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Potential Antimalarial Activity from Alcoholic Extracts of Wild *Salvia palaestina* Leaves

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Abstract: Malaria threatens the lives of more than one third of the world's population; it is a major cause of human deaths. As a result of the emergence of resistant strains of *Plasmodium falciparum* to common antimalarial drugs, the search for new antimalarial drugs is urgently needed. Hemozoin synthesis is an indispensable process for the parasite survival and is the target of action for several known antimalarial drugs. Sage, *Salvia palaestina*, is an aromatic Mediterranean plant. Its leaves have been used over centuries in Palestinian traditional medicine and are now being investigated for potential antimalarial activity. This study reveals the antimalarial activity of crude and HPLC separated fractions tested using two methods; the inhibition of ferriprotoporphyrin IX (FP) bio-mineralization: semi-quantitative micro-assay used by Deharo and a previously self-developed quantitative *in vitro* method. Reversed phase preparative liquid chromatography coupled to Photo Diode Array (PDA) detector was used to isolate and enrich eight fractions. Three fractions showed promising antimalarial activity. The crude alcoholic extract of sage leaves seems to have the potential of an antimalarial drug; it prevents β-hematin formation with an efficiency of about 72% when compared to the standard Chloroquine which gave 93% at comparable concentrations of chloroquine and extract.

Keywords: Antimalarial drugs, β-Hematin, chloroquine, ferriprotoporphyrin (IX), hemozoin, *Salvia palaestina*

INTRODUCTION

Malaria is a disease of poverty, where it is concentrated in the tropical areas mostly of developing countries; it is one of the most prevalent parasitic diseases. According to the latest WHO estimates, there were about 219 million cases of malaria in 2010 and an estimated 660,000 deaths. Africa is the most affected continent with about 90% of all malaria deaths (World Malaria Report (WMR), 2012).

Plasmodium; the parasite causing malaria, is the single most devastating protozoan (Bannister and Sherman, 2009). There are five species of plasmodium that may cause human malaria, but *Plasmodium falciparum* is the most highly fatal species (Rathore, 2006).

In nature, *Plasmodium* is transmitted from one host to another by a bite of an infected female *Anopheles* mosquito to a human victim where thousands of sporozoites may enter the body. Once inside, sporozoites travel to the liver, producing thousands of merozoits within a week then migrating back into the blood stream where they invade and infect erythrocytes. During this intra-erythrocytic stage the parasites consume intercellular proteins, mainly hemoglobin, as a source of amino acids. Digestion of hemoglobin takes place within food vacuoles of the parasite where pH ranges between 5.0-5.4 (Goldberg *et al*., 1990). Inside the RBC the parasites multiply to form more merozoits, the infected red blood cells then burst releasing the newly formed merozoits thus infecting new cells (Bannister and Sherman, 2009).

The intra-erythrocytic stage appears to be fundamental to the Plasmodium life cycle. Massive degradation of hemoglobin results the liberation of monomeric heme which is highly toxic to the parasite. In order to survive, this parasite, as other blood parasites, have evolved a unique mechanism for heme detoxification through its conversion into an uncreative, nontoxic, insoluble crystalline pigment, known as hemozoin (Egan *et al*., 2002).

Structurally, hemozoin consists of reciprocal head-to-tail dimeric units of heme bound through propionate oxygen-Iron (III) (Slater *et al*., 1991; Pagola *et al*., 2000). The propionic acid groups of the heme dimer then hydrogen-bond with other dimers to form extended

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crystals. Disruption of this crystallization process results in parasite death (Rathore, 2006), this serves as an important strategy for drug design and a target for action of several known antimalarial drugs (Hawley *et al*., 1998; Sullivan *et al*., 1996).

A synthetic analouge to hemozoin called β-hematin is considered to be structurally and spectroscopically identical to purified hemozoin (Slater *et al*., 1991), making it an excellent target for biochemistry studies (Tekwani and Walker, 2005; Ncokazi and Egan, 2005).

Since Plasmodium species have become resistant to many antimalarial drugs; risk of infection is increasing. Plasmodium has developed resistance to drugs in the aminoquinoline group: chloroquine, amodiaquine and mefloquine. Recently resistance to Artemisinin derivatives used in combination therapy has been reported (Dondorp *et al*., 2009), making the development of new antimalarial drugs much more urgent.

We had previously attempted to find new anti-malarial drugs in concentrating on the effect of Pyrimidine derivatives in the *in-vitro* inhibition of β-hematin (Aljazzar *et al*., 2010) and *cis*-platin complexes (Akkawi *et al*., 2012). In this study however we concentrate on the effect of new isolated molecules from Palestinian herbal plants.

Palestinian sage, which is known as *Salvia palaestina* belongs to the family Lamiaceae is an important aromatic medicinal herb that is widely used today in the form of herbal tea. Previous studies showed that *Salvia palaestina* essential oils and extracts have excellent anti-oxidant (Gürsoy *et al*., 2012), anti-bacterial (Karataş and Ertekin, 2010) and anti-proliferative (Fiore *et al*., 2006) properties. . However, no studies on the *in-vitro* antimalarial activity of this plant have been carried out thus far. In a continuation to verify the efficacy of traditional medicines against malaria, *Salvia palaestina* was investigated.

The target of this investigation is to develop a rapid analytical HPLC method that scans the active constituents from 35% ethanolic crude *Salvia palaestina* followed by enrichment and isolation of the active antimalarial fractions by using reversed phase preparative chromatography coupled with photo diode array detector. The isolated fractions were tested for their antimalarial activity.

MATERIALS AND METHODS

Plant collection: Fresh wild sage leaves (*Salvia palaestina*) were collected towards the end of March from different areas around Jerusalem and the West Bank of Palestine far from agricultural lands. Samples were air dried in the shade for ten days.

Materials: Glacial acetic acid (HPLC grade), acetonitrile (ACN) and Ethanol (EtOH) solvents were purchased from Merck (Germany). Hemin chloride and all other products were analytical reagent grade obtained from Sigma. Highly purified water was prepared by using a Millipore Milli-Q plus water purification system.

Extraction of the nonvolatile secondary metabolites of *Salvia palaestina*: Dried leaves were grinded into powder, extraction was performed by soaking (1:10) (*wt*/*vol*) of dried plant leaves in 35% ethanol, left for about 24 h at room temperature. The extract was then filtered using what man No 42 filter paper. The crude ethanol extract was obtained after the solvent was rotary evaporated at 60-80°C under reduced pressure, followed by lyophilization using a Labconco freeze drier until constant weight was obtained then stored at -20°C until use.

***In-vitro* semi-quantitative method:** The method adopted was according to Deharo *et al*. (2002). A mixture containing 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 potential anti-malarial drug solution or solvent, was incubated in a non-sterile 96-well flat bottom plate at 37ºC for 18-24 h. The solutions were added to the plate in the above 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 was 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 followed by discharging the supernatant. The β-hematin remaining was then dissolved in 200 μL of 0.1 M NaOH to form an alkaline hematin that can be measured spectrophotometrically at 405 nm using ELISA reader.

Ultra pure water was used as negative control, positive controls and tested extracts were dissolved in ultra-pure water.

***In-vitro* quantitative method:** According to Blauer and Akkawi (1997), freshly prepared stock solution of hemin chloride was prepared by dissolving the salt in 0.4 M aqueous NaOH and incubated for 30 min at 37°C, stock solution of the leaf extract used was prepared using ultra-pure water. The final concentration of hemin and *Salvia palaestina* leaf crude ethanol extract were 0.5 and 1 mg/mL respectively, aqueous HCl was also included in order to obtain the required pH (ionic strength was 0.1235 M). The reaction was equilibrated at 37°C for 10 min, finally 4 μL of glacial acetic acid were added with gentle mixing (Blauer and Akkawi, 2000). The whole mixture was left for 2 h at 37°C without stirring. The total volume of the reaction mixture was 4 mL and the final pH was 4.9 to 5.2. Samples were centrifuged for 10 min using (Jouan B4) centrifuge. The supernatant was discarded and the precipitate was washed with ultra-pure water and quantitatively transferred to a Millipore Swinnex 13 filter containing Whatman filter paper No. 50, already

lyophilized to a constant weight in freeze-drying machine (Labconco Freezone). DMSO was passed slowly through the filter until the filtrate remained feebly colored and then washed again with ultra-pure water. The remaining precipitate was then lyophilized to a constant weight.

HPLC instrumentation systems: The Analytical High Pressure Liquid Chromatography (HPLC-PDA) system consists of an alliance 2695 HPLC, 2996-Photo Diode Array (PDA). Data acquisition and control were carried out using Empower™ software (Waters Company, Germany). The Preparative High Pressure Liquid Chromatography (Prep-HPLC-PDA) system consisted of 3535 quaternary gradient module which provides a flow rate of up to 50 mL/min, equipped with 996 PDA detectors.

Chromatographic conditions: The HPLC analytical experiments were run on octadecyl silane C18 chemically bonded column (Waters XBridge, 4.6×150 mm, 5 μm). The mobile phase was a gradient of (0.5% acetic acid aqueous solution) (eluent A) with ACN (eluent B). The gradient elution was set for a liner gradient starting from 90% of eluent A and 10% eluent B up to 40% of eluent A and 60% of eluent B for 50 minutes. Before the analysis, the column was equilibrated with the starting mobile phase for about 20 min. All the solvents and sample were filtered with a 0.45 μm micro porous filter and were degassed by sonication prior to use. Wavelengths of 260 nm and 350 nm were chosen since it was found that the *Salvia palaestina* compounds possess maximum absorption at these wavelengths. The flow rate was set at 1 mL/min. The injection volume was 10 μL of 3 mg/mL and the temperature of the column was at room temperature.

The HPLC Preparative experiments were run on ODS column (Agilent PrepHT C18, 22.2×250 mm, 10 μm). The same mobile phase was used and the gradient elution was set for a liner gradient starting from (90% of eluent A and 10% eluent B) up to (50% of eluent A and 50% eluent B) for 20 minutes and then the preparative HPLC system was washed for 10 min with 90% ACN to elute any lipophilic compounds from the column. The flow rate used was 15 mL/min, the injection volume was 1000 μL and the temperature of the column was at room temperature.

Sample preparation: The sample solution of crude *Salvia palaestina* was prepared by dissolving about 75 mg in 3 mL of 50% EtOH, shaken by mechanical means for 1 min, sonicated for 2 min and then was filtered using 0.45 μm membrane filter before injection. The obtained final solution contained 25 mg/mL. One ml of this solution was directly injected to the preparative HPLC and eight fractions were collected.

RESULTS AND DISCUSSION

The results are shown as comparisons to positive and negative controls. Figure 1 shows the antimalarial activity of different dilutions of the alcoholic crude leaves extract of *Salvia palaestina*. The absorption is inversely proportional to drug efficiency; the lower the absorption is the more efficient the drug is.

The antimalarial activity testing was repeated using a quantitative method; the results are presented in percentage yield (Fig. 2). The efficiency of crude extract as compared to positive control (chloroquine) and our positive internal control (2-MP) is shown in Fig. 3.

Regardless of the mechanism of action of the extract of *Salvia palaestina,* it is clearly seen that the extract inhibits β-Hematin formation, however we believe it acts through a similar mechanism to chloroquine.

In an attempt to isolate an active ingredient(s), several HPLC separation protocols were carried out resulting in eight fractions (Fig. 4 and 5), their individual effects are shown in Fig. 6.

Sage is a rich source of polyphenols and has the ability to accumulate high levels of active flavonoids (Lu and Foo, 2002; Ulubelen and Tuzlaci, 1990). These classes of compounds are known to exhibit a range of biological activities including; anticancer, antibacterial, anti-oxidant and anti-inflammatory properties. High Performance Liquid Chromatography (HPLC) is the best technique to separate phenolic and triterpenoid compounds.

Typical analytical HPLC chromatogram of the 35% ethanolic crude mixture of *Salvia palaestina* revealed fourteen compounds at 260 and 350 nm. The overlaid UV spectra of the eluted peaks are shown in Fig. 4.

Further step of purification was attained by loading 1000 μL (25 mg/mL) 35% ethanolic crude extract of *Salvia palaestina* to inch preparative column and eluted subsequently using gradient binary solvent mixture of acidic water and ACN. Eight major fractions (I-VIII) with different concentrations were obtained as shown in Fig. 5. The aliquots collected were dried by using rotary evaporator to remove the acetonitrile solvent followed by direct freeze drying to get rid of the water and ending up with few mg's of fluffy powdered material.

As shown in Fig. 6 only three fractions were found to exhibit antimalarial activity when assayed *in-vitro*. We noticed that the most pharmacological potent fraction was fraction VII which was eluted between 14.8-19 min. As shown in Fig. 5, this fraction contains a major peak at 15 min and to a lesser extent some other few peaks that lack chromophores which strengthens our believe that this compound is the one responsible for inhibitory effect on β-Hematin formation. Previous studies on other species of salvia leaves have proven the presence of phenolic compounds which possess strong UV-absorption chromophores (Lu and Foo, 2002).

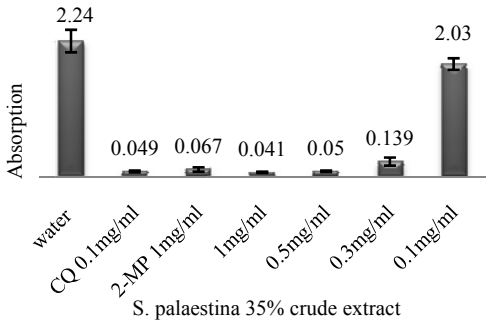

Fig. 1: Column diagram representing the efficiency of 35% ethanol extract of S. palaestina leaf compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to Deharo *et al*. (2002) semi-quantitative method. Each result represents the average of 16 individual experiments

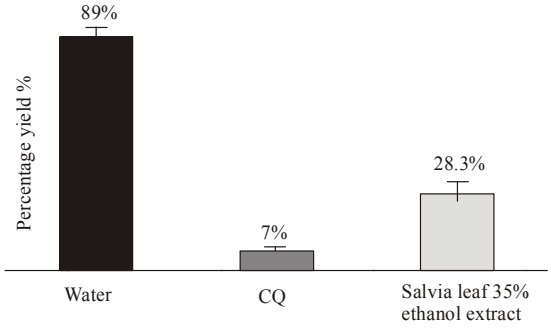

Fig. 2: Column diagram representing the percentage yields of 35% ethanol extract of *Salvia palaestina* leaf as potential anti-malarial drug, compared to CQ and water. Yields are inversely proportional to drugs efficiency. Each result is an average of 6 individual experiments. Concentration of Hemin chloride was 0.5 mg/mL while the concentration of CQ and *Salvia palaestina* was 1.0 mg/mL

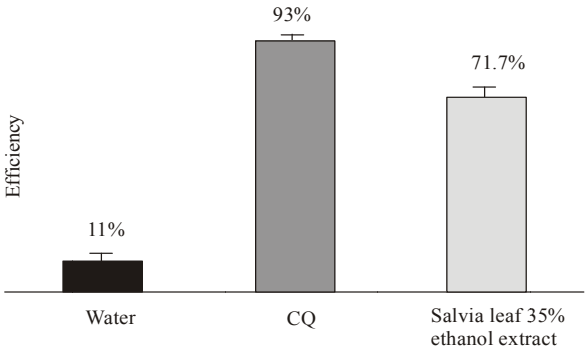

Fig. 3: Column diagram representing the efficiency of 35% ethanol extract of *Salvia palaestina* leaf as potential anti-malarial drug, compared to CQ and water. Each result is an average of 6 individual experiments. Concentration of Hemin chloride was 0.5 mg/mL while the concentration of CQ and *Salvia palaestina* was 1.0 mg/mL

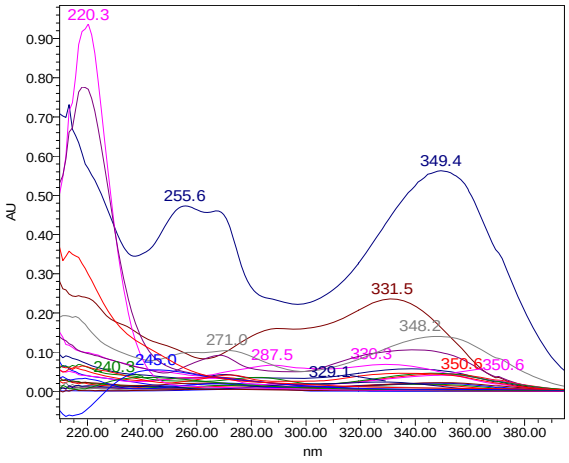

Fig. 4: Overlaid UV spectra of the eluted peaks from 35% ethanolic crude mixture of *Salvia palaestina*

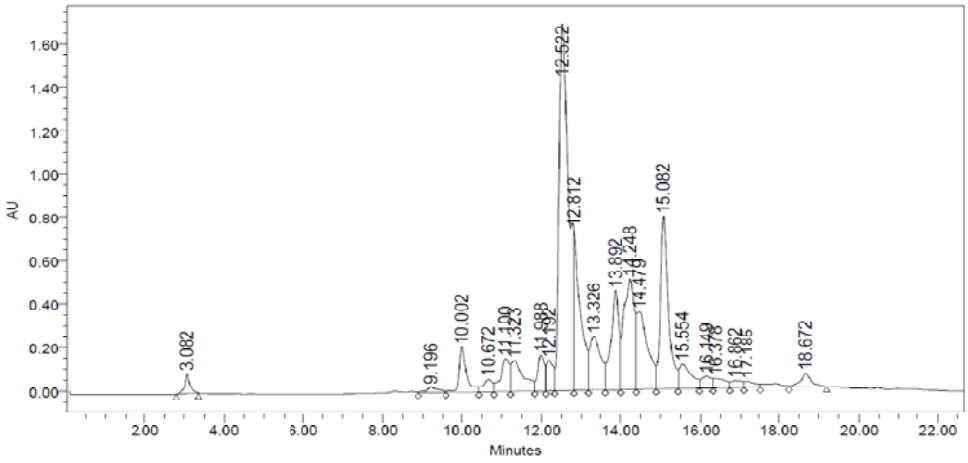

Fig. 5: Typical preparative HPLC chromatogram of 35% ethanolic crude mixture of *Salvia palaestina*. 1000 µL were injected at flow rate 15 mL/min and the monitoring λ of 260 and 350 nm were used. Fraction I was collected from (2-3.2 min), fraction II (3.3-7 min), fraction III (7-9.6 min), fraction IV (9.6-12.1 min), fraction V (12.2-13 min), fraction VI (13-14.8 min), fraction VII (14.8-19 min) and fraction VIII (19-22)

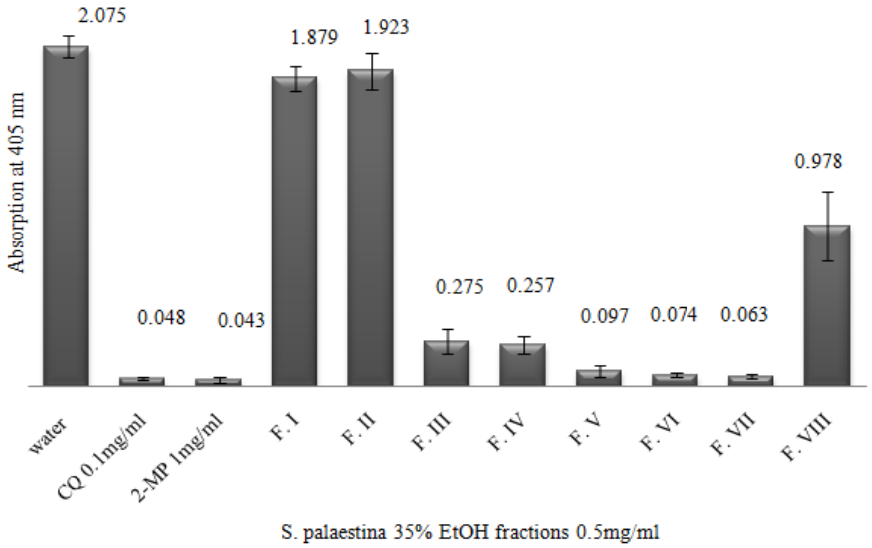

Fig. 6: Column diagram representing the efficiency of the eight fractions obtained from separation of S. palaestina leaf 35% ethanol extract, dissolved in water at concentration 0.5mg/mL, compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to Deharo *et al*. (2002) semi-quantitative method. Each result represents the average of 16 individual experiments

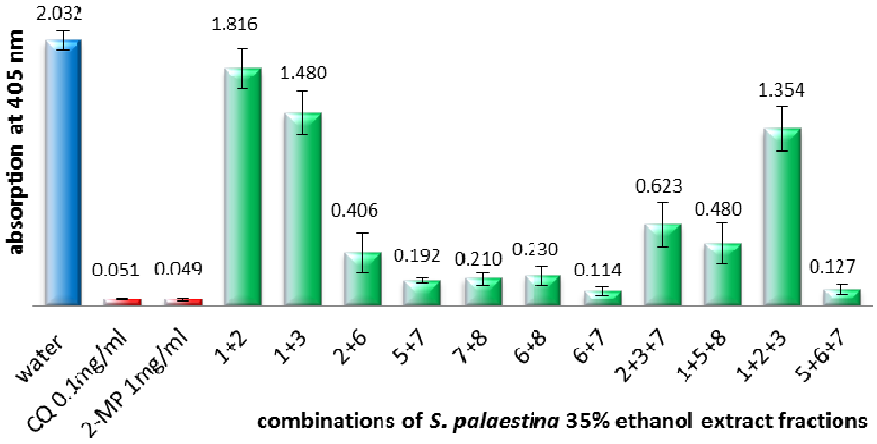

Fig. 7: Column diagram representing the efficiency of combinations made of the fractions obtained from separation of *S. palaestina* leaf 35% ethanol extract, dissolved in water, compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to Deharo *et al*. (2002) semi-quantitative method. Each result represents the average of 6 individual experiments

To investigate any possible synergistic effects, different and random combinations of the separated fractions were assayed for their *in-vitro* inhibitory effect on β-Hematin formation. Results for these combinations are shown in Fig. 7.

CONCLSUION

Malaria is a global disease causing millions of deaths mostly amongst African children. Due to the rise of resistant strains of *Plasmodium falciparum* to available drugs there is an urgent need to find new cheap and easily available anti-malarial drugs preferably from natural products.

Hence attention is given to *Salvia palaestina*, a safe and widely available herb. It is clear from the outcome of this investigation that *Salvia palaestina* is an inhibitor of β-Hematin formation, both as a crude extract and HPLC fractions and hence must be given further attention as a possible antimalarial drug. Further fractionation, purification and identification of possible active ingredients as well as *in- vivo* testing is currently under investigation.

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