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First-time comparison of the in vitro antimalarial activity of *Artemisia annua* herbal tea and artemisinin

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ABSTRACT

Artemisia annua tea has been proven to be a very effective treatment for malaria in various clinical trials, but to date its efficacy has not been investigated in vitro. A study was therefore performed to evaluate the effects of *A. annua* tea on *Plasmodium falciparum* cultures in vitro. The concentration of artemisinin in the herbal tea preparation was also determined. The herbal tea extract was tested against chloroquine (CQ)-sensitive D10 and CQ-resistant W2 strains of *P. falciparum* using the parasite lactate dehydrogenase assay. Quantification of artemisinin in the extract of leaves of *A. annua* was performed using proton nuclear magnetic resonance (^{1}H-NMR). Results of the in vitro tests were consistent with the clinical efficacy of *A. annua* tea [50% inhibitory concentration (IC_{50}) for strain D10 = 1.11 ± 0.21 μg/ml; IC_{50} for strain W2 = 0.88 ± 0.35 μg/ml]. The concentration of artemisinin in *A. annua* tea (0.18 ± 0.02% of dry weight) was far too low to be responsible for the antimalarial activity. The artemisinin present in the tea is probably co-solubilised with other ingredients, some of which also have antimalarial activity and act synergistically with it. These compounds also merit further research to determine whether their presence hinders the development of parasite resistance compared with pure artemisinin.

1. Introduction

Malaria is the world's most important parasitic infection, causing 250–300 million clinical cases annually and with 3.3 billion people at risk and nearly a million deaths, mostly among children under 5 years of age in sub-Saharan Africa.[1,2]

The classic symptoms of malaria are characterised by extreme exhaustion associated with episodes of high fever, sweating, shaking chills and anaemia.[3] Malaria control requires an integrated approach comprising mainly prevention, including vector control and the use of effective prophylactic antimalarials, as well as treatment of infected patients with effective antimalarials.[4]

Among the different species of parasites belonging to the *Plasmodium* genus that cause malaria, *P. falciparum* is the most virulent and is considered the most lethal form.[5] Resistance of *P. falciparum* to most antimalarial drugs is a major obstacle to eradication of the disease.[6] The antimalarial chloroquine (CQ), which in the past was a mainstay of malaria control, is now ineffective in most *P. falciparum* malaria-endemic areas.[7] The rapid increase in

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resistance and multiresistance to many of the antimalarial drugs used at the moment makes it necessary to find and test new drugs or new molecules with antimalarial properties.[8] Furthermore, the difficulty of creating efficient vaccines as well as adverse side effects of existing antimalarial drugs highlight the urgent need for novel, well tolerated antimalarial drugs both for prophylaxis and treatment of malaria.[9]

Interest in plants as potential sources of new antimalarial drugs has been stimulated since the isolation and clinical use of artemisinin from a Chinese plant, *Artemisia annua* L. (Asteraceae).[10] This plant has been used in traditional Chinese medicine for the treatment of febrile diseases and malaria for many centuries and is included in the current pharmacopoeia of China.[11] The active compound, artemisinin, was isolated by Chinese researchers in the early 1970s.[12] In the last two decades, artemisinin and its semisynthetic derivates artemether and artesunate have been established as safe and effective antimalarials.[13] Artemisinin derivatives are now recommended by the WHO worldwide in combination with other drugs (artemisinin-based combination therapies) such as lumefantrine, amodiaquine, mefloquine and sulfadoxine/pyrimethamine as the first-line treatment of malaria.[14]

Artemisinin is poorly soluble in oil and water and is therefore usually administered orally, although it can be given rectally[15] and, when suspended in oil, intramuscularly.[16] Synthetic derivatives that are water soluble (artesunate) and oil soluble (artemether) have been developed to enable i.v. and i.m. administration, respectively.[17] It is now universally accepted that this family of compounds is among the most powerful antimalarial drugs ever discovered. The pharmacological and clinical evidence is well documented.[14]

Artemisia annua is usually used to prepare a tea and, if it contains effective amounts of artemisinin, it might be used today as a self-reliant treatment of malaria.[18] This aqueous preparation of the dried herb has been proven to be an effective treatment for malaria in various clinical trials. Treatment resulted in quick resolution of parasitaemia and clinical symptoms, and artemisinin was absorbed faster from herbal tea preparations than from oral solid dosage forms, although bioavailability was similar.[19,20] The fast-growing herb *A. annua* can be cultivated with relative ease in poor countries so it may offer an additional tool for the control of malaria, especially in these countries where modern antimalarial drugs are often unavailable.[21]

However, the efficacy of herbal tea has not been investigated in vitro. A study was therefore performed to evaluate the effects of *A. annua* tea on *P. falciparum* cultures in vitro. The concentration of artemisinin in the herbal tea preparation was also determined.

2. Materials and methods

Artemisia annua L. plants were grown in experimental fields at the Consiglio per la Ricerca e Sperimentazione in Agricoltura (CRA), Lecce, Italy. Aerial parts were collected at the flowering stage during September 2008. The leaves were air-dried at temperatures below 40 °C.

2.1. Herbal tea preparation

Dried *A. annua* leaves were used to prepare herbal tea. Boiling water (1000 ml) heated in glass was added to approximately 5 g of plant material and the mixture was left to cool, manually stirred from time to time, and filtered after 15 min. The aqueous extracts were freeze-dried, weighed and stored at 5 °C until used for chemical analysis by nuclear magnetic resonance (NMR) spectroscopy and for drug susceptibility assay against *P. falciparum* cultures.

2.2. NMR spectroscopy

Proton NMR (^{1}H-NMR) was used to determine the concentration of artemisinin in the extract of leaves of *A. annua*.[10,22–24] ^{1}H-NMR and carbon NMR (^{13}C-NMR) spectra were recorded on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with probes for inverse detection and with *z* gradient-accelerated spectroscopy. Standard Bruker automation programs were used for two-dimensional (2D) NMR experiments. 2D correlation spectroscopy (COSY) experiments were performed using COSYDFTP (double-quantum-filtered phase-sensitive COSY) and COSYG (gradient-accelerated COSY) sequences. Inverse detected one bond and long range ^{1}H and ^{13}C heterocorrelated (HETCOR) 2D NMR spectra were obtained by using the gradient-sensitivity enhanced pulse sequences INVIEAGSSI and INV4GPLRND, respectively. For $CDCl_3$ solutions, residual ^{1}H and ^{13}C peaks of the solvent were used as internal standards to calculate chemical shifts referred to tetramethylsilane. Standard ^{1}H-NMR analysis was first performed on 20 mg aliquots of the freeze-dried total extract dissolved in 500 μl of heavy water (D_2O) containing 1 mg/ml 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as the chemical shift reference. One-dimensional ^{1}H-NMR spectra used for quantification were acquired according the following conditions: pulse program 'zg'; 32 K data points; 5995 Hz spectral window; recycle delay 5 s; 128 scans; and a 90° pulse. The free induction decays (FID) were exponentially multiplied using a 0.1 Hz line broadening parameter and 32 K zero filling and were Fourier transformed to give a 0.18 Hz per point final resolution. Artemisinin presence was assessed on the basis of comparison of the chemical shift of the H12 proton (δ 6.20 ppm in D_2O-saturated solution for the authentic standard). Spectra were then baseline-corrected and the peaks of DSS methyls' signal and the H12 proton of artemisinin were integrated. The molar concentration of artemisinin was therefore calculated based on the ratio of the integrals for the two signals. The presence and quantity of artemisinin in the extract was confirmed by additional experiments. The freeze-dried infuse used for quantification was extracted with hexane, the solvent was dried under vacuum and the residual was re-dissolved in 500 μl of $CDCl_3$ containing a weighted amount of 1,4-dinitrobenzene as internal standard to acquire NMR spectra. The FIDs were Fourier transformed, baseline-corrected, and the peak of 1,4-dinitrobenzene protons signal and the H12 proton of artemisinin (5.84 ppm in $CDCl_3$) were integrated. Also in this case, the molar concentration of artemisinin was calculated starting from

the ratio of the integrals for the two signals. This method is analogous to a published one,[10] with the additional advantage that 1,4-dinitrobenzene has a single, intense signal at a chemical shift of 8.46 ppm owing to four equivalent aromatic protons, in a spectral area that is free from overlapping signals due to other species found in the hexane extract of *A. annua*. The reliability of the ^{1}H-NMR-based method for determining the artemisinin content of the aqueous solutions was also independently confirmed by comparison with the results obtained on *A. annua* herbal tea by a recently developed quantitative method based on HPLC using an evaporative light scattering detector.[22]

2.3. Parasite cultures

CQ-sensitive D10 and CQ-resistant W2 strains of *P. falciparum* were used in this study. All cultures were sustained in vitro as described by Trager and Jensen.[25] Parasites were maintained at 5% haematocrit (human type A-positive red blood cells) in RPMI 1640 (Gibco-BRL, Gaithersburg, Maryland, USA; 24 mM $NaHCO_3$) medium with the addition of 10% heat-inactivated A-positive human plasma, 20 mM HEPES (EuroClone, Pero, Milan, Italy) and 2 mM glutamine (EuroClone). All cultures were placed in a humidified incubator at 37 °C in a standard gas mixture of 1% O_2, 5% CO_2 and 94% N_2. When parasitaemia exceeded 5%, subcultures were taken; the culture medium was changed every second day.

2.4. In vitro antiplasmodial assay

Herbal tea was assessed for antiplasmodial activity in vitro using a modified parasite lactate dehydrogenase (pLDH) method as described by Makler et al.[26] The assay was made using asynchronous cultures with parasitaemia of 1–1.5% and 1% final haematocrit. Parasitaemia was determined by light microscopy using Giemsa-stained thin films by counting ≥1000 cells. In all experiments, standard artemisinin (Sigma-Aldrich S.r.l., Milan, Italy) was used as the control. Herbal tea extract was dissolved in sterile distilled water, whilst artemisinin was dissolved in dimethyl sulphoxide (final concentration <1%, which is non-toxic to the parasite). Drugs were placed in 96-well flat-bottom microplates (Costar, Cambridge, Massachusetts, USA) and seven two-fold dilutions were made starting at 8 μg/ml for the tea, whilst for artemisinin the initial concentration was 100 nM. In each plate, drug-free unparasitised erythrocytes and parasitised erythrocytes were used, in triplicate, as a blank control and a positive control, respectively. Plates were incubated for 72 h at 37 °C.

2.5. Lactate dehydrogenase measurements

Parasite growth was determined spectrophotometrically at 650 nm by measuring the activity of pLDH. pLDH activity is distinguishable from human LDH using 3-acetylpyridine adenine dinucleotide (APAD) as co-factor. At the end of incubation, cultures were re-suspended and 20 μl aliquots were removed and added to 100 μl of Malstat reagent in a 96-well microtitre plate. Spectrophotometric assessment of pLDH activity was obtained by adding 25 μl of a solution of 1.9 mM nitroblue tetrazolium and 0.24 mM phenazine ethosulfate to the Malstat reagent. As APADH (reduced APAD) is formed, nitroblue tetrazolium is reduced and forms a blue formazan product that can be measured in the spectrophotometer.

2.6. Determination of the 50% inhibitory concentration (IC_{50}) and dose–response curve construction

The antimalarial activity of the test compound was expressed as the 50% inhibitory concentration (IC_{50}); each IC_{50} value is the mean ± SD of the five separate experiments performed in duplicate. IC_{50} values were obtained from the dose–response curves using the log–logit method with SoftMax software (Molecular Devices, Sunnyvale, California, USA).

The antiplasmodial activity of crude extracts was qualified as 'very good' when the IC_{50} was <2.0 μg/ml, 'good' for IC_{50} values between 2.0 μg/ml and 5.0 μg/ml, 'good to moderate' for IC_{50} values between 5.1 μg/ml and 10 μg/ml, 'weak' for IC_{50} values between 11 μg/ml and 25 μg/ml, 'very weak' for IC_{50} values between 26 μg/ml and 50 μg/ml and 'inactive' when the IC_{50} was >100 μg/ml.[27]

3. Results

Using the pLDH assay, herbal tea was assayed against CQ-sensitive D10 and CQ-resistant W2 strains of *P. falciparum*. In total, 10 experiments were carried out (5 for each strain).

The herbal tea extract inhibited parasite growth in a dose-dependent manner, with IC_{50} values of 1.11 ± 0.21 μg/ml and 0.88 ± 0.35 μg/ml against CQ-sensitive and CQ-resistant strains, respectively (Figure 1). The IC_{50} values of standard artemisinin were determined as 25.0 ± 9.2 nM against strain D10 and 21.6 ± 3.7 nM against strain W2 (Figure 2).

Quantification of artemisinin in the extract of leaves of *A. annua* by ^{1}H-NMR analysis was 0.18 ± 0.02% of dry weight.

On the basis of the results of chemical analysis, the IC_{50} values based only on the artemisinin contained in the tea would result in about 7.08 ± 1.38 nM for strain D10 and 5.60 ± 2.23 nM for strain W2.

4. Discussion

Results of the in vitro tests conducted in this study are consistent with the clinical efficacy of the tea as observed independently by Räth et al. (2004) and Mueller et al. (2004).[19,20]

Artemisinin itself is poorly soluble in water, but its solubility may be improved by the presence of other plant constituents with amphiphilic properties such as flavonoids, glucosides or saponins.[20,21] Nevertheless, in this work we refer to the overall artemisinin concentration as obtained from NMR data. The activity of the tea was found to be approximately three-fold greater than would be expected from the artemisinin content alone, implying that there must be synergistic interactions with other compounds in the tea, as has been suggested for flavonoids.[28]

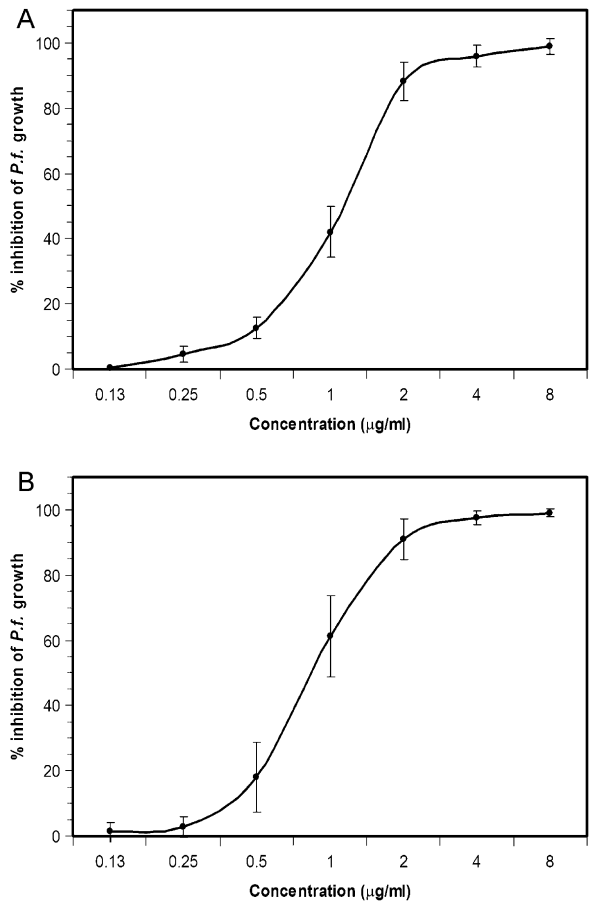

Figure 1. Dose–response curve of the effect of herbal tea on the growth of (A) chloroquine (CQ)-sensitive *Plasmodium falciparum* (*P.f.*) strain D10 and (B) CQ-resistant *P.f.* strain W2. Each point represents the mean ± SD of five different experiments conducted in duplicate.

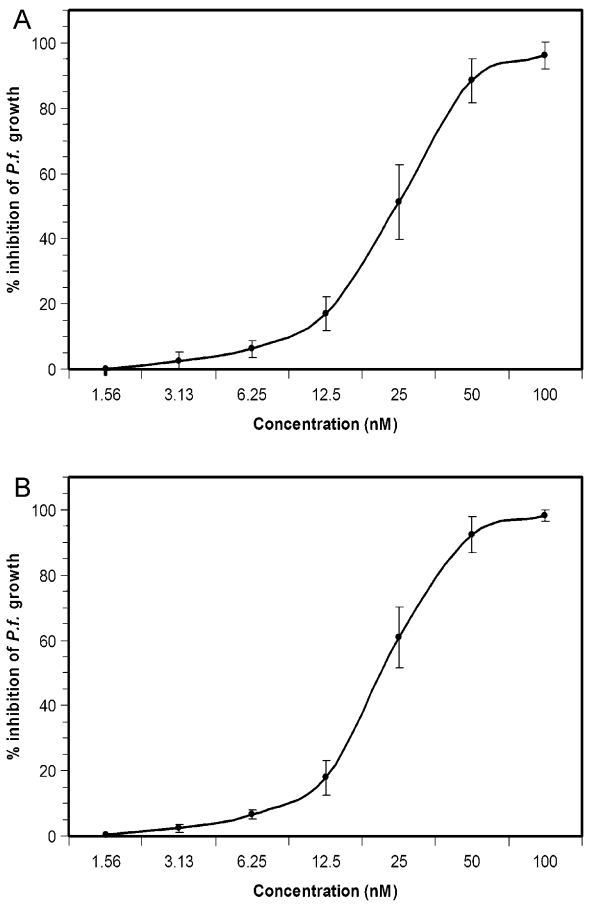

Figure 2. Dose–response curve of the effect of artemisinin on the growth of (A) chloroquine (CQ)-sensitive *Plasmodium falciparum* (*P.f.*) strain D10 and (B) CQ-resistant *P.f.* strain W2. Each point represents the mean ± SD of five different experiments conducted in duplicate.

In principle, saponins could also elicit an antiplasmodial effect due to erythrocyte lysis. Nevertheless, the solubility of saponins in the herbal tea preparation, an aqueous solution, is expected to be very low. This has been confirmed by recently published metabolic profiles of *A. annua* teas, which do not report the presence of substantial quantities of saponins.[22,23]

The presence of many possible active ingredients suggests that *A. annua* is a natural artemisinin combination therapy.[29] These other ingredients also merit further research to assess their synergistic effect as well as whether and how their presence could hinder the development of parasite resistance compared with pure artemisinin.

Tea preparations are currently recommended by several non-governmental organisations but not by the WHO. There is controversy, with a range of opinions on this issue. Further research will have to evaluate whether modifications of this therapy, for example combination with other antimalarials, may result in effective, safe and affordable treatment options for combating malaria, especially in poor countries. Such remote areas are particularly problematic for malaria control programmes and are often neglected. Herbal medicines may not be as perfect as the exact dosages administered in industrially produced formulations, but may be better than no treatment.

To reduce costs of malaria therapy, the international scientific community and funding agencies should invest in local production of raw materials. The *A. annua* plant grows well in parts of East Africa, where farmers should be motivated to increase acreage while agriculturalists assist them to produce high-yield leaves.[14] Therefore, local cultivation and preparation of *A. annua* could be considered part of a malaria control strategy, especially in a remote areas with poor access to health facilities and poor availability of effective antimalarial drugs.

Authors' contributions: FPF, MG, AM and LV conceived and designed the study; ADD, TG, AI, PP, AC and FB analysed and interpreted the data and drafted the manuscript; FPF, ADD, LV, MG and AM critically revised the manuscript for intellectual content. All authors read and approved the final version. ADD is guarantor of the paper.

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