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Investigation of Traditional Palestinian Medicinal Plant *Inula viscosa* as Potential Anti-malarial Agent

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Abstract: Malaria is a life threatening parasitic disease which is prevalent mainly in developing countries; it is the main cause of global mortality and morbidity. Development and search of novel and effective anti-malarial agents to overcome chloroquine resistance have become a very important issue. Most anti-malarial drugs target the erythrocytic stage of malaria infection, where hemozoin synthesis takes place and is considered a crucial process for the parasite survival. Throughout last decades, natural products have been a significant source of chemotherapeutics especially against malaria. *Inula viscosa*, is a shrub that grows around the Mediterranean basin and considered as an important Palestinian traditional medicinal herb. In this research it was found that the Palestinian flora *Inula viscosa* alcoholic extract has a significant and promising anti-malarial effect in both *in vitro* and *in vivo* systems. The crude alcoholic extract of *Inula viscosa* has the capability to impede the formation of β-hematin *in-vitro*; with an efficiency of about 93% when compared to the standard chloroquine which gave 94% at comparable concentrations. *In vivo* studies showed that this crude extract inhibited the growth of *Plasmodium* parasites in the red blood cells at a rate of about 96.6%, with an EC50 value of 0.55 ng/mL. Several secondary plant metabolites may be responsible for this anti-malarial activity; the effect also may be most probably due to the presence of high concentrations of nerolidol which has often been found at high concentrationsin this plant. Nerolidol shows a stronger inhibition of hypoxanthine incorporation than quinine. Its anti-malarial effect is potentiated by other essential oils. Nerolidol is also found in several Artemisia species and in *Cymbopogon citratus* (lemongrass) and *Virola surinamensis*, all plants known for their anti-malarial properties.

Keywords: β-hematin, anti-malarial drug resistance, anti-malarial drugs, chloroquine, ferriprotoporphyrin (IX), hemozoin, *Inula viscosa*

INTRODUCTION

In a recent statement WHO called WHO Traditional Medicine Strategy (www.who.int/ medicines/publications/traditional/trm_strategy 14_23/ en) 2014-2015 encourages the development of anti-malarial drugs based on herbal medicine and confirms that 80% of the people have only access to these drugs. Malaria is caused by a microscopic protozoan called *Plasmodium* (Bannister and Sherman, 2009). There are about 120 different species of *Plasmodium*, but only four of these can transmit the malaria disease to humans; *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum* the most common and accounts for 90% of overall deaths caused by this disease. During the intra-erythrocytic stage, *Plasmodium* merozoites utilize hemoglobin as a source of amino acids. Digestion of hemoglobin takes place inside food vacuoles within the parasite (Spiller *et al*., 2002; Klones *et al*., 2007) (where pH range is between (5.0-5.4).

Enzymes such as plasmepsins (Liu *et al*., 2005; Klemba *et al*., 2004) digest hemoglobin releasing oxygen and an oxidized ferric form of protoporphyrin IX (Sullivan Jr., 2003; Slater *et al*., 1991; Goldberg *et al*., 1990; Pagola *et al*., 2000; Egan *et al*., 2002; Tekwani and Walker, 2005) which is highly reactive and toxic to the parasite. As a usual law of survival, the *Plasmodium* parasite has evolved a mechanism for detoxification of free heme through its bio-mineralization (Slater *et al*., 1991) into an un-reactive, insoluble crystalline form called hemozoin which is famously known as "Malaria Pigment" (Goldberg *et al*., 1990; Pagola *et al*., 2000). The formation of hemozoin is crucial for the parasite's survival making it a major target of action for several known anti-malarial drugs (Blauer and Akkawi, 1997). For studying hemozoin, a synthetic analogue called β-hematin is used, that is structurally, morphology and spectroscopically identical to purified hemozoin. β-hematin is constituting of cyclic heme dimers; where the central iron of one hematin is linked to the oxygen of the

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carboxylate side chain of the adjacent hematin arranged in an crystalline ordered structure through intermolecular hydrogen bonding (Pagola *et al*., 2000), β-hematin can be produced in acidic solutions and used in assay for anti-malarial compound screening. Natural compounds were historically used as drugs. The history of anti-malarial drugs is intimately linked with the history of herbal medicinal products (Bowden *et al*., 2003). Inappropriate use of anti-malarial drugs contributed to widespread resistance of the malaria parasite to many drugs (Waller *et al*., 2003; Dondorp *et al*., 2009; Orjih and Fitch, 1993). Resistance to anti-malarial drugs has proved to be a challenging problem to control malaria in most parts of the world and highlights the need for new drugs. Earlier attempts showed the effect of cisplatin complexes (Akkawi *et al*., 2012a) and wild sage (Akkawi *et al*., 2012b; Jaber *et al*., 2013) in *in-vitro* inhibition of β-hematin formation. In this study however we concentrate on the effect of new promising Palestinian herbal plant called *Inula viscosa (I. viscosa).*

Inula viscosa is a medicinal shrub that grows in the wild around the Mediterranean basin, It belongs to the Compositae family. It is well known for its medicinal properties in Middle Eastern cultures and has been used in traditional medicine as a cure for many diseases.

There is published evidence that *Inula viscosa* possesses many important medicinal benefits, including anti-inflammatory (Máñez *et al*., 1999) anti-oxidant (Schinella *et al*., 2002) acting as superoxide anion scavengers, anti-bacterial (Talib and Mahasneh, 2010) and anti-fungal activities (Hawley *et al*., 1998; Kanan and Al-Najar, 2008). Previous studies on this plant led to the isolation and characterization of many compounds present in *Inula viscosa* like hispidulin and nepetin (Talib *et al*., 2012) as flavonoids and 3β-hydroxyilicic acid as sesquiterpenoids (Wollenweber *et al*., 1991).

In this study light was shed on the activity of *Inula viscosa* ethanolic extract as potential anti-malarial drug in both *in vitro* and *in vivo* systems.

MATERIALS AND METHODS

Plant collection: Fresh wild leaves of *Inula viscosa* were collected towards the end of October from different areas around Jerusalem and the West Bank of Palestine far from agricultural lands. Samples were air dried in the shade for 10 days.

Materials: 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 *Inula viscosa*: 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 Whatman 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 37°C 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 as well as the 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 *Inula viscosa* 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.

***In vivo* study:**

Plasmodium parasite culture: NF54-luc parasites (Salazar *et al*., 2012) were cultivated at 5% haematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate and 0.01 mg/mL gentamycin. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen. The level of parasitemia was calculated by counting three independent blood smears stained with Giemsa under light microscope.

Plant extract treatment: Parasites (200 µL) were incubated with 5 ng/mL of plant extracts for 48 h (parasite life cycle), Blasticidin was used as positive control for obtaining full inhibition, no treatment wells were included as negative control and for calculating the Inhibition percentage. IC50 values were determined for plant extract by measuring growth inhibition in serial dilutions (5.00, 2.50, 1.25, 0.625, 0.3125 and 0 ng/mL, respectively). All assays were carried out in triplicate.

Luciferase assays: Luciferase activity was measured using 200 µL of culture. Infected Red Blood Cells (iRBC) were collected by centrifugation then lysed with saponin (0.05%). Lysed cells were removed and the pellet was dissolved in 50 µL PBS. Luciferase activity was measured immediately after adding 50 µL Bright-Glo® luciferase reagent (Promega, Madison, USA) suing FLUOROSKAN FL luminometer (Thermo, USA). The luciferase activity assays were carried out in triplicate. GraphPad Prism version 4.0b was used to calculate the Inhibition percent. Sigmoidal dose-response linear regression curve fitting comparing fixed and variable slope by GraphPad Prism version 4.0b was used to calculate the IC50.

RESULTS AND DISCUSSION

This study is aimed at developing new anti-malarial drugs from herbal origin to eliminate this dreadful disease. This study is the first to show the inhibitory effect of Palestinian *Inula viscosa* alcohol extracts on the formation of the β-hematin. We were unable to find another scientific paper having documented the anti-malarial properties of this plant. It was found in this investigation that *Inula viscosa* has a strong inhibitory effect similar to that of chloroquine in both in vitro and *in vivo* systems.

Results of the in vitro semi-quantitative tests done at different concentrations of the *Inula viscosa* alcoholic extracts are viewed below in Fig. 1 which summarizes the efficacy of 35% alcoholic crude leaves extract of *Inula viscosa* as anti-malarial agent in inhibiting β-hematin formation. These results were compared to positive controls of Chloroquine (CQ) and 2-Mercaptopyrimidine (2-MP) as well as the negative control, water. According to the semi-quantitative

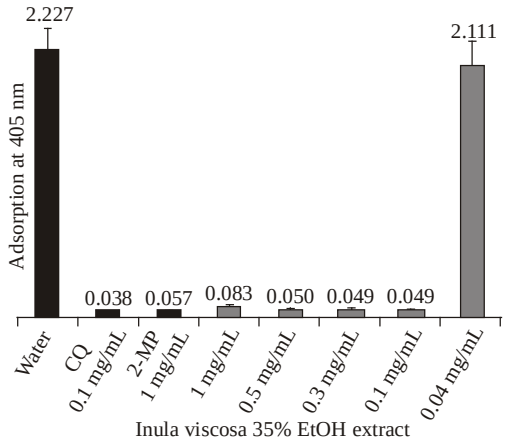

Fig. 1: Column diagram representing the efficacy of potential anti-malarial drug 35% ethanol extract of *Inula viscosa* leaves; compared to the control water and positive controls CQ-chloroquine 0.1 mg/mL, 2MP-2mercaptopyrimidine 1 mg/mL; showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader; according to E. Deharo semi-quantitative method; each result represents the average of 24 individual experiments

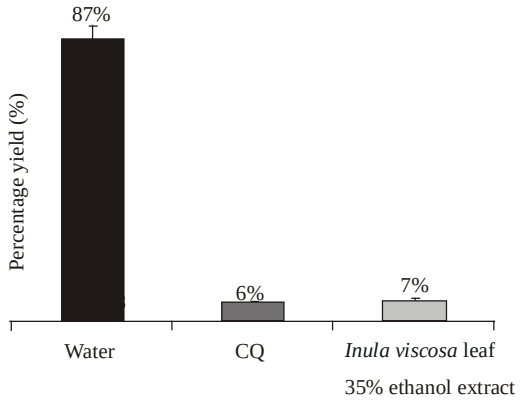

Fig. 2: Column diagram representing the percentage yields of 35% ethanol extract of *Inula viscosa* leaves as potential anti-malarial drug, compared to CQ and water; yields are inversely proportional to drugs efficiency. Each result is an average of 6 individual experiments; concentration of hemin chloride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

method the absorption is inversely proportional to drug efficiency; the lower the absorption is the more efficient the drug is.

The anti-malarial activity testing of the *Inula viscosa* leaves was repeated using another in vitro quantitative method; the results are presented in percentage yield as in Fig. 2. While the efficiency of crude extract compared to positive control (chloroquine) is shown in Fig. 3.

Regardless of the mechanism of action of the *Inula viscosa* extract, it is clearly seen that the extract extremely inhibits β-hematin formation. We believe it

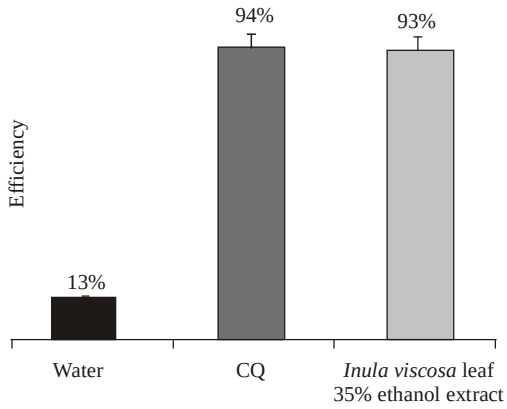

Fig. 3: Column diagram representing the efficiency of 35% ethanol extract of *Inula viscosa* leaves as potential anti-malarial drug; compared to CQ and water; each result is an average of 6 individual experiments; Concentration of hemin chloride was 0.5 mg/mL while the concentration of CQ and *Inula viscosa* was 1.0 mg/mL

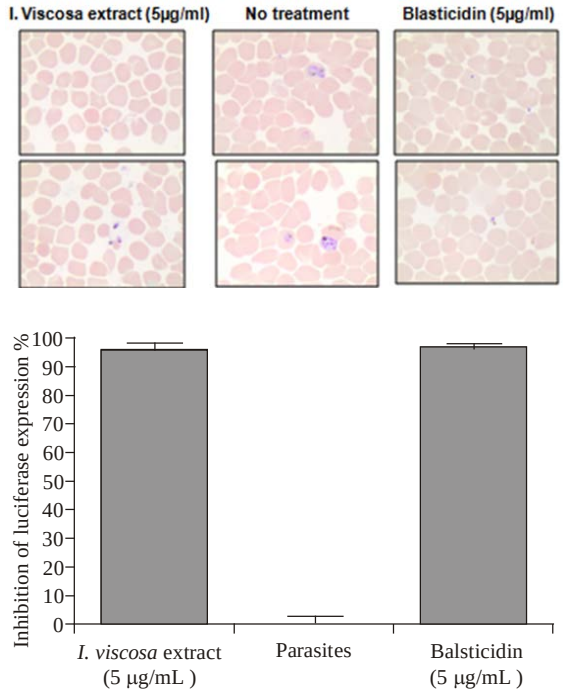

Fig. 4: *In vivo* effect of *Inula viscosa* leaves ethanol extract on plasmodium cultured parasite
5 µg/mL of the plant extract was used compared to 5 µg/mL of blasticidin anti-malarial drug

acts through a similar mechanism to chloroquine, probably through formation of a complex between active compounds in this extract and ferriheme; this complex prevents the formation of β-hematin.

The effect of *Inula viscosa* 35% alcoholic leaf extract over the development of culture maintained *Plasmodium* parasites with human red blood cells was

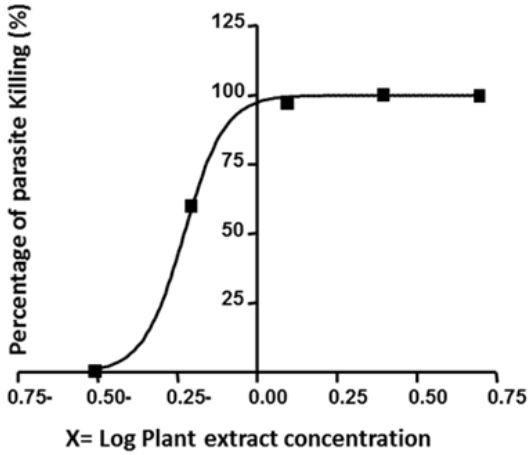

Fig. 5: The maximum half Effective drug Concentration (EC50) calculation using different concentrations of *Inula viscosa* leaves extract

Table 1: Calculation of the maximal effective concentration of *Inula viscosa* leaves extract on the survival of cultured *Plasmodium* parasites in red blood cells

Plant extract concentration (ng/mL)	Killing percentage (%)	S.D.
0.3125	0	3.163860
0.6250	59.72072	1.357593
1.2500	96.65255	1.066239
2.5000	99.64877	0.199455
5.0000	99.45297	0.173498

S.D.: Standard deviation

tested. An important finding in this research study was the fact that the tested extract inhibited the growth of the parasites in the red blood cells in a rate of about 96.6%. Results were similar to the most experimentally used anti-malarial drug, Blasticidin, which reached up to 96.1% inhibition. The extracted compounds from the *Inula viscosa* leaves are totally a natural product that was stored at room temperature and succeeded to inhibit the survival of *Plasmodium* parasites in red blood cells at a concentration of 5 µg/mL without causing lysis of the red blood cells in the PF culture (Fig. 4).

Plant extract EC50 calculations: Based on the *in vivo* results that shows an effective killing of the *Plasmodium* parasite by *Inula viscosa* leaves extract, the maximum half effective concentration of the plant extract was calculated. This was done by incubating the cultured *Plasmodium* parasites with different concentrations of the plant extract; starting from 5 ng/mL with two fold serial dilutions down to 0.3 ng/mL (Table 1). Figure 5 shows the effective killing of the different concentrations of *Inula viscosa* leaves extract and the calculated EC50 was found to be 0.55 ng/mL.

Many candidate pharmacologically active secondary compounds present in *Inula viscosa* extract that may be able to hinder the growth of the

plasmodium parasite in the red blood cells, such as alkaloids, flavonoids and sesquiterpene lactones. (Saxona *et al*., 2003; Bohlmann *et al*., 1978; Grande *et al*., 1985).

The inhibition of β-hematin may be due at least in part to the fact that the sesquiterpenes are known to be present at higher concentrations in this plant (Abu Zarga *et al*., 1998; Fontana *et al*., 2007), probably through formation of a complex between active compounds in the extract and ferriheme; this complex impedes the formation of β-hematin.

Polyphenols are known to be antioxidants, considerable interest was given to these compounds recently because of their potential beneficial effects on human health in fighting diseases such as cancer and cardiovascular disease (Scalbert *et al*., 2005; Shohaib *et al*., 2011) and the most common types of polyphenols are flavonoids which are known to present in this plant (Danino *et al*., 2009; Grande *et al*., 1985). Different non-covalent interactions may be responsible for this inhibition. For example it is possible to form hydrogen bonding between propionic acid in ferri-heme dimer with the active compounds in the extract probably those containing for example a carbonyl group.

Another possibility for this anti-malarial activity may be related to the high concentrations of nerolidol in this plant. Nerolidol shows a stronger inhibition of hypoxanthine incorporation than quinine (Seatlholo, 2007). Its anti-malarial effect is potentiated by several other essential oils. It is present in many anti-malarial shrubs: *Cymbopogon citratus*, *Artemisia absinthium*, *Virola surамerinensi* and *Aframomum zambesiacum* (Kenmogne *et al*., 2006).

Following the results above, we could infer that the anti-malarial activity of *Inula viscosa* ethanolic extract may be due to the synergistic effect of its different components. This investigation provided evidence that *Inula viscosa* extracts is a potential natural anti-malarial agent and it may be used in the field of pharmaceutical industries for treating malaria patients. Evaluation of cytotoxicity of *Inula viscosa* leaf extracts must be done.

CONCLUSION

Malaria is the most common lethal infectious disease in the world. The emergence of resistant strains of *Plasmodium falciparum* to common anti-malarial drugs, ranging from chloroquine, sulphadoxine-pyrimethamine to ACTs (artemisinin combined therapy) has been noticed not only in Asia but also in Africa., The search for new anti-malarial drugs is urgent. Herbal medicines would constitute a true polytherapy less likely to be affected by resistance to monotherapy.

According to the results above, it is seen for the first time that the Palestinian flora *Inula viscosa* has a strong anti-malarial activity both *in vivo* and *in vitro* systems. Several secondary plant metabolites are responsible for this anti-malarial activity. More attention must be given to this plant, further fractionation, purification and identification of possible active ingredients is currently under investigation, results will be published in the near future.

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