

## Research Article

### The effect of *Artemisia sieberi* extracts on the Formation of $\beta$ -Hematin

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**Abstract:** The aim of the study is to assess the effect of Palestinian flora *Artemisia sieberi* extract against the formation of  $\beta$ -hematin *in-vitro*.  $\beta$ -hematin is a synthetic polymer made of ferriprotoporphyrin-IX and is structurally, chemically and spectroscopically identical to purified hemozoin formed during the intra-erythrocytic stage of Plasmodium life cycle. As strains of the malaria parasite Plasmodium emerged gaining resistance to the known drugs used such as chloroquine, amodaquine, artemisinin, the search for new anti-malarial drugs has become a must. Natural compounds were used throughout time as drugs and the history of anti-malarial drugs is linked with the history of herbal medicinal products. As an attempt to find new anti-malarial drugs, the potential inhibitory effect of *Artemisia sieberi* plant parts were studied using a semi-quantitative method. Leaves, flowers and stems were collected from various areas around Jerusalem, dried at room temperature and separately ground. Extraction was performed by soaking dried plant parts in 35% ethanol or ultrapure water. Extracts were filtered, rotary evaporated at 50°C and then lyophilized to a constant weight. Results of this study showed the alcoholic stem extract was the strongest in preventing the formation of  $\beta$ -Hematin when compared to that of the leaf or flower extracts. However the need to find the active components of this herbal extract requires further studies.

**Keywords:** *Artemisia sieberi*,  $\beta$ -hematin, chloroquine, ferriprotoporphyrin (IX), hemozoin

## INTRODUCTION

Malaria remains a major global health issue harvesting the lives of many. As reported by the World Health Organization and according to the World Malaria Report of 2011, 91% of total malaria deaths occurred in the African Sub-Saharan region, a region well known of its high poverty rate and a major settlement for this parasitic disease (Goldberg *et al.*, 1990; Kumar *et al.*, 2007; WHO, 2012).

Humans are prone to the infection by five species of the genus *Plasmodium*; *P. vivax*, *P. ovale*, *P. malariae*, occasionally *P. knowlesi*, *P. falciparum* is the most dangerous, contributing to 90% of total malarial deaths (Rathore, 2006; Weissbuch and Leiserowitz, 2008).

These unicellular eukaryotes undergo a series of remarkable morphological transformations during their life cycle. As they enter a human host, the parasites start the journey with an invasion of the liver; there they undergo maturation before being released into the bloodstream, starting yet another stage called the intra-erythrocytic stage.

During this stage, the malaria parasite multiplies and changes forms into what looks like a ring called the

"ring stage" (Pagola *et al.*, 2000). At this stage, the parasite degrades hemoglobin for its biosynthetic requirements, large amounts of free heme also known as Ferriprotoporphyrin (IX) (FePPIX) are released (Pagola *et al.*, 2000; Rathore, 2006).

Ferriprotoporphyrin (IX) is considered to be highly reactive and toxic to plasmodium. If allowed to accumulate it will cause the generation of Reactive Oxygen Species (ROS) which may induce oxidative stress leading to parasitic death (Kumar *et al.*, 2007). To avoid the toxic effects of heme, trophozoite stage parasites polymerize these molecules within their food vacuole of an estimated pH between 4.5 to 5.0, into a non-toxic, un-reactive, insoluble crystalline compound called *Hemozoin* or "Malaria pigment" (Pagola *et al.*, 2000; Rathore, 2006; Kumar *et al.*, 2007). *Hemozoin* formation thus is considered an important target in the search and finding of new antimalarial drugs (Slater *et al.*, 1991; Pagola *et al.*, 2000; Sullivan, 2000).

To mimic the exact environments and for further studies on hemozoin *in-vitro*, a synthetic polymer structure made from Ferriprotoporphyrin (IX) named  $\beta$ -Hematin is believed to be structurally, chemically and spectroscopically identical to purified hemozoin, making it an excellent alternative for the purpose

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(Slater *et al.*, 1991; Blauer and Akkawi, 1997, 2000; Pagola *et al.*, 2000).

Drugs from plant sources have been widely used for medical purposes throughout history and still continue to serve as the basis for many pharmaceuticals used today. The history of anti-malarial drugs is strongly allied with the history of herbal medicinal products. One of the novel natural products used as an anti-malarial therapeutic base was quinine, identified and isolated by French scientists Pelletier and Caventou from *Cinchona* tree bark. Quinoline-ring would accumulate inside the food vacuole of the parasite preventing the formation of hemozoin, killing the parasite (Slater *et al.*, 1991; Kayser *et al.*, 2003; Kumar *et al.*, 2007). Artemisinin is an endoperoxide which is the active ingredient of the *Artemisia annua* plant. This herbal remedy was used in the treatment of many illnesses by Chinese folk medicine for 2000 years, it was known at the time as *Qinghaosu* (White and Olliaro, 1996; He *et al.*, 2009; Willoughby *et al.*, 2009; Arsenault *et al.*, 2008).

The resistance to pharmaceutical products derived from these natural molecules, like chloroquine and artemether, highlights the need for new drugs. Earlier attempts showed the effect of pyrimidine derivatives (Aljazzar *et al.*, 2010), cis-platin complexes (Akkawi *et al.*, 2012) and wild sage (Akkawi *et al.*, 2012) in *in vitro* inhibition of  $\beta$ -hematin formation.

We concentrate on finding other new molecules from natural product. *Artemisia afra*, *Artemisia herba alba*, *Artemisia sieberi*, *Artemisia absinthium* have been and are still widely used as antimalarials (Lutgen, 2012). They all contain a broad range of essential oils, polyphenols, coumarins, polysaccharides, saponins which differ from species to species. It was found that  $\alpha$ -thujone and  $\beta$ -thujone represent the major constituent of all these artemisia varieties (Lutgen, 2012). Thujone is known for the augmentation of humoral and cell mediated immune responses (Siveen and Kuttan, 2011). *Artemisia sieberi* has shown positive antimalarial effects on *Plasmodium berghei* with minimum to no toxicity even when high concentration of herbal extract were administrated, A significant reduction percentage of parasitaemia was observed (Nahrevanian *et al.*, 2012). Another study showed that oral delivery of dried *Artemisia annua* leaves in the form of tablets or capsules would reduce parasitaemia more effectively than a dose of pure artemisinin drug (Hassanali, 2005; Elfawal *et al.*, 2012). In addition the powdered leaves mixed with other herbs have been proven in Uganda to have immune stimulating and prophylactic properties (Ogwang *et al.*, 2011).

In the current investigation carried out, a screening of the *in vitro* potential inhibitory effect of extract of *Artemisia sieberi* on  $\beta$ -hematin formation is done in order to detect new sources of antimalarial agents.

## MATERIALS AND METHODS

### Collection and extraction:

**Plant collection:** *Artemisia sieberi* was collected from different areas around Jerusalem and West Bank far from agricultural lands in Palestine and air dried in the shade for 10 days.

**Extraction of *Artemisia sieberi* components:** Dried leaves were ground into coarse powder; extraction was performed by soaking (1:10) wt./vol. of dried plant part, in 35% ethanol or boiling distilled water 90°C, left for 20 to 24 h at room temperature. The extract was then filtered using MN 615.Ø110 mm filter paper.

The crude 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.

***In vitro* Semi-Quantitative test for screening of anti-malarial activity:** According to Deharo *et al.* (2002) a mixture containing of 50  $\mu$ L of 0.5 mg/mL hemin chloride freshly dissolved in dimethylsulphoxide (DMSO), 100  $\mu$ L of 0.5 M sodium acetate buffer (pH 4.4) and 50  $\mu$ L 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 min 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  $\mu$ L DMSO per well to remove free hemin chloride. The plate was centrifuged again, discharging the supernatant afterwards. The  $\beta$ -hematin remaining was then dissolved in 200  $\mu$ L of 0.1 M NaOH to form an FP that can be measured spectrophotometrically. Finally the absorbance read at 405 nm using ELISA reader.

Ultra pure water was used as negative control meanwhile chloroquine and 2-mercaptopyrimidine were dissolved in ultra pure water and both used as positive controls. The extracts tested as well were dissolved in ultra pure water. All items were analytical reagent grade purchased from Sigma-Aldrich.

## RESULTS AND DISCUSSION

The objective of this research was to find new potential anti-malarial drugs to eradicate this outrageous global disease. This investigation is the first report on the effect of Palestinian *Artemisia sieberi* extracts on the formation of the  $\beta$ -hematin. It was found

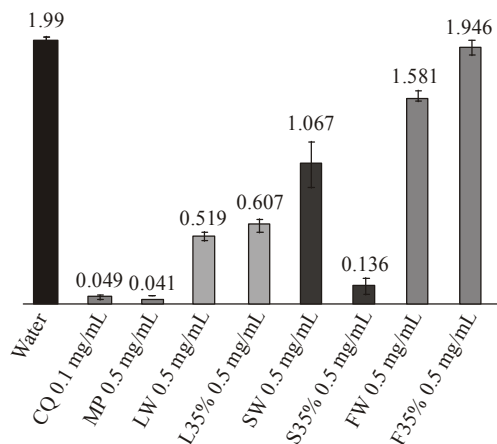


Fig. 1: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* 35% ethanolic and water extracts compared to the negative and positive controls, showing the absorption values of dissolved  $\beta$ -Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficacy, the lower the absorption is, the more efficacious the drug is considered. Each result represents the average of 16 individual experiments

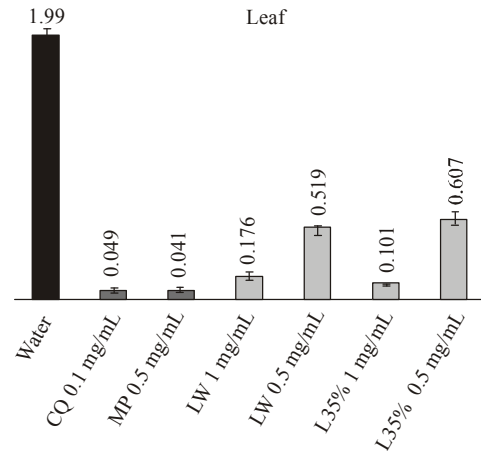


Fig. 3: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* 35% ethanolic and water extracts of the leaf compared to the negative and positive controls, showing the absorption values of dissolved  $\beta$ -Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficacy, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments

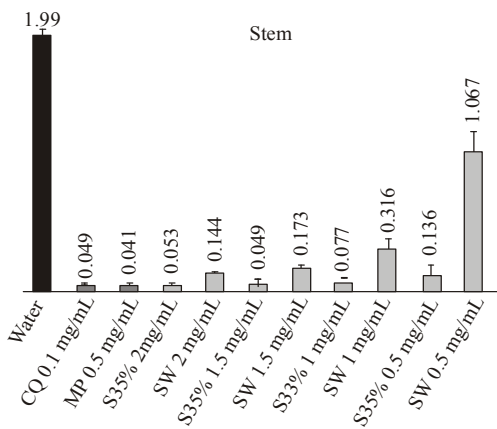


Fig. 2: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* 35% ethanolic and water extracts of the stem compared to the negative and positive controls, showing the absorption values of dissolved  $\beta$ -Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficacy, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments

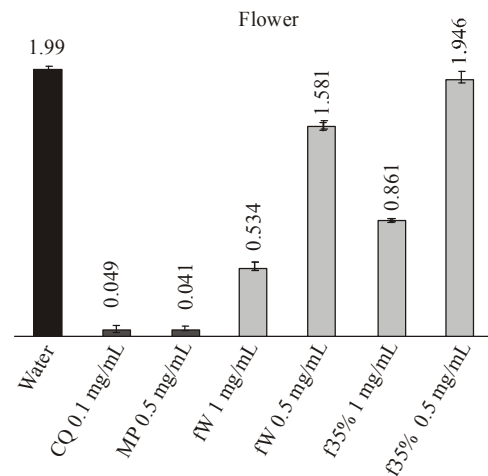


Fig. 4: Column diagram representing the efficacy of potential anti-malarial drug *Artemisia sieberi* 35% ethanolic and water extracts of the flower compared to the negative and positive controls, showing the absorption values of dissolved  $\beta$ -Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficacy, the lower the absorption is, the drug is considered to be more efficient. Each result represents the average of 16 individual experiments

in this research that *Artemisia sieberi* has the capability to impede the formation of  $\beta$ -hematin in *in-vitro* systems. This finding deserves further studies.

Results of the semi-quantitative experiments done on different plant material extracts are shown below in

Fig. 1 to 4. Figure 1 summarizes the efficacy of different parts of *Artemisia sieberi* for different solvents used (Water (W) and 35% ethanol (35%)) in inhibiting  $\beta$ -hematin formation. These results were

compared to positive controls of chloroquine (CQ) and 2-mercaptopyrimidine (MP) as well as negative control water (W).

This study revealed that the most pronounced effect was that of *Artemisia sieberi* stem extract in 35% ethanol (S35%) followed by the leaf extracts while the least effect was that of flowers extracts. There were no significant differences between the water (W) extract or the ethanolic (35%) extracts for both the leaf and flower.

Each absorption value is the average of 16 experiments and each value is inversely proportional to the absorption; low absorption indicates higher efficacy and vice versa. The mechanism of inhibition of  $\beta$ -hematin formation by these different crude extracts is probably through formation of a complex between active compounds in these extracts and ferriheme; this complex prevents the formation of  $\beta$ -hematin.

Details of the efficiency of different concentrations of the *Artemisia sieberi* stem, leaf and flowers are shown in Fig. 2 to 4.

The finding that the extract of stems is more efficacious than the extract of leaves or flowers in the inhibition of  $\beta$ -hematin may be due at least in part to the fact that the sesquiterpenes (Goel *et al.*, 2007) are known to be present at higher concentrations in the stems. This might also be the case for scopoletin, saponins, thujone.

The mechanism of hemozoin inhibition of these different molecules is not necessarily the same. Scopoletin for example has a strong anti-inflammatory effect. *Plasmodium falciparum* infected erythrocytes contain uric acid precipitates in the cytoplasm of the parasitophorous vacuole, which are released when erythrocytes rupture. Uric acid precipitates are highly inflammatory molecules mediators for inflammatory cytokines IL-6, IL-8 and are considered a danger signal for innate immunity (Van de Hoef *et al.*, 2013). The situation seems more complex for saponins since saponins are amphipathic (lyophilic and hydrophilic). In the recent past there has been unforeseen interest in the clinical utilization of saponins as chemotherapeutic agents. They have a biological role as membrane permeability enhancers, immunostimulant and hypocholesterolaemic properties (Francis *et al.*, 2002). They however have hemolytic properties and develop 40-50 Angstrom pores in erythrocyte membranes and this might explain why they are extensively used as adjuvants in vaccines or to enhance the bioavailability of other drugs. They also modulate the sodium pump and ATPase which are target sites for most drugs. Lipids provide a scaffold for the hemozoin formation. Heme degrades inner membranes of the RBC to generate these lipids. It has been shown (Carter,

2009) that detergents like Tween may strongly interfere with this mechanism and saponin is a detergent. Evidence that thujone is the active antimalarial molecule in many artemisia species is confirmed by the fact that *Artemisia dracunculus* which contains no thujone has no noticeable antimalarial activity (Sponza and Chizzola, 2013). Many recent studies also report that the anti-cancer properties of several plants, including *Artemisia absinthium*, are proportional to the concentration of thujone (Biswas *et al.*, 2011).

Other plants like wild sage (*Salvia officinalis*) contain large amounts of thujone and are potential anti-malarial drugs. In a previous study (Akkawi *et al.*, 2012) we have shown that this plant prevents the formation of  $\beta$ -hematin.

And as a reminder: the well known antimalarial quinine, extracted from the Cinchona tree, also inhibits the hemozoin formation.

## CONCLUSION

The herb *Artemisia sieberi* (alias *herba alba*) has been used in folk medicine with no reported toxicity.

Crude plant extracts of *Artemisia sieberi* may also have enhanced antimalarial activity due to synergistic effect which may be derived from the presence of different anti-malarial compounds in *Artemisia sieberi*. Although the concentration for the extracts are higher than for the positive controls, it is important to keep in mind that the concentration of plant extract tested used in each experiment represents the crude concentration which means that the active components concentration could be much lower. We are already working on isolating the ingredients; results will be published in the near future.

*Artemisia sieberi* results index:

S	: Stem
S 35%	: Stem extracted in 35% ethanol
S W	: Stem extracted in distilled water
CQ	: Positive control chloroquine
WATER	: Ultrapure water
MP	: Mercaptopyrimidine
LW	: Leaf extracted in distilled water
L35%	: Leaf extracted in 35% ethanol
FW	: Flower extracted in distilled water
F35%	: Flower extracted in 35% ethanol

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