Medicinal & Aromatic Plants
ISSN: 2167-0412

Akkawi et al., Med Aromat Plants 2014, 3:1
DOI: 10.4172/2167-0412.1000150

Research Article Open Access

Investigations of *Artemisia Annua* and *Artemisia Sieberi* Water Extracts Inhibitory Effects on β-Hematin Formation

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Abstract

Malaria is the most prevalent infectious disease in the world, killing 1-2 million people each year. New drugs are urgently needed to treat drug-resistant strains of malaria. In a previous study we found that extracts from *Salvia palestinia* leaves inhibited the formation of β-hematin with efficiency similar to that of chloroquine. The objective of this study was to investigate the effect of other plant extracts on hemozoin formation. A comparison between the efficiency of aqueous extracts or infusions of *Artemisia annua* from Luxembourg and *Artemisia sieberi* from Palestine in inhibiting β-hematin formation was done. Although it was found that the *Artemisia sieberi* leaf tea infusion was less effective than that of the *Artemisia annua*, the stem infusion of *Artemisia sieberi* was found to be better than that of *Artemisia annua* stems. Results obtained with infusions prepared with tap or well water may be different from results obtained in the laboratory with distilled water. *Artemisia annua* leaf infusions prepared using salt water (0.5g salt/150ml water) had higher efficiency in inhibiting β-hematin formation than those infusions done with distilled water. Mixing equal amounts of *Artemisia annua* leaf and *Artemisia sieberi* stem water extract showed an increase in their inhibitory effect on β-hematin formation. An important finding in this investigation was that the *Artemisia annua* lyophilized extracts lost activity with time, which may have an impact not only on *in vitro* laboratory results but also on *in vivo* treatment efficiency obtained with old extracts. In light of this finding it might be advisable to use *Artemisia annua* in the form of dried leaf powder and not in the form of extracts or infusion. Stored in dry, ventilated conditions the plant keeps its properties for many years.

Keywords: Ferriprotoporphyrin (IX); β-Hematin; *Artemisiaannua; Artemisia sieberi;* Chloroquine; Hemozoin

Introduction

Malaria is a major global health issue taking many lives daily. As reported by the World Health Organization and according to the World Malaria Report of 2012, Africa is the most affected continent with about 91% of all malaria deaths [1-3].

Infections are mainly caused by five species of the genus *Plasmodium,* making *P. falciparum* the most infectious parasite, contributing to 90% of total malarial deaths [4,5].

These parasites undergo a series of morphological transformations during their life cycle. In the human host the parasites enter the liver cells, where they continue maturation before they are released into the bloodstream, where another stage called the intra-erythrocytic stage is formed.

Inside of the erythrocyte the malaria parasite changes into a form called the "ring stage", at which the parasite degrades hemoglobin for its biosynthetic requirements. Large amounts of free heme known as ferriprotoporphyrin (IX) (FePPIX) are released [4,6]. The accumulation of ferriprotoporphyrin (IX) causes the generation of reactive oxygen species which may induce oxidative stress leading to parasitic death [2]. The parasite avoids these toxic effects by polymerizing these heme molecules within the food vacuole at a pH between 4.5 to 5.0, into a non-toxic, un-reactive, insoluble crystalline compound called hemozoin or "malaria pigment" [4]. Hemozoin formed in this unique life cycle is considered an important target in the search of new antimalarial drugs [7,8]. A synthetic analogue to hemozoin called β-hematin is considered to be structurally and spectroscopically identical to purified hemozoin [7] making it an excellent target for biochemistry studies [6,7]. Recently, many strains of *Plasmodium falciparum* formed resistance to classes of pharmaceutical antimalarial drugs.

Plants sources of drugs have been used for medical purposes throughout history, and still continue to serve as the basis for many pharmaceutical drugs used today. Quinoline-ring containing drugs accumulate inside the food vacuole of the malaria parasite preventing the formation of hemozoin [2,7,9].

We had previously attempted to find new antimalarial drugs in concentrating on the effect of pyrimidine derivatives in the *in vitro* inhibition of β- hematin [10] and *cis*-platin complexes [11] and also on the effect of *Salvia officinalis* [12]. In this study however we investigate the effect of different water extracts of the herb *Artemisia annua* in comparison to that of *Artemisia sieberi*, a Palestinian herb.

Artemisia annua (sweet wormwood), an annual herb, belongs to the family of the Asteraceae with great therapeutic importance. Chiefly spread in temperate areas of mid to high latitudes of the northern hemisphere [13]. The *Artemisia annua* plant, a herbal remedy used in the treatment of many illnesses by Chinese folk medicine for 2000 years, known at the time as *Qinghaosu* [14-16]. *A. annua* is considered as a good source of antimalarial compounds: the sesquiterpene lactone

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Received December 11, 2013; **Accepted** February 13, 2014; **Published** February 17, 2014

Citation: Akkawi M, Jaber S, Abu-Remeleh Q, Engeu OP, Lutgen P (2014) Investigations of *Artemisia Annua* and *Artemisia Sieberi* Water Extracts Inhibitory Effects on β-Hematin Formation. Med Aromat Plants 3: 150. doi: 10.4172/2167-0412.1000150

artemisinin, and many other medicinally active compounds including essential oils, flavonoids, coumarins, polysaccharides, phytosterols. Tea infusions of *A. annua* have been used for millennia to treat malaria and other aliments [17]. It is the most potent and efficacious compound against chloroquine and quinine-resistant *Plasmodium falciparum* and other malaria-causing parasites [18].

Beside antimalarial effects [19] *A. annua* has antibacterial [20], anti-HIV [21] and anti-inflammatory [22] properties, and is also active against a number of viruses [23], against a variety of human cancers [24-26], and other human parasites [27,28]. WHO and pharmaceutical companies show high interest for the active constituent artemisinin and its chemical derivatives, mainly in the form of artemisinin combination therapies (ACT) which is used worldwide as antimalarial drug [29-31]. Other studies showed that oral delivery of dried *Artemisia annua* leaves would reduce parasitemia more effectively than a dose of pure artemisinin [32]. In the current investigation carried out, a screening of the *in vitro* potential inhibitory effect of different water extracts of *Artemisia annua* and *Artemisia sieberi* on β- hematin formation is done.

Materials and Methods

Collection and extraction

Plant collection: *Artemisia sieberi,* collected from Palestine in 2012

Artemisia annua, collected from Luxemburg, leaf in 2012 and stem in 2011.

Certificates of origin for both plants are available.

Materials: DMSO, Dimethyl sulfoxide, purity 99.5% was obtained from Sigma Aldrich. Chloroquine diphosphate salt was obtained from Sigma. Glacial acetic acid was obtained from Fluka. Sodium acetate, purity 99% was obtained from Aldrich. Hemin chloride was purchased from Sigma.

Extraction of plant *components*

A. **Preparation of *Artemisia annua* extract (Method A)**

Dried leaves and stems were separately ground into coarse powder; extraction was performed by soaking (1:10) wt. /vol. of dried plant part, in distilled hot water at 90°C, and then left for 20 to 24 hours at room temperature. The extract was filtered using MN 615.Ø110 mm filter paper.

The crude water extract was obtained after the solvent was evaporated at 60-80°C under reduced pressure using (IKA WEREK RV06-ML) rotary evaporator, followed by lyophilization using (Labconco freeze drier) until constant weight was achieved. The final dried extract was stored in opaque bottles and kept in a desiccator until use.

The results of Figures 1 and 4 correspond to this extraction method.

A. **Infusion of *Artemisia annua* (Method B)**

2 g of the plant material were soaked in 150 ml of distilled hot water at 90°C, left for 20 minutes at room temperature, then filtered using MN 615.Ø110 mm filter paper.

All other figures correspond to this extraction method

In vitro semi-quantitative test for screening of anti-malarial activity

According to Deharo [33] a mixture containing of 50 µL of 0.5 mg/ml hemin chloride freshly dissolved in dimethylsulphoxide (DMSO), 100 µL of 0.5 M sodium acetate buffer (pH 4.4), and 50 µL of the tested potential anti-malarial drug solution or control, was incubated in a normal non-sterile 96-well flat bottom plate at 37°C for 18-24 h. It is important that the solutions be added to the plate in this order. The plate was then centrifuged for 10 minutes at 4000 rpm. The supernatant was removed and the pH of reaction was measured. The final pH of the mixture should be between (5.0-5.2). The wells were washed with 200 µl DMSO per well to remove free hemin chloride. The plate was centrifuged again, discharging the supernatant afterwards. The β-hematin remaining was then dissolved in 200 µl of 0.1 M NaOH to form an FP that can be measured spectrophotometrically. Finally the absorbance was read at 405 nm using ELISA reader.

Ultra pure water was used as negative control. Chloroquine (CQ) and 2-mercaptopyrimidine (2-MP) were dissolved in ultra pure water and both used as positive controls. Extracts prepared according to method A were also dissolved in ultra pure water.

Results and Discussion

The results are presented in comparison to positive and negative controls. Figure 1 shows the antimalarial activity of different dilutions of the crude water extract of *Artemisia annua* leaves (Method A). Please note that the absorption is inversely proportional to drug efficiency; the lower the absorption, the more efficient the drug.

The antimalarial activity testing of the *Artemisia annua* water extract was repeated using (Method B). *In-vitro* activity was studied under different conditions and was compared to positive control (CQ) chloroquine 0.1mg/ml and (2-MP) 2-mercaptopyrimidine 1mg/ml. Results of infusions and dilutions made in distilled water are shown in Figure 2.

Activity of infusions made using salt water are shown in Figure 3. Experiments done in salt (NaCl) water showed an increase in activity. This is obvious at low concentrations of infusions (10%) as seen in

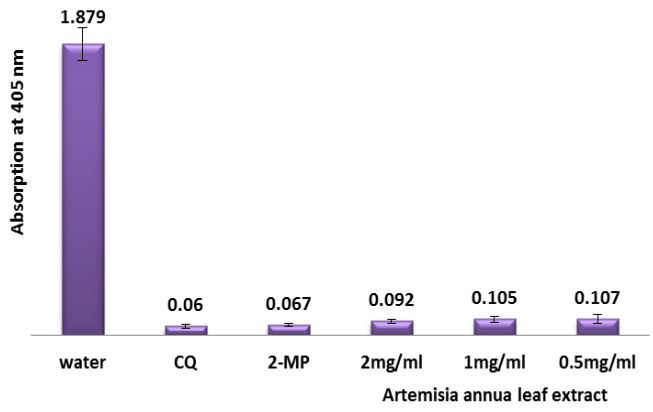

Figure 1: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia annua* water extract using method A, compared to the negative and positive controls: CQ-chloroquine 0.1mg/ml and 2-MP(2-mercaptopyrimidine)1mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

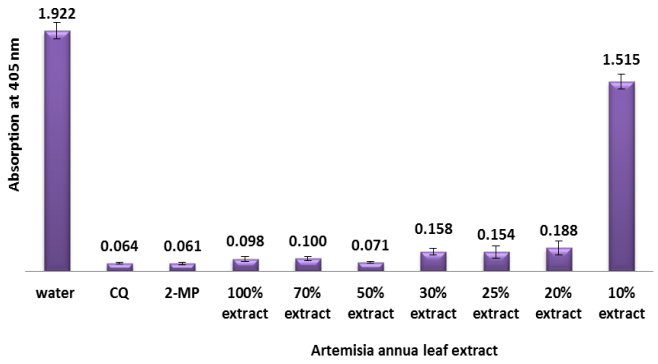

Figure 2: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia annua* leaf water extract using method B, compared to the negative and positive controls: CQ-chloroquine 0.1mg/ml and 2-MP (2-mercaptopyrimidine)1mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

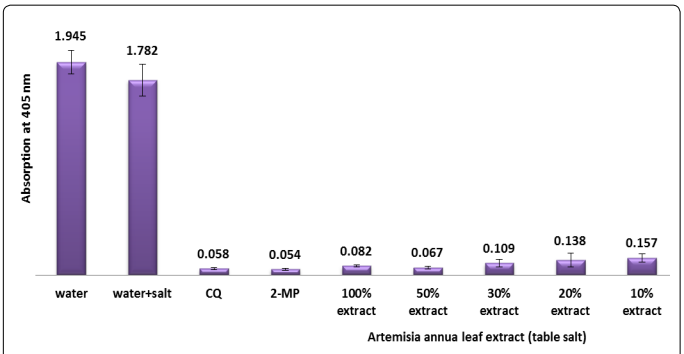

Figure 3: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia annua* leaf water extract using method B, using 0.5 g table salt/ 150 ml water, compared to the negative and positive controls: CQ-chloroquine 0.1 mg/ml and 2-MP (2-mercaptopyrimidine) 1 mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

Figure 3. What is important here is that water used in these dilutions has the same concentration of salt as that used for preparing the infusions and has no effect by itself when tested alone. That means that the effect may be due to an interaction with an active ingredient in the extract and not to the presence of the salt itself.

The effect of using table salt (0.5 g table salt/150 ml water) on the extraction process may decrease the solubility of organic plant constituents that are slightly soluble in water.

An important finding in this research work was the fact that the *Artemisia annua* lyophilized extracts (Method A) lose their activity with time as shown in Figure 4. This may have an impact not only on *in vitro* laboratory results but also on *in vivo* treatment efficiency obtained with aged extracts. That is to say it is essential to prepare fresh infusions rather than working with extracts. Or preferably to work with leaf powder, in the form of capsules for example [34]. The stability problem also exists for isolated molecules like artesunate or amodiaquine [35] especially under tropical climate.

Regardless of the mechanism of action of the extract of *Artemisia annua* it is clearly seen that it inhibits β-hematin formation. It is probably a pathway similar to chloroquine.

We have shown in a previous research study that extracts from *Artemisia sieberi* growing in Palestine have *in vitro* inhibitory effect on β-hematin formation. Results of a comparison done between *Artemesia sieberi* leaves and stem infusions are shown in Figures 5 and 6. *Artemesia sieberi* leaf water extract has better activity than that of *Artemisia sieberi* stem water extract at low concentrations.

But when comparing the *in vitro* activity of *Artemisia annua* and *Artemisia sieberi* leaf water infusions (Figures 2 and 5), the *Artemisia annua* leaf seems to better than *Artemisia sieberi* leaf in inhibiting the formation of β-hematin. To test a possible synergistic effect between these two species of Artemisia, a sample that contains an equal amount of both *Artemisia annua* and *Artemisia sieberi* water extract was assayed

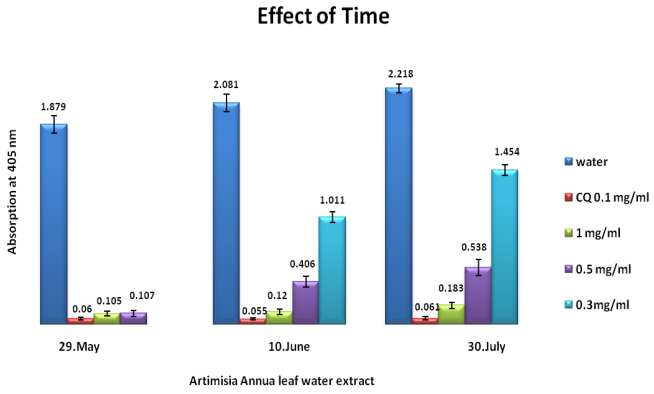

Figure 4: Column diagram representing the effect of time on the efficacy of *Artemisia annua* leaf water extract using method A, to inhibit β-hematin formation, compared to the negative (water) and positive(chloroquine) controls: CQ- chloroquine 0.1 mg/ml , showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 16 individual experiments.

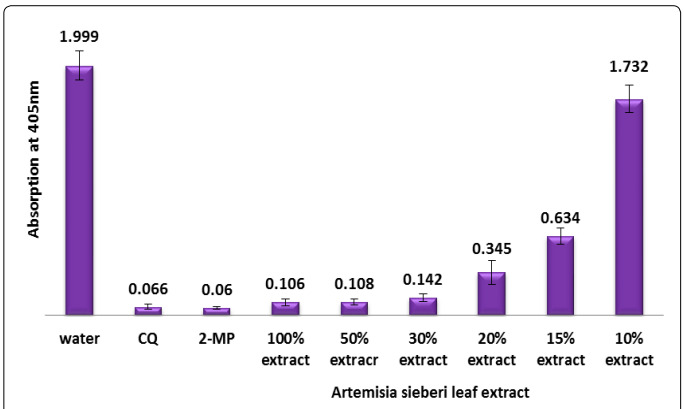

Figure 5: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* leaf water extract using method B, compared to the negative and positive controls: CQ-chloroquine 0.1 mg/ml and 2MP-2-mercaptopyrimidine1mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

for their *in vitro* inhibitory effect on β-hematin formation. There is an increase of the activity as shown in Figure 7.

A comparison between the *Artemisia sieberi* and *Artemisia annua* stems water infusions is shown in Figure 8. The stem water extract of the *Artemisia sieberi* was found to be more effective than that of *Artemisia annua*.

Conclusions

The human parasite *Plasmodium falciparum* enzymatically digests hemoglobin during its intra-erythrocytic developmental stages in acidic food vacuole compartments. The released heme is rapidly detoxified by polymerization into the chemically inert pigment, hemozoin. Several heme-binding anti-malarial compounds, such as chloroquine, efficiently inhibit this process, and this is believed to be the predominant mechanism by which these drugs work as antimalarials.

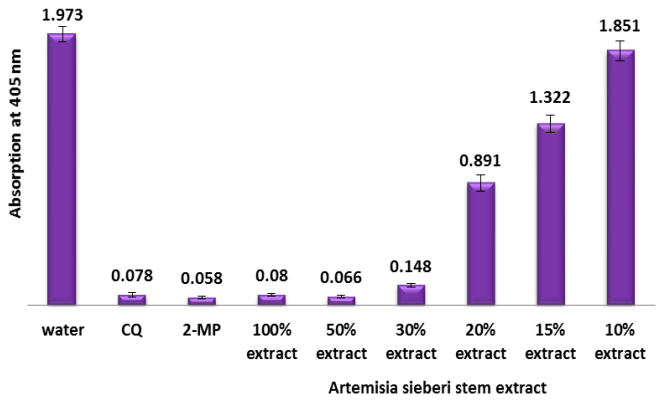

Figure 6: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* stem water extract using method B, compared to the negative and positive control: CQ-chloroquine 0.1 mg/ml and 2-MP(2-mercaptopyrimidine) 1 mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

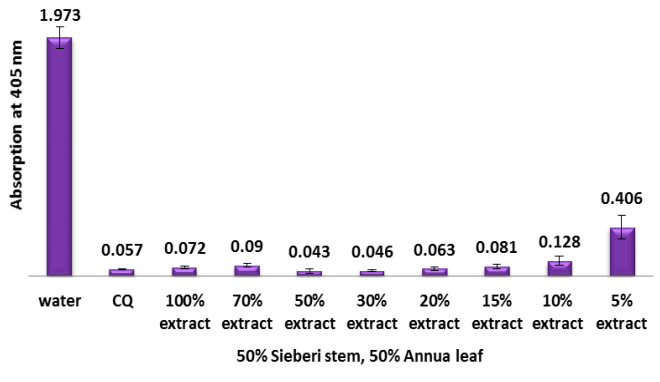

Figure 7: Column diagram representing the potential anti-malarial activity of a mixture made of 50% of *Artemisia sieberi* stem and 50% *Artemisia annua* leaf, water extract using method B, compared to the negative and positive control: CQ-chloroquine 0.1mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments.

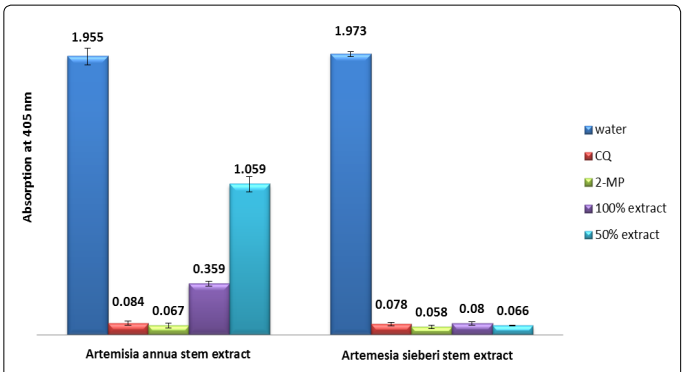

Figure 8: Column diagram representing the efficacy of Artimisiaannua and *Artemisia sieberi* stem water extract using method B, in inhibiting beta-hematin formation, compared to the negative and positive controls: CQ-chloroquine 0.1 mg/ml and 2-MP (2-mercaptopyrimidin), showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 16 individual experiments.

Malaria is one of the oldest diseases known to man. Despite huge investments in bed nets, drugs, pesticides it is still causing millions of deaths, mostly among African children and due to the rise of resistant strains of *Plasmodium falciparum* to available drugs, there is an urgent need to search for new, cheap and easily available anti-malarial drugs from natural products.

Using the ferriprotporphyrin bio-mineralisation inhibition test, this study compared aqueous extracts and infusions of two plants in a cell-free system. Both *Artemisia annua* and *A. sieberiact* as inhibitors of β-hematin formation. Our work confirms that artemisia herbal medicine could be an effective treatment for malaria infections, or at least as effective as chloroquine in the inhibition of hemozoin formation.

Due to the change of properties in the lyophilized extracts with time, which we have noticed for the first time in this work, for an effective treatment it is recommended that *Artemisia annua* be used in the form of dried leaf powder and not in the form of extracts or infusion.

Of course tea infusion is easier to prepare and thus of easier access in poor communities. So far the scientific literature fully confirms the absence of toxicity and resistance in humans. This is unfortunately not the case for quinine or artemisinin derivatives.

Fractionation, purification and identification of possible active ingredients as well as *in vivo* testing are currently under investigation.

Acknowledgment

We are grateful to Prof. Pamela Weathers for her helpful discussions and insightful comments.

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