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The antimalarial action of aqueous and hydro alcoholic extracts of *Artemisia annua L.* cultivated in Benin: *In vitro* and *in vivo* studies

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ABSTRACT

The aqueous and hydro alcoholic extracts of Artemisia annua plants acclimatized and set in culture in Benin, were investigated for their in vitro and in vivo antimalarial value. These extracts were prepared by decoction and maceration using water and ethanol, and were tested for in vitro activity against the strains of Plasmodium falciparum. Those extracts were also investigated in vivo against Plasmodium berghei NK 173 infected mice by the 4-day suppressive test. The action of these extracts was similar in vitro to the action of pure artemisinin at the same dosage. But, the in vivo studies on mice show that we have the same efficacy with aqueous extract of A. annua (artemisinin content of 20 mg/kg) than with pure artemisinin at a dosage of 140 mg/kg. The hydro alcoholic extract of A. annua (artemisinin content of 20 mg/kg) was better than the both other conditions. The others components of the plant Artemisia annua potentiate artemisinin activity on Plasmodium. The aqueous and hydro alcoholic extracts of Artemisia annua L. are very effective on malaria and they can be used by populations to cure this disease, especially in endemic areas where modern medicines are expensive.

Keywords: *Artemisia annua* extracts, in vitro studies, in vivo studies, artemisinin potentiation.

INTRODUCTION

Qinghaosu (artemisinin), isolated from the traditional Chinese antimalarial plant, *Artemisia annua L.*, has been shown to be active against the malaria, as well in the simple forms as in complicated ones and also effective in the chloroquino-resistant forms [1,2,3].

It is thus recommended by WHO since 2001, with its derivatives for the treatment of malaria in endemic areas [4]. But these drugs are relatively expensive for low income populations in African countries.

In this situation, it is judicious to consider the use of the entire plant *Artemisia annua L.* and its rough extracts to treat malaria. This is all the more justifying since infusions of the plant were found to be efficient on simple malaria [5]. In spite of the low quantity of artemisinin in these infusions, the patients were successfully cured.

Mueller et al. [5] have used an infusion (5 g of dried leaves of *A. annua* in 1 liter water per day during 5 days) containing 12 mg artemisinin, on patients in Congo. They found a 74 % cure rate.

In another work completed by Rath et al. [6], the patients received 1 liter of infusion obtained from 9g of dried leaves of a special variety of *Artemisia annua* containing 1,39% of artemisinin. This dose, equivalent to 94,5mg/l of artemisinin was delivered in one day. Although the artemisinin quantity was only 19% of the usual daily amount of artemisinin in an adult, the maximum plasmatic concentrations were 240±75ng/ml of artemisinin; what corresponds roughly to 40% of the maximum concentrations obtained with tablets of 500mg artemisinin. The data obtained show that artemisinin is more quickly absorbed from infusions than from tablets containing pure artemisinin; but the bioavaibility is similar in both cases. However, the use of *Artemisia annua* tea to treat malaria continues to be controversial [7, 8];

A recent study realized by Atemnkeng et al, [9], reports the evaluation of *A. annua* infusion efficacy for the treatment of malaria in mice. They conclude that *A. annua* tea could reduce parasitaemia but not to a curative level. But the doses delivered to mice in this study were exactly the same as in human cases; no correction factor relative to rodent was applied. In fact, human and rodents metabolisms are not the same.

In the present work, we study the *in vitro* and *in vivo* antimalarial action of aqueous and hydro alcoholic extracts of *Artemisia annua* plants cultivated in Benin.

The choice of the mice as experimental model can help to avoid influence of immunity to *Plasmodium* (which is frequent in endemic countries) and possibility of associated medication in human patients.

EXPERIMENTAL SECTION

2.1. Material

2.1.1. Solvents and reagents

The seedlings *of Artemisia annua* used in this study were provided by the Laboratory of Applied Ecology of the Agronomic Faculty of Sciences of Université d'Abomey Calavi in Benin. In fact, this Asian plant was acclimatized and set in culture in Benin. The seeds were obtained from Anamed® (Winnenden, Germany).

Ethanol (Ph Eur 96%) was acquired at VWR® (Fontenay-sous-bois, France). Methanol was HPLC-Grade and was purchased, with KOH from VWR®. All the other reagents (monopotassic and dipotassic phosphate and phosphoric acid 98%) were of analytical grade and acquired at Merck® (Darmstadt, Germany) and Alfa Aesar® (Karlsruhe, Germany), respectively.

2.2. Parasites and media

Crude extracts were evaluated for their antiplasmodial activity *in vitro* against a chloroquine-sensitive strain of Plasmodium falciparum (3D7) were obtained from Prof. Grellier (Musée d'Histoire Naturelle in Paris, France).

Plasmodium falciparum (3D7) asexual erythrocytic stages were cultivated continuously in vitro according to the procedure described by Trager and Jensen [10] at 37 ◦C and under an atmosphere of 5% CO2, 5% O2 and 90% N2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32mM NaHCO3, 25mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/l glucose (Sigma–Aldrich), 44 mg/ml hypoxanthin (Sigma–Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

2.3. Methods

2.3.1. Preparation of extracts

Plant material was dried in air conditioner room (approximately 25°C) and powdered before extractions.

Aqueous extract was prepared by adding 1 L of boiled water to 9 g of *A. annua* leaf powder. This infusion was left to cool for 15 minutes and then filtered. The filtrate was freeze-dried to obtain the dried aqueous extract of the plant. For the hydro alcoholic extract, portions of 35.12 g of *A. annua* leaf powder were macerated for 1 hour with 500 ml of ethanol 96%. The unit is agitated mechanically during extraction and all the system is kept at ambient temperature (approximately 30°C). The mixture is then filtered under vacuum. Filtrate was evaporated to dryness under reduced pressure.

All extracts were kept in a cold room (6°C) until their use for assays.

2.3.2 Artemisinin quantification in the extracts

UV detection of artemisinin is not easy, due to the lack of chromophores. So, it must be pre- or post-derivatized. We used in this study prederivatization inspired from Zhao and Zeng [11] method which has been modified and validated in hydro alcoholic extracts of *A. annua* [12].

This method was used to quantify artemisinin in the aqueous and the hydro alcoholic extracts of the plant and also to determine its artemisinin content (quantification on soxhlet extract of *A. annua* by petroleum ether).

For the prederivatization, 1ml of sample (extract solution or standard solution) was transferred into a 10 ml measuring flask. 4 ml of 0.2% (m/v) NaOH solution was added in the flask, and then left to react at 50ºC for 30 min. After cooling during 10 min, 1 ml of ethanol was added. Finally the flask was filled with acetic acid 0.2 N.

All solutions were filtered on a PTFE 0.45 µm membrane before HPLC analysis. The HPLC apparatus used for artemisinin quantification was an Hitachi Alliance from VWR® with LaChrom Elite software for data acquisition. Chromatographic separation was performed with a reversed phase RP-18 LiChroCART column (250 mm × 4 mm I.D.; particle size: 5 µm). Mobile phase consisted in a mixture of methanol and phosphate buffer (0,005 M; pH: 6.3) (45/55, v/v). A flow rate of 1 ml/min and detection at 260 nm were used. The column was maintained at 35°C and the injection volume was of 20 µl.

2.3.3. TLC analysis of the extracts

Analysis were carried out on TLC plates with a mobile phase consisting of dichloromethane- acetone (85:15) for the terpens and ethyl acetate – acetic acid – ac formic – water (100:11:11:26) for the flavonoids.

In terpens case, revelation was done with sulfurique vanillin and then dried at 100°C before observation of spots. The revelation of flavonoids plate was done under UV light (366 nm).

2.3.4. In vitro antiplasmodial activity

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the methods described by Makler et al. [13]. The in vitro test was performed as described by Bero et al. [14]. Artemisinin (98%, Sigma-Aldrich) was used as positive control. First stock solutions of crude extracts were prepared in ethanol, sterile water and DMSO respectly for hydro alcoholic extract, aqueous extract and pure artemisinin. The solutions were further diluted in medium. The highest concentration of solvent to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Each extract sample was applied in an eight serial threefold dilutions (final concentration range: on 2 rows of a 96-well microplate and was tested in triplicate. The parasitaemia and the haematocrite were 2% and 1%, respectively. According to the WHO guidelines and previous results [15, 16], antiplasmodial activity was classified as follows: highly active at IC50<5µg/ml, promising at 5-15 µg/ml, low at 15-50 µg/ml and inactive at >50 µg/ml

2.3.5. In vivo antimalarial activity

The present work was approved by the Ethical Committee for using animals at the University of Liège (n° 79). Aqueous and hydro alcoholic extracts of A. annua were assessed for *in vivo* activity in a 4-day suppressive test against *Plasmodium berghei berghei* infections in mice [17]. Female Swiss mice (10 weeks of age) mean body weight 25 ± 2 g obtained from Charles River Laboratories (Brussels) were inoculated (2×10^7 erythrocytes parasitized with *Plasmodium berghei berghei* NK 173) intraperitoneally on day 0 following the protocol described by Frederich et al. [18]. They were randomly divided in to five mice per group. All extracts before treatment were dissolved in 3% ethanol and 7% Tween 80. Each mouse receives 100µl/administration for all the product, except for hydro alcoholic extract for wich 200µl/administration was given because of its partial solubility.

The treatments dose, 1000mg/kg/day for aqueous extract and 500 mg/kg/day for hydro alcoholic extract, (both corresponding to 20 mg/kg/day of artemisinin) were given per os 4h after infection on day 0 and repeated twice daily for 5 days.

The artemisinin (positive control) dose administered to the mice was 140 mg/kg/day, corresponding to the WHO advice dosage for humans readjusted for rodents. The control group received distilled water during the 5 days of treatment.

On days 4, 5 and 7 post-infection, a thin blood smears were made from mouse tail blood and stained with Giemsa. Percent of parasitaemia reduction was determined microscopically using the following equation according to Fidock et al. [19]. % reduction of parasitaemia = 100−[(mean parasitaemia treated/mean parasitaemia negative control) × 100]. Mice stayed under observation and survivors on day 14 post-infection were considered as cured.

2.3.6. Statistical analysis

Differences between groups were analyzed by a student T-test. Statistical significance between treatments was set at $p < 0.05$.

RESULTS AND DISCUSSION

3.1. Artemisinin content of the plant and the extracts

The artemisinin content of the used *A. annua* batch was determined as 2.95 mg/g (0.295 % of dry leaves). This artemisinin content determined for the Artemisia annua seedlings cultivated in Benin is low compared to those obtained in other countries. Gaudin and Simonnet [20] found a content of 0.54% by analysing a sample cultivated in Switzerland. It is also reported values of 0.52% in Brazil [21] and 0.58% in Congo [5]. Researches are in progress to improve the contents by the modification of culture, harvesting and drying conditions [22].

Yields in artemisinin were 0.65 and 2.4 % respectively for the aqueous and the hydro alcoholic extracts. These values are not surprising as it is known that the ethanolic mixtures are more effective on artemisinin extraction than aqueous mixtures [21, 23].

3.2. *In vitro* antiplasmodial activity

The *in vitro* assays were done with equal quantities of artemisinin in the different products and the IC_{50} values are presented in table 1. The hydro alcoholic extract was found to be more active (IC_{50}= 3.27± 1.42 nM) than aqueous extract (IC_{50} = 4.95±2,07 nM and artemisinin (IC_{50}= 5.10 ± 1.89nM). No significant difference was observed between IC_{50} of artemisinin and the aqueous extract in these conditions. We can thus suggest that others components present in the hydro alcoholic extract contribute to potentiate the *in vitro* antiplasmodial activity of artemisinin.

Table 1: *In vitro* anti *P. falciparum* activity of aqueous and hydro alcoholic extracts of *A. annua* and artemisinin

Tested substance	*In vitro* antiplasmodial activity	IC_{50} (nM), average±SD
	3D7	
Artemisinin	5.10±1.89	
Aqueous extract of *A. annua*	4.95±2.07	
Hydro alcoholic extract of *A. annua*	3.27±1.42	

3.3. *In vivo* antimalarial activity

These *in vivo* antimalarial activities on mice were realized with level dose of artemisinin seven times weaker in aqueous extract and hydro alcoholic groups than the pure artemisinin group.

We could see on figure 1 that, despite the low artemisinin content in the extracts; they gave very good parasiteamia reduction (up to 80 to 100 % inhibition of parasiteamia). In fact, a comparable efficiency with positive control group is found for the aqueous extract and a better activity is noticed for the hydro alcoholic extract. Unsurprising, the mice receiving distilled water (control group) had no inhibition of their parasitaemia.

In all the treated groups (except the group receiving aqueous extract), we noticed a slight reduction of the activity on day 7 post infection. This could be due to the short half-life of artemisinin (1.9 to 2.6 hours) and the treatment period limited to 5 days in our study. This confirms the statement of other authors on the importance of taking *Artemisia annua* products for at least 7 days [5, 6, 21]. In the case of treatment with aqueous extract, an increase of curative action is noticed on day 7. So this extract present a better duration of activity, compared to the both other products.
Concerning the survival rate during the test, all the mice which received active products survived until day 14 post infection; excluded those who died of under cutaneous emphysema. Indeed, we recorded 2 deaths for each extract (at days 3 and 8 for the hydro alcoholic extract and at days 3 and 6 for the aqueous one) and 1 death at day 5 for positive control. However none of deaths mice presented a high parasitaemia. The under cutaneous emphysema were founded on the neck, the abdomen or the legs. According to literature, it is a possible consequence of the cramming which would have wounded the trachea. [24]

For the negative control group, only one mouse was alive at the day 14 and the all deaths mice presented a strong parasitaemia (more than 60%).

Our results are different for those of Atemnkeng et al, [9]. They worked on the evaluation of *A. annua* infusion efficacy for the treatment of malaria in *Plasmodium chabaudi chabaudi* infected mice and conclude that the infusion can reduce parasitaemia but not to a curative level. In fact, they noticed a 50 % parasiteamia inhibition after 6 days of treatment with *A. annua* tea. This difference result could be explained by the lack of dose readjustment between human and mice. They gave same quantity of infusion indicated for humans to the mice. But, the metabolism is

faster in rodents than humans. So, a calculation of dosage for the rodents must be done on the basis of human's dosage.

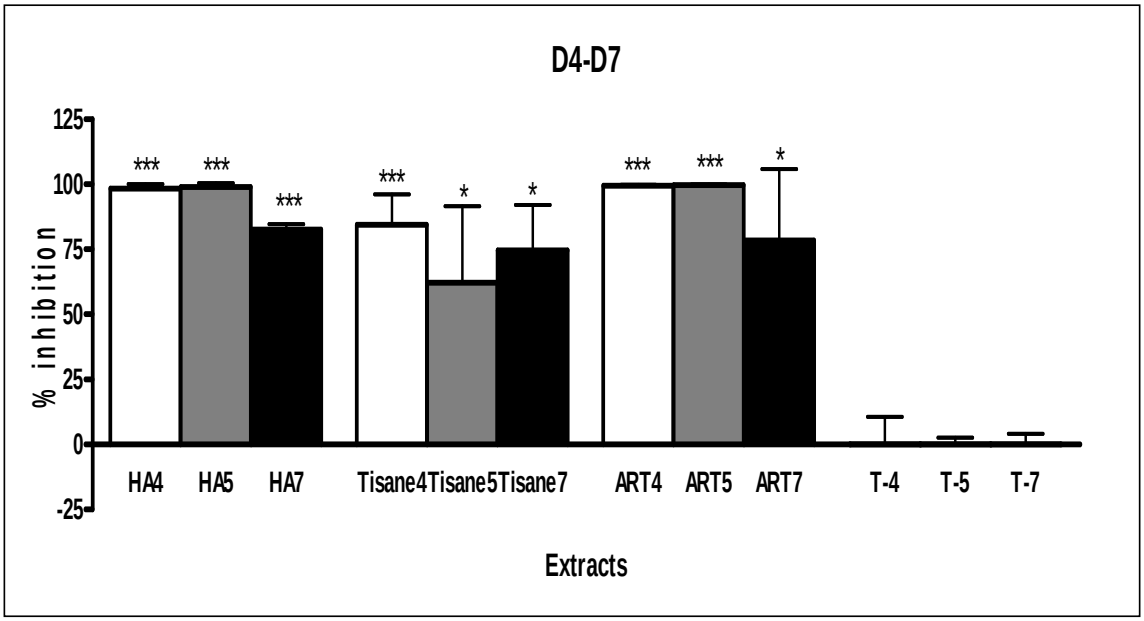

Figure 1: Curative action of aqueous (Tisane) and hydro alcoholic (HA) extracts of *A. annua* (extracts doses corresponding to 20mg/kg artemisinin per day) and pure artemisinin (140 mg/kg per day) against *P. Berghei* on infected mice

These results confirmed the presence in the extracts of others substances which enhance artemisinin activity as already shown by Elford et al [25] and Bhakuni et al [26].

We can go further and say that artemisinin is not the only active substance on malaria in *A. annua* extracts. This statement is in agreement with the results of Ramazani et al [27] in their work on the activity of four species of Artemisia (*A. vulgaris, A. absinthium, A. dracunculus and A. scoparia*). These others species included in Artemisia genus which are known free of artemisinin, even in traces, were found to be active against malaria. This activity was attributed to essential oils and other sesquiterpens that artemisinin [27]. Previous studies also established that artemisinin was not the only one antimalarial substance in A. annua extracts [8, 28, 29, 30, 31]. So treatment with the *A. annua* extracts can be considered as a real polytherapy and not a monotherapy as some authors said [7, 32]
Other advantage for use plant extracts is the fact that the artemisinin provided is faster absorbed after oral administration than pure artemisinin. In fact, aqueous solubility of artemisinin increased in presence of the others molecules of the plant like flavonoids and saponosides [5].

These results also show that level dose of artemisinin used in positive control treatment could be higher than what is needed. Rath et al [6] has already suggested that minimum plasmatic concentration enough to kill *Plasmodium falciparum* in human (9 ng/ml) was largely exceed with the WHO recommended treatment with artemisinin: 500 mg X 2 for the first day and 500mg for the four following days (corresponding to 20mg/kg and then 10mg/kg) [33]. In fact, this treatment leads to a C_{max} of 531 ng/ml in a mean time of 2.3 hours [6].

In this context the amounts of 500 mg per day appear too high. A work completed in Thailand by a team of Oxford [34] showed that it is useless to exceed 2 mg/kg body weight, that is to say 120 mg per day for a person of sixty kg. A Netherlander team Koopmans et al, [35] estimates that the necessary concentrations of artemisinin in plasma are effective starting from values of 3-30 ng/ml in plasma. In 1994 the University of Uppsala found that concentrations of 10 ng/ml led to a good effectiveness against *Plasmodium in vitro* [28].

The slight difference observed between the results of the aqueous and the hydro alcoholic extract can be explained by their composition. When we considered figure 2 showing comparative TLC chromatograms of these two extracts we can see that there are more sesquiterpens in the hydro alcoholic extract than in the aqueous one. For the flavonoids, we have the same bands but additionnal flavonoids are present in the hydro alcoholic extract. The findings of Elford et al [25] on artemisinin potentiation by flavonoids can thus explain the improved effect obtain with the hydro alcoholic extract.

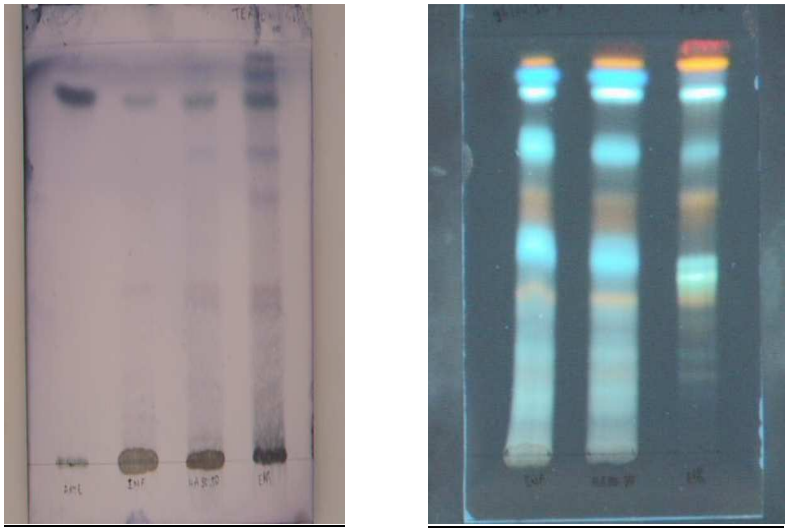

Figure 2: Comparative TLC of the aqueous and the hydro alcoholic extracts according to terpens (a) and flavonoids (b).
Art : Artemisinin ; Tis : Aqueous extract ; HAa : Hydro alcoholic extract (50-50) ; HAb : Hydro alcoholic extract 96%

CONCLUSION

At the end of this work on the *in vitro* and *in vivo* activities of *Artemisia annua* crude extracts, we can say that the others components of this plant potentiate artemisinin activity on *Plasmodium*. The aqueous and hydro alcoholic extracts of *Artemisia annua L* are very effective on malaria and they can be used by populations to cure this disease, especially in endemic areas where there is not enough money to pay modern medicines.

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