bottles in a refrigerator at 4ºC throughout the study period.

2.3 Induction of Mice Malaria and Administration of the Treatments

Healthy Swiss mice of both sexes (body weight, 20-30 g each) inbred at the animal house of Kenya Medical Research Institute (KEMRI) were used. The animals were randomly placed in plastic cages and given a pelleted diet and water at liberty. The animals were allowed to acclimatize to the cages over 7 days before being subjected to the experiments. A single donor mouse previously infected with *Plasmodium berghei* ANKA donated by the KEMRI lab was bled into sterile heparinized culture medium and the blood (0.4 ml) was diluted with physiological saline (7.6 ml). The healthy experimental mice were then each inoculated intraperitoneally with 0.2 ml of the diluted blood containing 10^7 parasitized erythrocytes [13]. The infected mice were then divided into eight groups (I to VIII) of six mice, each group comprising of 3 males and 3 females and the groups treated according to peter's test as described below[14].

After 2 hours post inoculation treatments were administered by gavage to groups as follows; group I 200 mg/kg of *A. annua* methanol extract, group II, 125 mg/kg of *V. amygdalina* methanol extract, group III, 200 mg/kg of *A. annua* pet ether extract, group IV, 125 mg/kg of *V. amgydalina* pet ether extract, group V, 125 mg/kg of *V. amygdalina* + 200 mg/kg of *A. annua* methanol extracts group VI, 125 mg/kg of *V. amygdalina* + 200 mg/kg of *A. annua* pet ether extracts, group VII, 15 mg/kg of artemisinin+ 3 mg/kg of naphthoquine and group VIII, 0.2 ml 10% Tween 80.

2.4 Determination of Parasitaemia Clearance and Survival Times

On day 5 post treatments, blood from the tail vein of each mouse was obtained and blood smears made. The smears were fixed with methanol, and then stained for 30 minutes with Giemsa 5% freshly prepared [15]. Parasitemia was determined microscopically by counting parasitized erythrocytes among 500 red blood cells in 4 fields per field per view of thin blood film [13]. Percent (%) parasitaemia for each mouse was calculated as a ratio of parasite infected RBCs counted to non infected RBCs counted (500 RBCs) x 100. The mean % parasitaemia of a group was thus obtained by summing the individual mouse % parasitaemia and dividing by the number of mice in the group. The survival times of the mice were documented over 30 days with survival beyond day 28 being an indicator of cure.

2.5 Data Analysis

The percent parasitaemia and the survival times means for each treatment group were computed and compared statistically using ANOVA followed by student's test. A difference was considered statistically significant if $P<0.05$. All analysis were done using STATA version 13.0 statistical package.

2.6 Ethical Considerations and Animal Care

Ethical approval was sought from the institutional review committee of the School of Health

Sciences, College of Health Sciences, Makerere University Kampala. The study animals were handled in conformity with the guidelines for the handling of laboratory animals. Experiments with animals were done according to the standards of the World Medical Assembly (WMA) and measures were taken to protect animals from discomfort and minimize pain as well as ensuring that the study animals were not subjected to stressful conditions. A clean environment for the animals was always maintained. Animals that survived after the experiment were humanely sacrificed under chloroform anesthesia and then incinerated.

3. RESULTS AND DISCUSSION

3.1 Results of Extract Yield, Parasitaemia Clearance and Survival Times

The extract yields were; *A. annua* pet ether extract =0.4%, *V. amygdalina* pet ether extract =0.8%, *A.annua* methanol extract=1.2%, and *V. amygdalina* methanol extract =2.4%.

The pet ether *A. annua* and *V. amygdalina* extracts combination like the ACT (artemisinin-napthoquine) achieved 100% parasite clearance by day 5. Other treatments failed to completely clear parasites in the blood of the study animals (Table 1). Only the mice in ACT treatment survived beyond day 28 indicating cure of malaria (Table 2).

3.2 Discussion

Mice have been used to study the antimalarial effects of various drugs including chloroquine, artemisinin, and several medicinal plants [16,17,18]. When a standard antimalarial drug is used in treating mice infected with *P. berghei*, it clears parasitemia to non detectable levels [19]. *Plasmodium berghei* is sensitive to the artemisinin and napthoquine combination, in this study, this drug was used as the standard drug because it contains artemisinin instead of its derivatives normally found in other ACTs. The artemisinin and napthoquine combination not only cleared parasitaemia but also cured the mice as indicated by survival times beyond day 28. Combination therapy is now recommended as the best way to treat malaria and this is the basis of ACTs. A number of medicinal plants have been shown to have antiplasmodial

Table 1. Parasitaemia mean clearance by treatments at day 5

Drug/extract	Dose mg $kg^{-1}day^{-1}$	Mean % parasitaemia (Mean± SEM)	Average % clearance	Pvalue
A. annua methanol extract	200	8.88± 2.61	61.21	0.030
V. amygdalina methanol extract	125	5.47± 2.55	76.12	0.015
A. annua+ *V. amygdalina* methanol extract	200 +125	0.28± 0.20	98.76	0.002
A. annua pet ether extract	200	0.48 ±0.82	98.25	0.002
V. amygdalina pet ether extract	125	6.60 ± 2.76	71.17	0.031
A. annua + *V. amygdalina* pet ether extract	200 +125	0.00 ± 0.00	100	0.0018
Artemisinin-naphthoquine (ACT)	18+5	0.00 ± 0.00	100	0.0018
10% Tween 80	0.2 ml	22.89± 3.77	-	-

Table 2. Group mean survival times of infected mice for different treatments

Drug/extract	Dose mg $kg^{-1}day^{-1}$	Survival time in days Mean±S.E.M	P value
A. annua methanol extract	200	9.67 ± 1.28	0.598
V. amygdalina methanol extract	125	8.83 ± 0.95	0.512
A. annua plus *V.amygdalina* methanol extract	200 +125	11.83 ± 2.33	0.625
A. annua pet ether extract	200	16.60 ± 3.61	0.742
V. amygdalina pet ether extract	125	11.50 ± 2.99	0.921
A. annua & *V.amygdalina* pet ether extract	200 +125	10.67 ± 1.09	0.683
Artemisinin-naphthoquine-ACT	18 +5 mg	30.00 ± 0.00	0.007
10% Tween 80	0.2 ml	7.83 ± 0.95	-

activities but many including *A. annua, V. amygdalina* etc used singly fail to clear parasites from the blood and also fail to cure malaria due to recrudescence of parasites after initial parasitaemia clearance. Combinations of medicinal extracts with known antimalarials or other plant extracts have been shown to have synergy [20] but combinations capable of causing complete parasitaemia clearance and cure remains to be discovered. In this study *V. amygdalina* which has been previously shown to enhance the *in vivo* activity of chloroquine [21] was studied in combination with *A. annua*. The antiplasmodial properties of *V. amygdalina* Del (Asteraceae) methanolic leaf extracts is attributed to the sesquiterpene lactones isolated from the leaves of the plant [22]. Other previously isolated constituents in *V. amygdalina* include flavonoids and steroid glycosides [23]. Artemisinin also a sesquiterpenoids found in *A. annua* of the asteraceae family of plants is currently the most potent natural antimalarial compound known [24]. Use of artemisinin or *A. annua* powder or infusion or extract thereof as a single agent in treatment of malaria is not encouraged due to high recrudescence rates and possible artemisinin resistance development. In this study neither plants used singly cleared parasitaemia completely however combination of the petroleum ether extracts of the two plants produced a parasitaemia clearance similar to that the standard ACT (Table 1). Parasitaemia clearance however without prevention of recrudescence is not useful in treatment of malaria. For a treatment to be considered effective it should not only clear parasitameia completely but it should also prevent parasite recrudescence post day 28. In the mice malaria model used in this study, recrudescence is indicated by mice death before day 28 and cure is indicated by mice survival beyond day 28. In this study only the mice in artemisinin-napthoquine (ACT) group survived beyond day 28 while all the mice in the other treatment groups died before day 28 (Table 2). This indicted that while the pet ether combinations cleared parasistaemia just like the ACT, it failed to prevent parasite recrudescence. It is also known that artemisinin or derivatives thereof generally clear parasitaemia but also suffer recrudescence challenge when used as monotherapies [25]. This is because though they are highly potent, they have short plasma half lives requiring frequent and long term administration if they are to prevent parasite recrudescence necessary to effect a cure [26]. This however becomes impractical due to high cost and poor patients' compliance to treatment. Artemisinin based combinations therefore in addition to containing artemisinin or derivative thereof do contain a second drug such as lumefantrine, napthoquine etc which have long plasma half lives helping in preventing recrudescence. Herbal artemisinin combinations capable of mimicking ACTs remains an urgent need in the fight against malaria in resource limited settings and the findings of this study offer hope for such alternatives.

4. CONCLUSIONS

Addition of *Vernonia amygalina* to *Artemisia annua* extracts achieves the required malaria parasitaemia clearance in mice models however modifications are needed to prevent parasite recrudescence.

CONSENT

It is not applicable because the study was done in animals following ethical clearance by the IRB of Makerere University College of Health Sciences.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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