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Plant-Derived Anti-Malarial Agents: *In vitro* Inhibition of Hemozoin Synthesis by Extracts of *Salvia Palaestina*

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Dedication

This thesis is dedicated to those dearest to me, my parents for their endless love, support and encouragement ….to my great teachers…. to my fiancé Nidal….. to my friends….

Thank you all

Declaration

I declare that the work presented in this thesis for the degree of Master was carried out by me under the supervision of Dr. Mutaz Akkawi, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed _____

Suhair Nabel Jaber

Date: 23/7/2013

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Plant-Derived Anti-Malarial Agents: *In vitro* Inhibition of Hemozoin Synthesis by Extracts of *Salvia Palaestina*

Abstract

Malaria is an infectious disease that has drawn worldwide attention due to the alarming rise of mortality rates particularly in developing countries, where young children and pregnant women are at higher risk. The malaria parasite is a multi-stage one celled organism, called *Plasmodium*, which has co-evolved in mosquitoes and vertebrates for millions of years. The most common and lethal type of malaria infection is caused by *Plasmodium falciparum*. According to world malaria report and the latest WHO estimates, there were more than 219 million cases of malaria in 2010 and an estimated 660, 000 deaths, where Africa is the most affected continent. Designing drugs that substantially and persistently interrupt the life cycle of this complex parasite will require a comprehensive understanding of its biology.

The major concern in the treatment of malaria at present is the increasing resistance of the malaria parasite *P. falciparum* to anti-malarial drugs, particularly chloroquine, and this situation has been intensified by the increasing spread of drug-resistant *P. falciparum* strains.

There are many targets in the fight against this dreadful disease, but our target is the intra-erythrocytic stage in the parasite's life cycle, during this stage, *Plasmodium* parasites reside inside the host erythrocytes degrading hemoglobin resulting in the accumulation of free heme; Ferriprotoporphyrin (IX), toxic to the parasite that is capable of generating oxygen radicals. The detoxification of the heme is through its incorporation into an insoluble crystal called hemozoin or malaria pigment.

β-Hematin, a synthetic polymer made from Ferriprotoporphyrin- IX is structurally, chemically and spectroscopically identical to purified hemozoin, a polymer for *in vitro* studies in anti-malarial drug research.

Natural products have played a key role in the discovery of leads for the development of drugs for malaria. Quinine, the original natural product used in anti-malarial therapy was isolated from Cinchona tree bark.

This investigation aims to explore the activity of Palestinian herb (*Salvia palaestina)* (Maramia) as anti-malarial agent that prevents the formation of β-hematin *in vitro* under specific chemical and physiochemical conditions.

In this study the alcohol extracts of; *salvia palaestina* (Maramia) and *Achillea Fragrantissima* (Qaysom), were all screened for potential anti-malarial activity using a semi-quantitative *in vitro* method developed by E. Deharo. These plants were collected from areas around Jerusalem. Two other different sea weeds; *Ulva fascianta* and *Cladophora aegagropila* were all collected from Jaffa coast. Any tested plant extract with a positive result was also tested for their anti-malarial activity by a self-developed quantitative method.

This investigation is the first report on the effect of Palestinian *salvia palaestina* extracts on the formation of the β–hematin. In this research it was found that *salvia palaestina* has the capability to impede the formation of β-hematin in *in vitro* systems. With very promising results, significant activity in inhibiting the formation of β-hematin *in vitro* at concentration of 0.3 mg/ml when compared to the negative controls, water and 35% ethanol and the positive controls chloroquine, Amodiaquine and 2-mercaptopyrimidine. Sage has been an important medicinal plant since earliest times, with the most typical form of application as infusion with

boiling water (sage tea). It has value as a carminative, spasmolytic, antiseptic, astringent, and is used against variety of complaints, and now being tested for anti-malarial activity.

Further investigations on the ability of *salvia palaestina* to inhibit β-hematin formation were performed using Blauer and Akkawi quantitative test which confirmed the previous results obtained, with β-hematin inhibition efficiency 71.7% compared to CQ 93% and negative control water 11%.

Attempts were done to try to separate the active compound from the crude extract by using preparative chromatography coupled with photo diode array (PDA) detector, eight fractions were obtained. In order to identify which fraction has the anti-malarial activity, they were screened using E. Deharo semi-quantitative method. Moreover, combinations of the fractions were also tested. Different combinations of these fractions were also tested using the same method.

Fourier Transform Infrared (FTIR) spectrum for the fractions was done as a first step in identification of the active ingredient. Further work is needed to identify the active compounds in *salvia palaestina* using Gas chromatography–mass spectrometry GC-MS, Nuclear magnetic resonance NMR and elemental analysis.

مشتقات النبات كعوامل ضد الملاريا: تثبيط بناء الهيموزوين في المختبر

بواسطة مستخلصات من الميرمية *سالفيا بالستينا*

الملخص بالعربية:

الملاريا هو أحد الأمراض المعدية التي جذبت اهتماما عالميا نظرا للارتفاع الخطير في معدلات الوفيات خاصة في البلدان النامية، حيث الأطفال الصغار والنساء الحوامل هم الاكثر عرضة لهذا المرض. وطفيل الملاريا هو كائن متعدد المراحل أحادي الخلية، يدعى البلازموديوم، الذي تطور في البعوض والفقاريات لملايين السنين. والذي يتسبب بالنوع الأكثر شيوعا وفتكا من عدوى الملاريا هو البلازموديوم فالسباروم *Plasmodium falciparum*. ووفقا لتقرير الملاريا العالمي وأحدث تقديرات منظمة الصحة العالمية، كان هناك أكثر من 219 مليون حالة إصابة بالملاريا في عام 2010 وما يقدر ب 000، 660 حالة وفاة، حيث أفريقيا هي القارة الأكثر تضررا. و تصميم أدوية تقوم بشكل كبير باعتراض دورة حياة هذا الطفيلي المعقد تتطلب فهما شاملا لوظائفه الحيوية.

مصدر القلق الكبير في علاج الملاريا في الوقت الحاضر هو تزايد مقاومة طفيل الملاريا فالسباروم للأدوية المضادة للملاريا، ولا سيما الكلوروكوين chloroquine، و هذا الوضع قد ازداد سوءا بسبب تزايد انتشار سلالات البلازموديوم فالسباروم المقاومة للأدوية.

هناك العديد من الأهداف في مجال مكافحة هذا المرض الخطير، ولكن هدفنا هو المرحلة داخل كريات الدم الحمراء في دورة حياة هذا الطفيل، ففي خلال هذه المرحلة، طفيليات البلازموديوم تقيم داخل الكريات الحمراء للعائل وتقوم بتحليل الهيموغلوبين مما يؤدي إلى تراكم الهيم (الحديد) الحر Ferriprotoporphyrin- IX, فهو سام للطفيل وقادر على توليد ذرات أكسجين نشطة. وتتم إزالة سمية الهيم من خلال إدماجه في بلورات غير قابلة للذوبان يسمى هيموزوين hemozoin أو صبغة الملاريا.

بيتا-هيماتين β-hematin، وهو بوليمر مصنع من مركب يدعى فيريبروتوبورفيريم 9 (Ferriprotoporphyrin- IX) والذي يعتبرتركيبه البنائي والكيماوي وتحليله الطيفي مطابق

للهيموزوين النقي, هذا البوليمر يتم استعماله في المختبر للدراسات في مجال البحوث للادوية المضادة للملاريا.

المنتجات الطبيعية لعبت دورا رئيسيا في اكتشافات تؤدي لتطوير عقاقير لمكافحة الملاريا. الكينين, أول ناتج طبيعي أصلي مستخدم في العلاج ضد الملاريا، قد تم استخلاصه من لحاء شجرة الكينا.

ويهدف هذا البحث لاستكشاف الفعالية للعشب الفلسطيني (الميرمية) *Salvia palaestina* كمضاد للملاريا وقيامه بمنع تكوين البيتا هيماتين في المختبر تحت ظروف فيزيائية وكيميائية.

في هذه الدراسة تم فحص المستخلص الكحولي للميرمية *Salvia palaestina* والقيصوم *Achillea Fragrantissima* واحتمالية فعاليتها كمضاد للملاريا في المختبر باستخدام طريقة النصف كمية التي وضعها E. Deharo . وهذه النباتات تم جمعها من مناطق حول القدس. وهنالك عشبتين بحريتين أخرتين *Ulva fascianta* و *Cladophora aegagropila* تم جمعهما من ساحل يافا. وأي مستخلص نباتي يعطي نتيجة ايجابية في الفحص يتم اختبار نشاطه كمضاد للملاريا باستخدام طريقة كمية تم بنائها ذاتيا بالمختبر.

وهذا البحث هو الاول الذي يبين تاثير مستخلصات النبتة الفلسطينية *Salvia palaestina* على تكوين البيتا هيماتين, ففي هذا البحث وجد ان الميرمية *Salvia palaestina* لديها القدرة على تثبيط تشكيل البيتا هيماتين في المختبر حتى تركيز 0.3 مغ/مل بالمقارنة مع الضوابط السلبية , الماء ومحلول كحولي بتركيز 35% , والضوابط الايجابية الكلوروكوين (CQ) والامودياكوين(AQ) و المركبتو بايرمدين-2) (MP. الميرمية من اهم النباتات الطبية منذ اقدم العصور, والطريقة الامثل لاستخدامها بواسطة نقعها بالماء المغلي (شاي الميرمية). ولهذا الشاي قيمة بوصفها طارد للريح, مزيل للتشنجات, مطهر, قابض, وتستعمل ضد مجموعة متنوعة من الامراض, والان يتم اختبار فعاليتها كمضاد للملاريا.

تم عمل فحوصات إضافية بشأن قدرة الميرمية على منع تكوين البيتا هيماتين باستخدام الاختبار الكمي لبلور Blauer وعكاوي Akkawi , التي اكدت النتائج التي تم الحصول عليها سابقا. بفعالية في تثبيط البيتا هيماتين بقيمة 71.7% مقارنة مع الكلوروكوين 93% والماء كضابط سلبي 11%.

كما جرت محاولات لفصل المركب النشط من المستخلص الخام باستخدام الفصل الكروموتوغرافيا مزود بكاشف photo diode array (PDA), تم الحصول على ثمانية اجزاء. وبهدف التعرف على الجزء

الذي لديه فعالية ضد الملاريا, تم فحص الاجزاء باستخدام الطريقة النصف كمية للعالم E. Deharo, اضافة الى عمل تركيبات مختلفة من هذة الاجزاء وفحصها باستعمال نفس الطريقة.

كخطوة اولى للتعرف على المكونات الفعالة في الأجزاء المفصولة, تمت قراءة امتصاص الطيف لتحويلات فوريير للأشعة تحت الحمراء(FTIR), هنالك فحوصات اضافية بحاجة لعملها للتعرف على المادة الفعالة في الميرمية باستخدام كروموتوغرافيا الغاز مع قياس الطيف الكتلي GC-MS , الرنين المغناطيسي النووي (NMR) والتحليل العنصري elemental analysis.

Abbreviations and Units:

Table of abbreviations

Abbreviation	Full word
FTIR	Fourier Transform Infrared spectroscopy
DMSO	Dimethylsulfoxide
ELISA	Enzyme Linked Immuno Sorbent Assay
Fe PPIX	Iron protoporphyrin IX
UV	Ultra violet
λ	Lambda
ACN	Acetonitrile
NaOH	Sodium hydroxide
EtOH	Ethanol
PDA	Photo diode array detector
P.	Plasmodium
min	Minute
HPLC	High Performance Liquid Chromatography
S. palaestina	Salvia palaestina
WHO	World Health Organization
RBCs	Red blood cells
CQ	Chloroquine
AQ	Amodiaquine
QN	Quinine
QD	Quinidine
MQ	Mefloquine
2-MP	2-Mercaptopyrimidine
ACT	Artemisinin based Combination Therapy
KBr	Potassium bromide
NMR	Nuclear magnetic resonance

Abbreviation	Full word
GC-MS	Gas chromatography–mass spectrometry
β	Beta
ODS	octadecyl silane
IC50	The half maximal inhibitory concentration
F.	Fraction

List of Units:

mg/ml: milligram per milliliter.

M: Molar.

µM: micro-molar.

µL: microliter.

µm: micrometer.

mm: millimeter.

nm: nanometer.

% : percent.

wt. / vol : weight per volume.

Rpm : Round per minute.

ml/min: milliliter per minute.

mbar: millibar.

AU: absorption unit.

˚C: degree centigrade.

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Chapter 1

Introduction

Malaria is a mosquito borne parasitic disease of the blood caused by a Protozoan belonging to the genus *Plasmodium* which is widely distributed in many poor tropical and subtropical regions of the world. The disease is most prevalent in Africa, whose main victims are children under five years of age [WMR, 2012]. It is considered one of the major public health concerns globally where about 40% of the world's population are currently at risk for malaria disease, in addition to killing thousands of poor people in developing countries each year; primarily African children because of the widespread drug resistance [White, 2004]. The complexity of malaria parasite mechanism together with the growing resistance to anti-malarial drugs presents several difficulties in the development of effective drugs against this disease. Besides, in poor areas where people are unable to afford and access effective anti-malarial drugs, traditional medicines could be an important source of treatment.

This present work contributes to the fight against malaria by providing research on a new potential active compound from herbal origin. Species of the genus *Salvia* (Lamiaceae) are well known throughout the world for their medicinal properties which have made it an attractive choice for our research.

According to [Knols, 2013] who wrote about Malaria in Palestine, that, Palestine was one of the malaria hopeless countries especially Jerusalem, Jaffa and Valley of Jordan termed

‘Soaked in Malaria’, see Figure 1.1 showing Huleh Lake in Palestine which was a breeding ground for malaria in the 50’s, and within 45 years Palestine was declared as ‘Malaria eradicated’ area. The steps that were taken to eradicate Malaria were based in eradicating the parasite carrier; data collection, education the population about the disease, drying streams and cleaning the pools where the parasite lives, the last step was the most important one in eliminating the parasite.

Figure 1.1*: Huleh Lake in Palestine, it was a breeding ground for malaria but drained in the 1950s.*

1. Literature Review

1.1. The Disease: Malaria

Malaria, one of the most important parasitic infections, it is even one of the oldest diseases known to mankind; recent studies showed that King Tutankhamun died of malaria [Hawass et al., 2010]. Malaria is caused by a one-celled parasite called *Plasmodium* [Bannister and Sherman, 2009], belonging to the parasitic phylum Apicomplexa. It is an entirely preventable and treatable disease but it is still a global health problem. According to the World Health Organization (WHO) and the World Malaria Report [WMR, 2012] an estimated 3.3 billion people were at risk of malaria in 2011, and about 219 million new cases arise per year making it the most prevalent serious infectious disease. More than 660, 000 people die from it, 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. It presents a major disease hazard for travelers to warm climates [Snow et al., 2005]. Figure 1.2 shows the geographic distribution of malaria that is endemic in the tropics and subtropics, spreading in countries of Africa, Asia and Latin America.

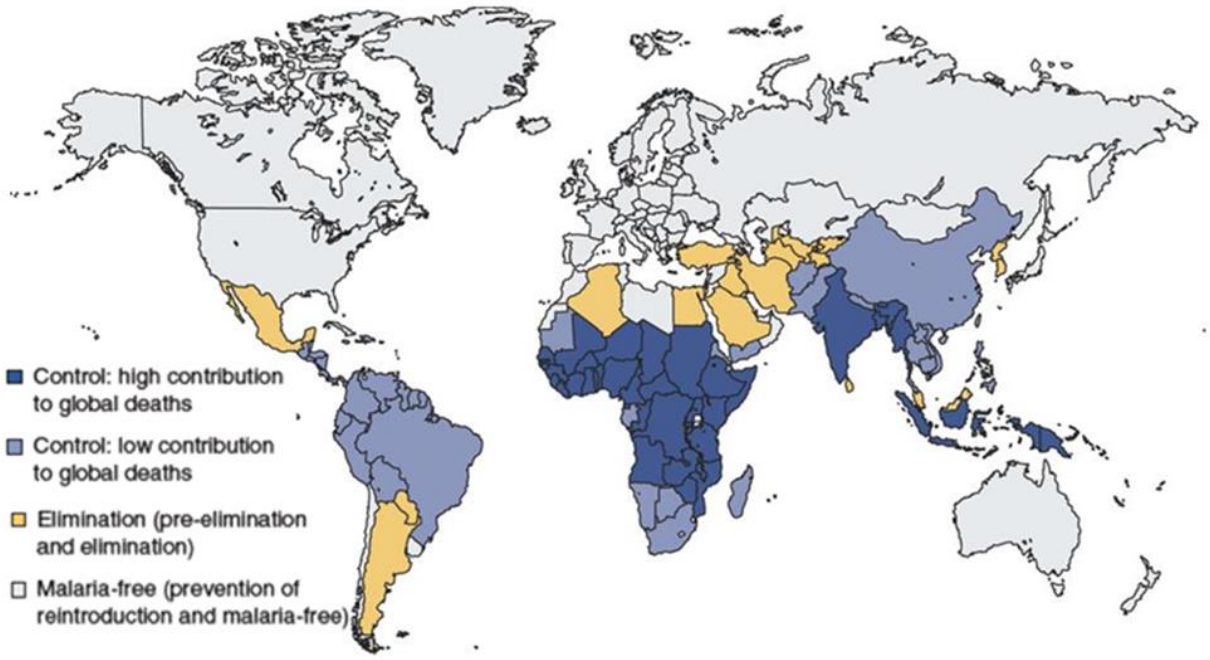

Figure 1.2: *Geological distribution of malaria around the world.*

After being infected with malaria by the female mosquito, symptoms usually begin within 10–15 days, depending on the type of malaria. The clinical symptoms of malaria appear as a consequence of the parasite multiplication in the RBC, which are similar to the flu, such as: headache, fever, vomiting, muscular aching, weakness, vomiting and abdominal pain; it may therefore be commonly mistaken for flu. In more serious cases, infected red blood cells can clump together and block blood flow. At worst, the disease can damage internal organs, including the brain, and cause breathing difficulties, coma and death [Church et al., 1996; Beare et al., 2006; Clark et al., 2004].

1.2. Malaria parasite*: Plasmodium*

Plasmodium is a genus of unicellular parasitic protozoa which infect vertebrates and cause malaria. There are nearly 200 *plasmodium* species that are parasitic to mammals, birds, and reptiles.

At present, only five major species commonly infect humans causing malaria; *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* recently *Plasmodium knowlesi* [Cox-Singh et al., 2008] and the most dangerous *Plasmodium falciparum* [Bannister and Sherman, 2009]. All of these are transmitted to hosts solely through the bite of female Anopheles mosquito vector. Of these, *P. falciparum* causes the most severe disease and mortality as a result of its prevalence, virulence, and drug resistance, is responsible for the majority of malaria cases worldwide [Snow et al., 2005].

1.3. Plasmodium Life-cycle

Plasmodium parasites have an elaborate lifecycle with multiple stages, which exhibit three lifecycle stages—gametocytes, sporozoites, and merozoites. The life cycle of the human malaria involves two hosts, firstly: the female anopheles mosquito, secondly: humans, see

Figure 1.4. Transmission of the parasite between the two hosts occurs only during the blood feeding of the mosquito vector, which needs blood to nurture its eggs [Levinson W., 2008].

Plasmodium species inhabit the salivary gland of the female anopheles mosquito in the form of sporozoites. When a female anopheles mosquito with plasmodium bites humans, it transmits the sporozoites into the human body. Once in the blood, the sporozoites travel to the liver to invade the liver cells (hepatocyte), where the parasite undergoes an asexual replication forming merozoites, this stage is called the exo-erythrocytic stage, throughout this stage an infected person shows no signs or symptoms of the disease, and malaria cannot be detected in the routine microscopic examination of blood films.

When Merozoits burst out of the liver cells, they journey back to the blood stream and infect healthy red blood cells. This stage of the infection is also called the intra-erythrocytic cycle. Inside red blood cells merozoits become enlarged ring shaped trophozoites, termed the ring stage due to its morphology in Geimsa-stained blood smears [Sherman, 1979], see Figure 1.3. Asexual replication occurs during the intra-erythrocytic stage releasing newly formed merozoites from the red blood cells, which in turn infect other RBC leading to massive destruction of RBC. When red blood cells destroyed by the merozoites liberate toxins, the continuous proliferation of malaria parasite inside the RBC's is a distinct feature of malaria infection, and is chiefly responsible for the pathology of the disease [Pandey and Tekwani, 1997], with each replication, some of the merozoites, instead of producing new merozoites,

develop into gametocytes, which can then infect susceptible mosquitoes, bringing the transmission cycle full circle.

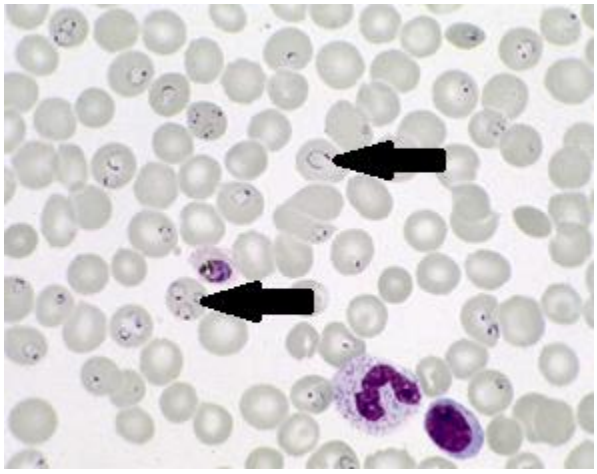

Figure 1.3: *Ring-form trophozoites of P. falciparum in a blood smear.*

In turn, when an uninfected female mosquito bites an infected human the plasmodium gametophytes are taken, and after a series of divisions resulting in formation of active sporozoits which will inhabit the salivary gland of the mosquito waiting for its next victim. Thus the mosquito carries the disease from one human to another. Unlike the human host, the mosquito vector does not suffer from the presence of the parasites.

Other transmission routes are possible, including blood transfusions [Slinger et al, 2001], congenital transmission from mother to fetus [Valecha et al. 2007], and through needle sharing. Understanding the complete lifecycle is important in understanding the pathogenicity of plasmodium parasite.

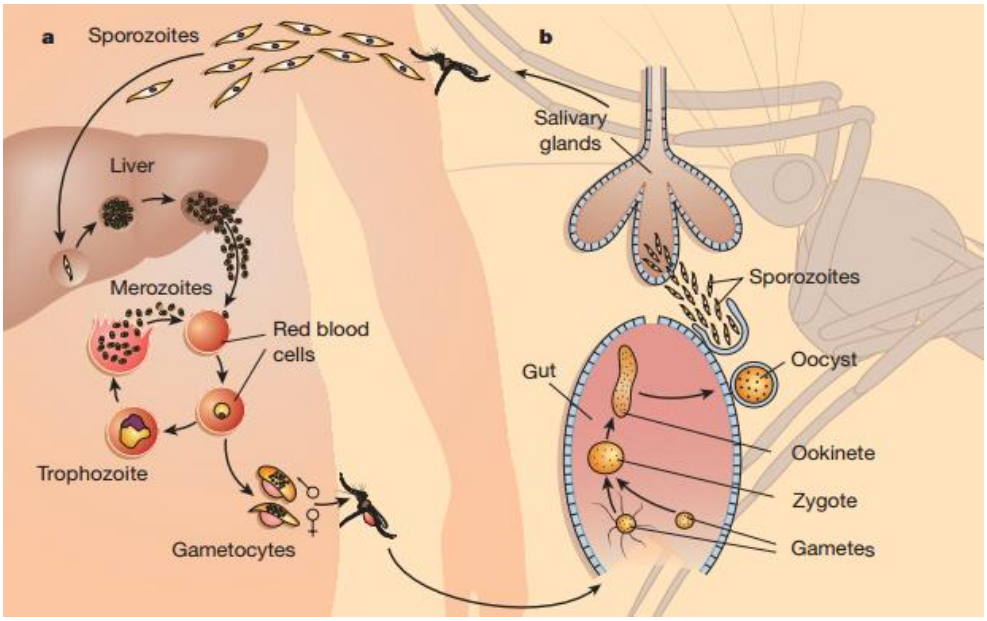

Figure 1.4: *A diagram of the lifecycle of the plasmodium parasite showing the role of mosquito that is responsible for the spread of malaria.*

1.4. Hemozoin and β-hematin

During the intra-erythrocytic stages the parasite consumes and degrades hemoglobin - iron containing metalloprotein and the major protein available in erythrocytes - as source of amino acids for its own nutrition, the digestion takes place inside the parasite's food vacuole [Spiller et al., 2002; Klones et al., 2007] – an acidic proteolytic compartment – where the pH 5.0 - 5.4, with the aid of special type of enzymes called plasmepsins [Klemba et al.,2004; Liu et al., 2005], it digests 60−80% of the available hemoglobin.

The byproduct of hemoglobin digestion is the release of large amounts of Iron protoporphyrin IX (FePPIX) in the ferrous form along with oxygen, causing its oxidation to the ferric form, or Ferri-heme which is highly reactive and toxic to the *plasmodium* [Egan, 2008; Sullivan, 2000], these heme groups are able to induce oxidative stress that can damage the parasite biological membranes. It needs only about 0.1% of total hemoglobin present in RBC's to be destroyed in order to produce enough free heme (20 mM) that can lyse malaria parasites within 10 minutes. However, malaria parasite has evolved a mechanism for detoxification of free heme through its mineralization [Slater et al., 1991] into an unreactive, insoluble crystalline form known as hemozoin or malaria pigment [Sullivan, 2000; Slater et al.,1991; Goldberg et al.,1990; Pagola et al.,2000; Egan et al., 2002; Tekwani and Walker 2005; Egan, 2008]. Formation of hemozoin is crucial for parasite survival and has been the focus of many studies mainly because of its role in the pharmacological activity of several known anti-malarials [Hawley et al., 1998].

β-hematin (FeIII-protoporphyrin-IX) is a synthetic analogue of purified hemozoin, constituting of cyclic heme dimers -where the central iron of one heme is linked to the oxygen of the carboxylate side chain of the adjacent heme- arranged in an ordered crystalline structure through intermolecular hydrogen bonding [Slater el al.,1991; Gildenhuys et al., 2012]. Figure 1.6 shows the structure free heme, β-hematin dimers and Hemozoin.

β-Hematin is structurally, chemically and spectroscopically identical to purified hemozoin [Pagola et al.,2000], and can be produced in acidic solution and used in an assay to screen for anti-malarial compounds.

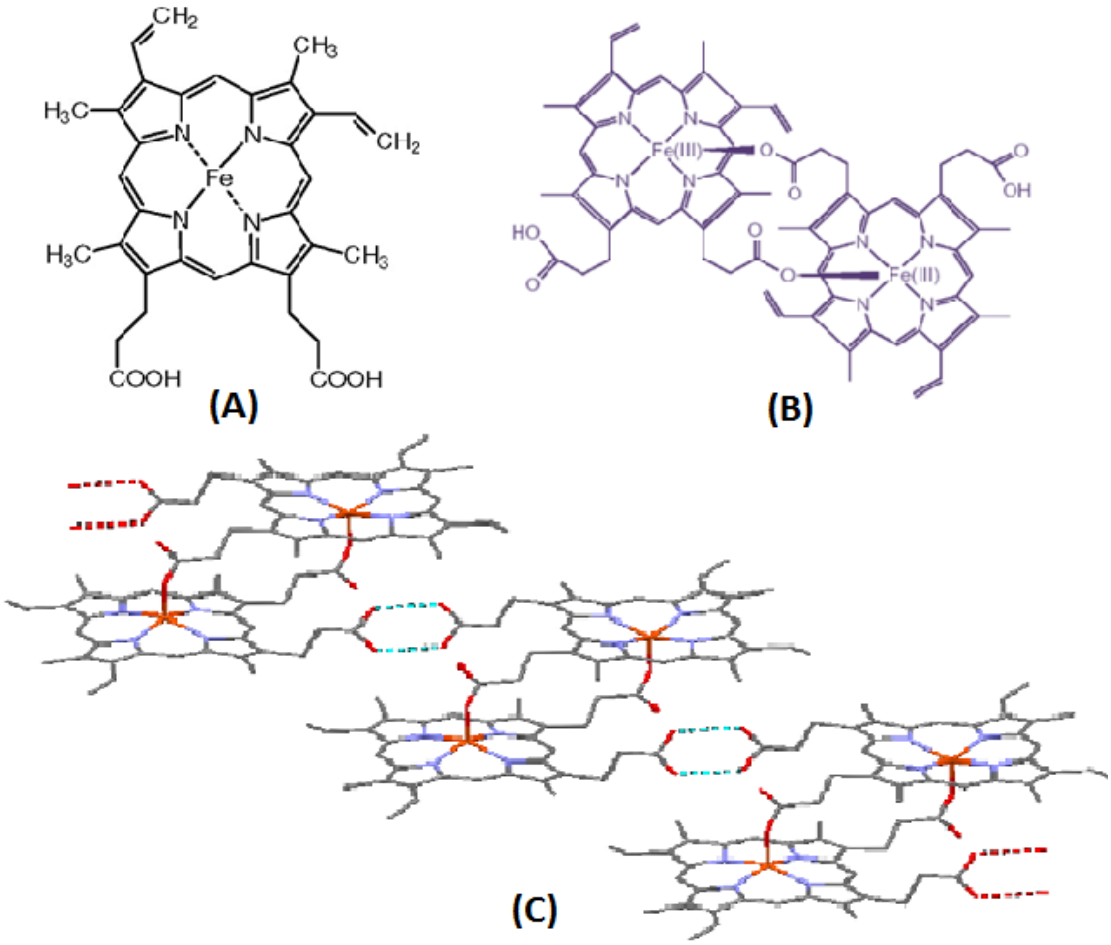

Figure 1.5*: Presenting the structure free heme (A), the structure of β-hematin dimers (B), and structure of hemozoin, showing hydrogen bonds between hematin units as dotted blue lines, and coordinate bonds between iron atoms and carboxylate side chains as red lines (C).*

1.5. Drugs Available for Treatment of Malaria

Malaria may be cured with prescription drugs. The type of drugs and length of treatment depend on stage of parasite, which kind of malaria is diagnosed, where the patient was infected, age of the patient, and how severely ill the patient was at the beginning of the treatment.

1.5.1. Quinoline Anti-malarial Drugs

Quinoline anti-malarials originate from the compound quinine, which is an active anti-malarial natural product found in the bark of *Cinchona* tree. The quinolone containing antimalarial drugs, such as chloroquine (CQ), amodiaquine (AQ), quinine (QN), quinidine (QD), and mefloquine (MQ) all have been vital therapeutic agents against malaria [Meshnick and Dobson,2001].

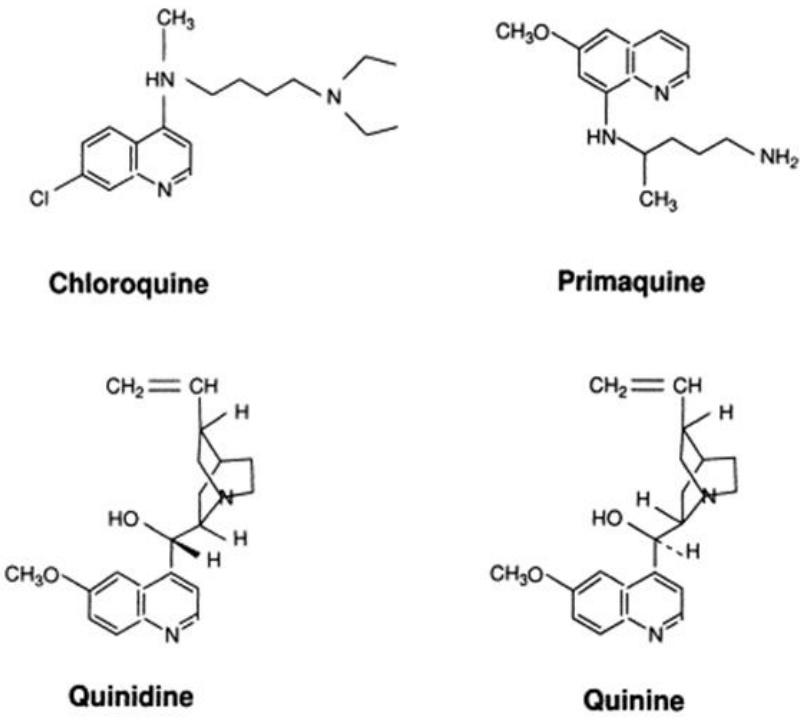

Figure 1.6*: The chemical structure of main classes of anti-malarial drugs, chloroquine, primaquine, quinidine and quinine.*

Chloroquine (4-aminoquinoline) the most common and effective drug used to treat malaria, its main advantage was its fast action against the blood stages, low toxicity, as well as its good bioavailability making it the ideal drug for Africa [krafts et al.,2012].

CQ targets the intra-erythrocytic stage particularly the hemozoin formation pathway, inhibition of heme detoxification of the malaria parasite leads to accumulation of toxic heme which in turn would kill the parasite [Hawley et al., 1998]. This is the mechanism of action of many known anti-malarials such as chloroquine, which accumulates inside the parasites food vacuole and forms heme-chloroquine complex that is incorporated into the growing hemozoin polymer to terminate chain extension [Gildenhuys et al., 2012] see Figure 1.7, resulting in the accumulation of free heme-chloroquine complex in the food vacuole, which is more potent to Plasmodium than free heme causing the parasite's death [Sullivan et al., 1996; 1998].

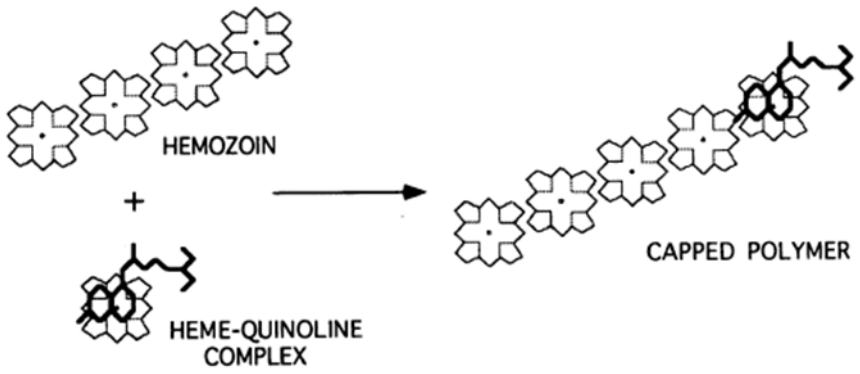

Figure 1.7*: A model showing chloroquine mode of action in inhibiting hemozoin formation.*

1.5.2. Artemisinin and Its Derivatives

Artemisinin is a powerful and the most effective anti-malaria drug, extractes from *Artemisia annua,* it is even effective against highly drug resistant *plasmodium* strains [Willcox et al., 2004; Das, 2012], Chemically, artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge ,see Figure 1.8. This peroxide is believed to be responsible for the drug's mechanism of action. Semi-synthetic derivative Artemeter was found to be more active than its precursor and is the most frequently used artemisinin derivative as a first line treatment for malaria [Meshnick and Dobson, 2001].

Artemisinin, in the form of Artemisinin-based Combination Therapy (ACT), (combination of artemisinin and its derivatives with existing antimalarial drugs) is currently the best effective drug treatment option against those malaria parasites that have evolved resistance to drugs such as chloroquine, [Douglas et al.2010] for example; Artemisinin-Aminoquinoline hybrids showed good anti-malarial activity.

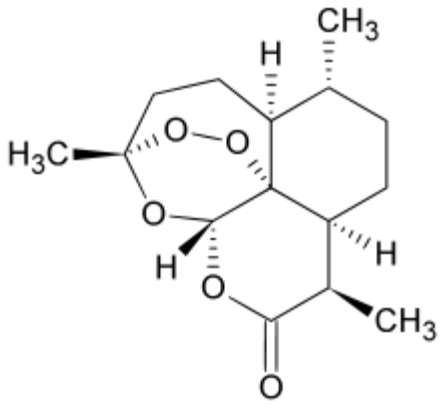

Figure 1.8: *Anti-malarial drug, Artemisinin chemical structure.*

1.6. Anti-malarial Drug Resistance

Malaria parasites are putting up a strong fight against anti-malarial drugs and recently have developed resistance against several drugs diminishing the therapeutic efficacy of drugs [Waller et al., 2003; Orjih et al., 1993; Reddy et al., 2012], which means the ability of a parasite species to survive and multiply despite the administration of a drug in doses equal to or higher than those usually recommended.

Although CQ is still a safe, inexpensive and widely available drug, resistance to it now occurs across the tropical world, so it is no longer effective in many areas, see Figure 1.9. The genetic events that confer anti-malarial drug resistance are mutations in or changes in the copy number of genes encoding for influx/efflux pumps that affect intra-parasitic concentrations of the drug, for example; Chloroquine resistance in *P. falciparum* may be multi-genic and is initially conferred by mutations in a gene encoding a transporter (*PfCRT*) [Waller et al., 2003].

Resistance to anti-malarial drugs is proving to be a challenging problem in malaria control in most parts of the world. Since the early 60s the sensitivity of the parasites to CQ has been on the decline. This resistance began with *P. falciparum* and today is extremely common, especially in Africa, Newer anti-malarials have been developed in an effort to tackle this

problem [Agarwal et al., 2005; Aljazzar et al., 2010; Akkawi et al., 2012], but all these drugs are either expensive or have undesirable side effects.

In recent years, artemisinin, has emerged as a new anti-malarial drug, and is being promoted as a new tool in the fight to eradicate the disease, though resistance is already reportedly spreading [Dondorp et al., 2009]. Although scientists worldwide are testing new malaria drugs, there are as yet no realistic alternatives to artemesinin combination drugs, which are considered the most effective.

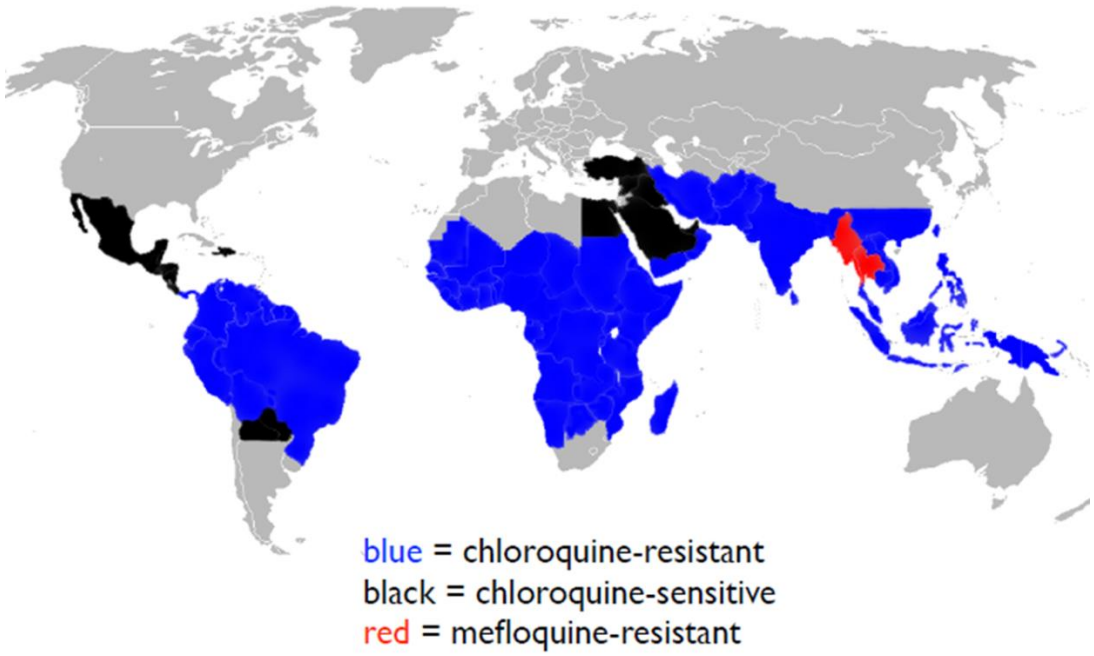

Figure1.9: *World map showing the spread of anti-malarial drug resistance.*

1.7. Natural Compounds as Anti-malarials

Natural compounds were historically used as drugs. Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today as they can be found to be accessible and affordable. The history of anti-malarial drugs is intimately linked with the history of herbal medicinal products. For example, the bark of the Cinchona tree was one of the anti-fever herbs that led to the discovery of natural quinine (an alkaloid). In 1820 identified and isolated by the French scientists Pelletier and Caventou [Benedek T.], the first anti-malarial drug that is still used today as well as several synthetic quinolones, particularly chloroquine.

Another example includes artemisinin, which is a terpene, the active ingredient of the Chinese herb, sweet wormwood (*Artemisia annua*) [Willcox et al., 2004; Das, 2012], which represents one of the great discoveries in medicine by Chinese scientists in 1972, and its derivatives have become essential components of anti-malarial treatment. This plant has been used as a traditional remedy for chills and fevers for more than 2000 years by the Chinese.

1.8. *Salvia Palaestina*: tea sage (Maramia):

Salvia, the largest genus of the Lamiaceae family, includes about 900 species and is common throughout the world. *Salvia palaestina* is an important genus widely cultivated in Palestine Turkey, Syria, Iraq, Iran, and northeastern Egypt. This plant is an evergreen perennial herb that has been an important medicinal plant for centuries; and still used up to this day as herbal remedy. The Latin name Salvia comes from the Latin verb "salvare" meaning to save or to heal in reference to the curative properties of the plant.

S. palaestina is an important domestic herbal remedy for disorders of the digestive system, also used in cooking and in the cosmetics industry. An infusion of dried sage leaves with boiling water is the most typical form of preparation, proven to be effective against many ailments [Duke et al., 2002].

The range of traditional applications of this herb in domestic medicine seems to be endless: it has been used as a medication against perspiration and fever; as a carminative; a spasmolytic; bactericidal; an astringent; as a gargle or mouthwash against the inflammation of the mouth, tongue and throat; a wound-healing agent; in skin and hair care; and against rheumatism and in treating mental and nervous conditions among others [Duke JA, 2001].

Figure 1.10*: leafs of Salvia palaestina, Maramia (sage tea)*

Previous studies showed that *S. 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.

1.8.1 Secondary metabolites in Salvia species

Plants in general constitute chemicals known as primary and secondary metabolites. Primary metabolites include compounds that are necessary for cellular processes such as amino acids, nucleic acids, lipids and simple sugars; whereas secondary metabolites are non-nutritional compounds which include compounds which are produced in response to stress factors on the plant or involved in cell metabolism [Kennedy and Wightman,2011]. Often, plant's secondary

metabolites are considered to be an excellent reservoir of new medical compounds. There are two major groups of secondary metabolites terpenoids, phenolics.

Essential oil composition of Salvia species has previously been reported, the main secondary metabolite constituents are terpenoids [Cioffi et al., 2008; Vassallo *et al*., 2008] and flavonoids, also contain large amounts of thujone, [Gürsoy et al., 2012], which are considered potential anti-malarial agent. In addition, sage leaf contains numerous other compounds, including cineol, borneol, tannic acid; bitter substances like cornsole and cornsolic acid; fumaric, chlorogenic, caffeic and nicotinic acids; nicotinamide; flavones; flavone glycosides, thymol and camphor among many others [Gürsoy et al., 2012; Walch et al., 2011].

The largest class of plant secondary metabolites are alkaloids which are physiologically-active heterogeneous compounds containing nitrogen, and are one of the most important natural products, which offers drugs that exhibit anti-malarial activity, the outstanding example in this group is quinine [Oliveira et al.2009].

Terpenoids are derived from five-carbon isoprene units (C5H8) see Figure 1.11, classified by the number of isoprene units used to construct them. For example, monoterpenoids consist of two isoprene units, sesquiterpenoids (three units). Monoterpenoids and sesquiterpenoids are the primary components of essential oils, which are highly volatile compounds that contribute to the fragrance.

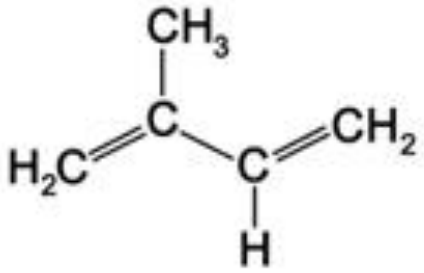

Figure 1.11: *structure of isoperene unit.*

Flavonoids are one of the largest classes of phenolics, which are considered as ketone-containing compounds, the basic structure of a flavonoid is shown in Figure 1.12.

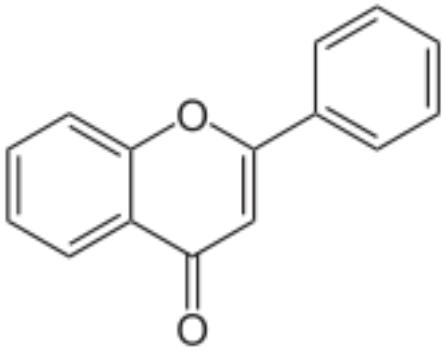

Figure 1.12: *Structure of the flavone backbone.*

1.9 Achillea Fragrantissima (Qaysom):

A member of compositae family that habitat dry areas, flowering time starts from May until August, see Figure 1.13, its antibacterial activity was studied by [Barel et al.,1991].this plant has been used in traditional medicine as disinfectant, gargle ,carminative, insect repellent and for chest complaints among many others.

***Figure 1.13**: Achillea Fragrantissima (Qaysom).*

1.10 Seaweeds as anti-malarials:

Seaweeds are micro-algae that live in the sea. It includes members of the red, brown and green algae. They all have many uses in different as food, medicine, industrial and fertilizer. Recent studies on seaweeds from Fiji Island showed a compound called Bromophycolide A considered as antifungal agent, had the ability to prevent the formation of β-hematin [Stout et al., 2011].

1.11 Purpose of this work:

The main purposes of this study are:

- To find a new potential anti-malaria drugs to target the hemozoin synthesis pathway during the intra-eryrthorcytic stage from natural sources.

- To investigate the activity of water and ethanolic extracts of *Salvia palaestina* as potential anti-malarial agents.

- To perform both in vitro semi-quantitative (E. Deharo, 2002) and quantitative method (Blauer and Akkawi, 1997) on the plant extracts to test β-hematin formation.

- To develop a rapid analytical HPLC method that scans the active constituents from 35% ehanolic crude *salvia palaestina* followed by enrichment and isolation of the active anti-malarial fractions by using preparative chromatography coupled with photo diode array (PDA) detector.

- To retest the fractions obtained from the HPLC separation, in order to identify the fraction with anti-malarial activity.

Chapter 2

Materials and Methods

2.1. Instrumentation:

- The analytical HPLC system consisted of Waters 2695 Alliance® equipped with 2996 PDA model detector and Empower data software. The preparative HPLC consisted of 3535 quaternary gradient module which provide a flow rate of up to 50 ml/min, equipped with 996 PDA detector.
- Freeze Drying machine (Lyophilizer) from Labconco.
- Rotary Evaporator from IKA WEREK RV06-ML.
- Bench top pH meter from (WTW).
- Analytical balance from Sartorius CP.
- Water bath from Jouan.
- Bench top B4- centrifuge from Jouan.
- Stabili therm incubator from thermo.
- Stat Fax-2100 ELISA reader.
- Ultrasonic cleaner DC150H Sonicator

- Nicolet Avatar 370DTGS FTIR.
- Carver compressor.

2.2. Materials and chemicals:

- DMSO, Dimethyl sulfoxide, purity 99.5% was obtained from Sigma Aldrich.
- Chloroquine diphosphate salt was obtained from Sigma.
- Hemin from porcine was obtained from Sigma.
- Absolute Ethanol (EtOH) was obtained from Sigma Aldrich.
- Glacial acetic acid was obtained from Fluka.
- Sodium acetate. Purity 99% was obtained from Aldrich.
- ACN, acetonitrile, HPLC grade, was purchased from Merck (Germany).
- Highly Purified water was prepared by using a Millipore Milli-Q plus water purification system.
- KBr, potassium bromide 99+%, FTIR grade was purchased from Sigma Aldrich.
- Chloroform

2.3. Methods

2.3.1 Plant collection:

Salvia palaestina and *Achillea Fragrantissima* were collected from areas far from agricultural land around Jerusalem on Mach and December 2012 respectively, while the two different seaweeds *Ulva fascianta, Cladophora aegagropila* were collected from Jaffa coast on October 2012. Plant leafs were air dried in the shade.

2.3.2 Extraction of the plant samples:

Leafs were grinded by electrical mixer into course powder. Extraction by the maceration method in 35% ethanol was carried out with the plant's powder to solvent ratio 1:10 (wt./vol), the mixture was left for a period of 20-24 hours at room temperature without stirring. The extract was then filtered using MN615.Ø110 mm filter paper. The filtrate was then evaporated using rotary evaporator at 80˚C under reduced pressure.

2.3.3 Lyophilization (Freeze Drying):

Lyophilization is the process of isolating a solid substance from aqueous solution by freezing the solution and evaporating the ice under vacuum, it is carried out using a simple principle of physics called sublimation.

The extract, was frozen overnight then it was placed in the freeze dryer machine at temp -55 °C and pressure 0.095 mbar, until a constant weight was achieved. The final dried extract was stored in bottles and kept in the freezer at -20 ˚C until use.

2.3.4 In vitro screening for anti-malarial activity using Semi-Quantitative Method:

According to [Deharo et al., 2002], the procedure for testing bio-mineralization hemin consists of:

1. Incubating a test mixture in a normal non-sterile flat bottom 96-well plate at 37 ºC for 18-24 hours.

   The mixture according to order of addition contained:

   50 µL (0.5 mg/mL hemin chloride) freshly dissolved in dimethylsulphoxide (DMSO).

   100 µL of 0.5 M sodium acetate buffer (pH 4.4)

   and finally 50µL of potential anti-malarial drug solution or solvent.

2. The plate was then centrifuged at 4000 rpm for 10 min. The supernatant was removed and pH of reaction was measured, the final pH between (5 - 5.2).

3. The remaining pellets were then suspended in 200 µL of DMSO to remove free hemin chloride.

4. The plate was centrifuged again, the supernatant was discharged.

5. The precipitate of β-hematin was dissolved in 150 µL of 0.1 M NaOH to give alkaline hematin for direct spectroscopic quantification at wavelength 405 nm using ELISA reader.

* *Salvia palaestina* and *Ulva fascianta* extracts were dissolved in pure water, while *Achillea Fragrantissima* and *Cladophora aegagropila extracts were dissolved in 35% ethanol, and camphor was dissolved in DMSO*.

**Ultra-pure water and 35% ethanol were used as negative controls and Chloroquine diphosphate salt, Amodiaquine and 2-mercaptopyrimidine as positive controls.*

2.3.5 Separation of the active anti-malarial fractions from crude *Salvia palaestina* by using reversed phase Preparative HPLC-PDA

2.3.5.1 Chromatographic conditions:

The HPLC analytical experiments were run on octadecyl silane C18 chemically bonded column (Waters XBridge, 4.6 x 150 mm, 5 µm). The mobile phase was a gradient of (0.5% acetic acid aqueous solution) (eluent A) with ACN 'Acetonitrile' (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 minutes. All the solvents and sample were filtered with a 0.45 μm microporous 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 room temperature. The HPLC preparative experiments were run on ODS column (Agilent PrepHT C18, 22.2 x 250 mm, 10 μm).

The same mobile phase was used, and the gradient elution was set for a linear 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 15ml/min, the injection volume was 1000 μl and the temperature of the column was at room temperature.

2.3.5.2 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 two 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 seven fractions were collected.

2.3.6 Evaporation of the fractions and lyophilization:

The aliquots collected were dried by using rotary evaporator to remove the acetonitrile solvent followed by freeze drying to get rid of the water and ending up with few mg's of fluffy powdered material.

2.3.7 Testing the fractions using the semi quantitative method:

The fractions were dissolved in distilled water and sonicated for 10 min in order to be tested for its ability to inhibit β-hematin formation using Deharo semi-quantitative assay. See Section 2.3.4.

2.3.8 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 N 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 *S.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 washed again with ultra-pure water. The remaining precipitate was then lyophilized to a constant weight [Blauer and Akkawi, 1997].

2.3.9 FTIR spectrum

0.4 mg of each fraction was milled with 400 mg of potassium bromide (KBr) to form a very fine powder. This powder was then compressed for 4 min under 9 tons of pressure to form a thin pellet using Carver compressor. The pellet was then analyzed using thermo FTIR.

FTIR measurement was taken against KBr disc as a background.

Chapter 3

Results

3.1 Semi Quantitative screening test results

Results of experiments done according to E. Deharo semi-quantitative method on different plant extracts are shown below in Tables 3.1A, 3.1B, 3.2A and 3.2B. Tables 3.1A and 3.1B show results of two different tested herbal extracts *Achillea Fragrantissima* and *Salvia palaestina*, collected from rural areas around Jerusalem, while Tables 3.2A and 3.2B show the results of other two sea weeds tested, *Ulva fascianta* and *Cladophora aegagropila*. The efficiency of these extracts in inhibiting β–hematin formation *in vitro* is compared to positive and negative controls. According to this semi-quantitative method, absorbance value measured at 405 nm is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient.

***Table 3.1A**: The efficiency of herbal leafs 35% ethanol extracts of Achillea Fragrantissima and Salvia palaestina 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 E. Deharo semi-quantitative method.*

	Negative controls		**Positive controls**		**Herbal extract in 35% ethanol**	
Test #	**Water**	**35% ethanol**	**2-MP 1mg/ml**	**CQ 0.1mg/ml**	***Achillea Fragrantissima* 1mg/ml**	***Salvia palaestina* 1mg/ml**
1	2.275	2.161	0.046	0.039	2.097	0.047
2	2.371	2.186	0.049	0.041	1.711	0.05
3	2.336	2.147	0.061	0.035	2.17	0.056
4	2.08	2.061	0.04	0.041	2.099	0.043
5	1.978	2.211	0.058	0.036	2.021	0.035
6	1.994	2.231	0.033	0.043	1.592	0.059
7	2.209	2.194	0.049	0.064	1.736	0.037
8	2.143	2.169	0.033	0.052	1.82	0.04
9	2.277	2.201	0.042	0.033	1.554	0.051
10	2.339	2.151	0.067	0.043	2.112	0.055

***Table 3.1B**: The efficiency of herbal leafs 35% ethanol extracts of Achillea Fragrantissima and Salvia palaestina 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 E. Deharo semi-quantitative method.*

Test #	**Negative controls**		**Positive controls**		**Herbs 35% ethanol extract**	
	Water	**35% ethanol**	**2-MP 1mg/ml**	**CQ 0.1mg/ml**	***Achillea Fragrantissima* 1mg/ml**	***Salvia palaestina* 1mg/ml**
1	2.215	2.271	0.076	0.039	2.112	0.037
2	2.208	2.23	0.068	0.037	1.612	0.035
3	2.052	2.281	0.056	0.040	1.789	0.042
4	2.094	2.208	0.045	0.058	2.011	0.053
5	2.291	2.174	0.057	0.060	1.599	0.051
6	2.15	2.139	0.061	0.054	1.652	0.046
7	2.222	2.109	0.079	0.038	1.784	0.032
8	2.152	2.118	0.058	0.053	1.697	0.038
9	2.263	2.306	0.05	0.041	1.595	0.037
10	2.27	2.22	0.041	0.048	1.928	0.040

Table 3.1 C below summarizes semi-quantitative results of Tables 3.1A and 3.1B showing the average and standard deviation values. These values are represented using column diagram in Figure 3.

Table 3.1C: *Average and standard deviation values for all absorption measurements at 405 nm of both Tables 3.1A and 3.1B using Deharo semi quantitative method done on A. Fragrantissima and S. palaestina 35% ethanol leaf extract.*

	Negative controls		**Positive controls**		**Herbs 35% ethanol extract**	
	water	**35% ethanol**	**2-MP 1mg/ml**	**CQ. 0.1mg/ml**	***A. Fragrantissima* 1 mg/ml**	**S. *palaestina* 1 mg/ml**
Average	**2.21**	**2.18**	**0.054**	**0.045**	**1.85**	**0.048**
STDEV	**0.113**	**0.06**	**0.013**	**0.009**	**0.210**	**0.008**

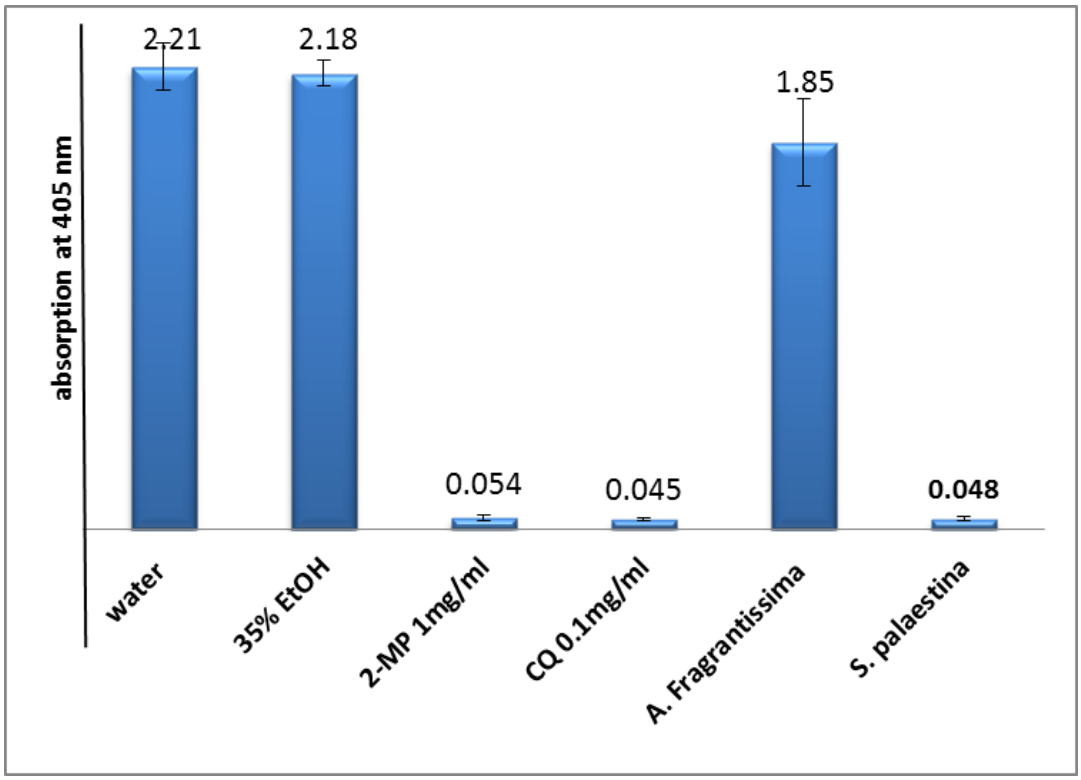

Figure 3.1: *Column diagram representing the efficiency of herbal leaf's ethanol extracts compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 20 individual experiments.*

Table 3.2A: *The efficiency of sea weeds 35% ethanol extracts of Ulva fascianta and Cladophora aegagropila 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 E. Deharo semi-quantitative method.*

Test #	**Negative controls**		**Positive controls**		**Sea-weed 35% ethanol extract**	
	Water	**35% ethanol**	**2-MP 1mg/ml**	**CQ. 0.1mg/ml**	***Ulva fascianta* 1mg/ml**	***Cladophora aegagropila* 1mg/ml**
1	2.102	2.105	0.061	0.036	1.954	2.119
2	2.238	2.142	0.08	0.044	2.151	2.173
3	2.226	2.28	0.076	0.054	2.006	2.16
4	2.22	2.223	0.064	0.059	1.991	2.118
5	2.245	2.266	0.072	0.057	2.026	2.104
6	2.34	2.267	0.051	0.071	2.269	2.258
7	2.165	2.271	0.059	0.056	2.037	2.108
8	1.922	2.163	0.042	0.055	2.093	2.238
9	2.254	2.215	0.056	0.048	2.093	2.221
10	2.306	2.044	0.045	0.05	2.239	2.162

Table 3.2B: *The efficiency of sea weeds 35% ethanol extracts of Ulva fascianta and Cladophora aegagropila 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 E. Deharo semi-quantitative method.*

Test #	**Negative controls**		**Positive controls**		**Sea-weed 35% ethanol extract**	
	Water	**35% ethanol**	**2-MP 1mg/ml**	**CQ. 0.1mg/ml**	***Ulva fascianta* 1mg/ml**	***Cladophora aegagropila* 1mg/ml**
1	2.275	2.01	0.068	0.039	2.175	2.216
2	2.171	2.209	0.048	0.041	2.094	2.162
3	2.236	2.075	0.045	0.035	2.029	1.979
4	2.08	2.001	0.052	0.041	1.926	2.174
5	1.977	1.933	0.055	0.036	2.152	2.026
6	1.974	1.857	0.061	0.043	2.037	2.269
7	2.011	2.16	0.069	0.064	1.974	2.157
8	2.183	2.087	0.045	0.052	2.168	2.093
9	2.277	1.963	0.057	0.059	2.041	2.187
10	2.32	1.932	0.039	0.043	2.085	2.1661

***Table 3.2C**: Average and standard deviation values for all absorption measurements at 405 nm of both tables 4.2A and 4.2B using Deharo semi quantitative method done on Ulva fascianta and Cladophora aegagropila 35% ethanol leaf extract*

	Negative controls		**Positive controls**		**Sea-weed 35% ethanol extract**	
	Water	**35% ethanol**	**2-MP 1mg/ml**	**CQ. 0.1mg/ml**	***Ulva fascianta* 1mg/ml**	***Cladophora aegagropila* 1mg/ml**
Average	**2.172**	**2.110**	**0.057**	**0.049**	**2.077**	**2.155**
STDEV	**0.116**	**0.130**	**0.012**	**0.010**	**0.093**	**0.072**

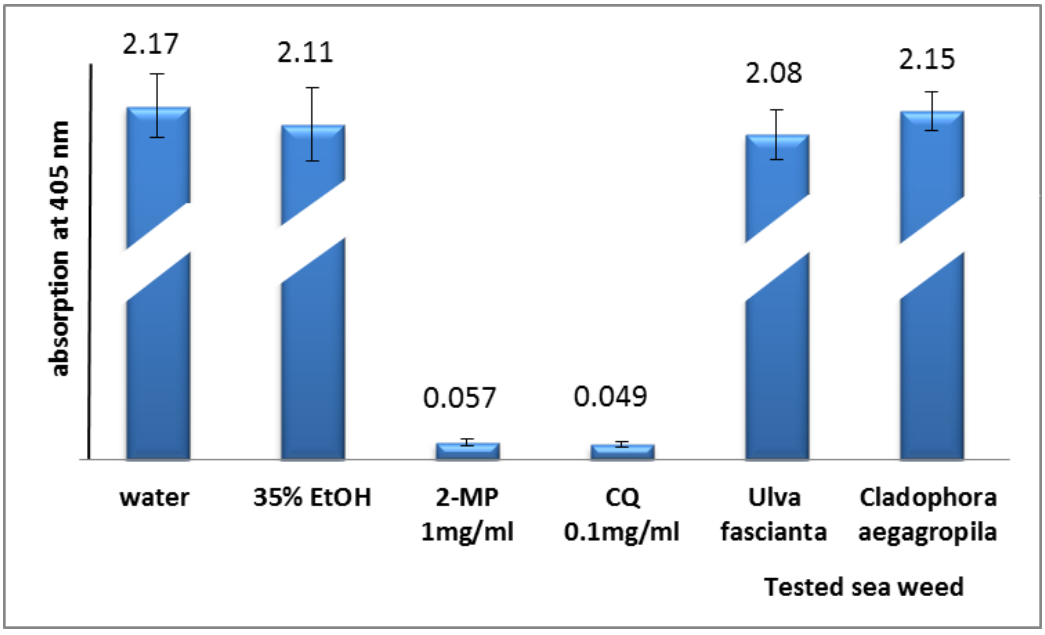

***Figure 3.2**: Column diagram representing the efficiency of different sea weeds extracts compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method.*

Different extracts of *S. palaestina* leaf were prepared using different solvents (water and 35% ethanol) and semi-quantitative test results are shown in Tables 3.3A.

***Table 3.3A**: The efficiency of Salvia palaestina leaf different extracts 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 E. Deharo semi-quantitative.*

Test #	Negative control		Positive control			Salvia in 35% ethanol	Salvia in water
	Water	DMSO	CQ. 0.1mg/ml	AQ. 0.1mg/ml	2-MP 1mg/ml	1mg/ml	1mg/ml
1	2.341	1.867	0.052	0.074	0.069	0.061	0.089
2	2.374	1.859	0.065	0.075	0.038	0.043	0.122
3	2.300	1.784	0.076	0.070	0.049	0.040	0.115
4	2.209	1.78	0.061	0.072	0.036	0.050	0.089
5	2.310	1.935	0.043	0.078	0.037	0.049	0.130
6	2.300	1.853	0.035	0.045	0.046	0.059	0.086
7	2.204	1.933	0.080	0.035	0.064	0.054	0.093
8	2.261	1.857	0.065	0.149	0.050	0.049	0.101
9	2.266	1.961	0.042	0.049	0.068	0.053	0.091
10	2.275	1.907	0.076	0.080	0.039	0.052	0.086
11	2.385	1.963	0.082	0.078	0.051	0.042	0.099
12	2.257	1.932	0.049	0.059	0.035	0.064	0.112
13	2.293	2.001	0.038	0.045	0.064	0.040	0.085
14	2.358	1.954	0.046	0.038	0.045	0.063	0.110
15	2.282	2.010	0.057	0.068	0.035	0.043	0.086
16	2.303	1.887	0.048	0.108	0.040	0.039	0.080
17	1.894	1.987	0.058	0.022	0.058	0.048	0.126
18	1.990	1.991	0.040	0.038	0.046	0.058	0.103
19	2.126	1.892	0.069	0.048	0.068	0.053	0.093
20	1.919	1.857	0.060	0.026	0.056	0.055	0.113

***Table 3.3B**: The average and standard deviation for Table 3.3A. Concerning the absorption measurements of dissolved β-Hematin at 405 nm using Deharo semi-quantitative method done on S. palaestina different extracts.*

	Negative control		Positive control			salvia in 35% ethanol	Salvia in water
	Water	DMSO	CQ. 0.1mg/ml	AQ. 0.1mg/ml	2-MP 1mg/ml	1mg/ml	1mg/ml
Average Abs. at 405nm	2.232	1.911	0.057	0.063	0.05	0.050	0.100
STDEV	0.142	0.067	0.015	0.030	0.012	0.008	0.015

Figure 3.3 below shows the efficiency of two different extracts of *salvia palaestina* leaf in inhibiting β-hematin formation according to E. Deharo semi-quantitative method, compared to positive and negative controls, lower values indicates higher efficiency.

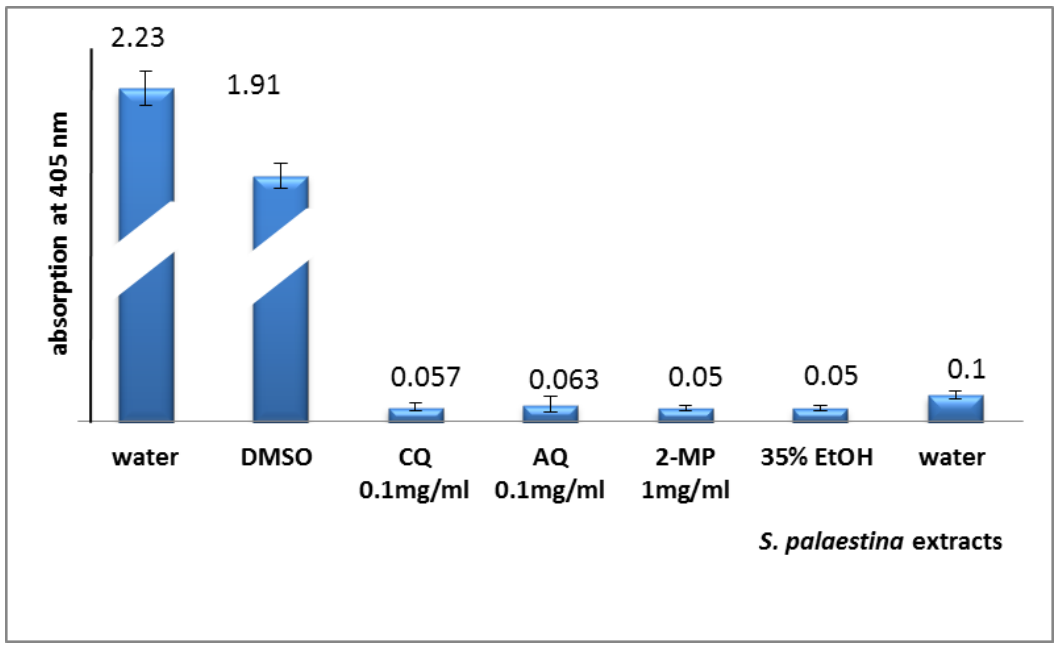

***Figure 3.3**: Column diagram representing the efficiency of two different salvia palaestina leaf extracts using 35% ethanol and water, both at 1mg/ml, compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method.*

Tables 3.4A, 3.4B, 3.5A and 3.5B show the efficiency of the *Salvia palaestina* leaf aqueous and 35% ethanol extract respectively, several dilutions were made in order to determine the lowest concentration that can inhibit β-hematin crystallization 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 E. Deharo semi-quantitative.

Table 3.4A: *The efficiency of Salvia palaestina leaf aqueous extract 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 E. Deharo semi-quantitative.*

	Negative controls		**Positive controls**		**crude extract of *S. palaestina* in water**	
Test #	**Water**	**35% ethanol**	**CQ 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**
1	2.140	2.220	0.073	0.062	0.099	0.209
2	2.050	1.927	0.046	0.054	0.112	0.251
3	2.100	2.210	0.044	0.060	0.085	0.232
4	2.137	2.005	0.031	0.042	0.110	0.267
5	2.150	2.230	0.032	0.041	0.086	0.302
6	2.113	2.186	0.042	0.039	0.080	0.322
7	2.158	1.936	0.045	0.048	0.126	0.243
8	2.010	2.167	0.041	0.045	0.103	0.331
9	2.040	2.161	0.059	0.049	0.093	0.299
10	2.107	2.126	0.050	0.046	0.113	0.319

***Table 3.4B**: The efficiency of Salvia palaestina leaf aqueous extract 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 E. Deharo semi-quantitative method.*

Test #	Negative controls		Positive controls		crude extract of *S. palaestina* in water	
	Water	35% ethanol	CQ 0.1mg/ml	2-MP 1mg/ml	1mg/ml	0.5mg/ml
1	2.025	2.147	0.055	0.054	0.089	0.256
2	2.068	1.891	0.039	0.052	0.122	0.182
3	1.983	2.241	0.057	0.061	0.115	0.197
4	2.029	2.126	0.04	0.068	0.089	0.239
5	2.122	2.032	0.033	0.057	0.130	0.293
6	2.131	2.098	0.043	0.048	0.086	0.301
7	2.198	1.997	0.055	0.07	0.093	0.291
8	2.263	1.958	0.046	0.067	0.101	0.199
9	2.101	2.147	0.062	0.047	0.091	0.324
10	2.164	2.011	0.037	0.059	0.086	0.261

***Table 3.4C:** Average and standard deviation values for all absorption measurements at 405 nm of both tables 3.4A and 3.4B using Deharo semi quantitative method, done on S. palaestina aqueous extract.*

	Negative control	Positive controls			crude extract of *S. palaestina* in water	
	water	35% ethanol	CQ 0.1mg/ml	2-MP 1mg/ml	1mg/ml	0.5mg/ml
Average Abs. at 405nm	**2.1**	**2.091**	**0.047**	**0.053**	**0.100**	**0.266**
STD deviation	**0.069**	**0.11**	**0.011**	**0.009**	**0.015**	**0.046**

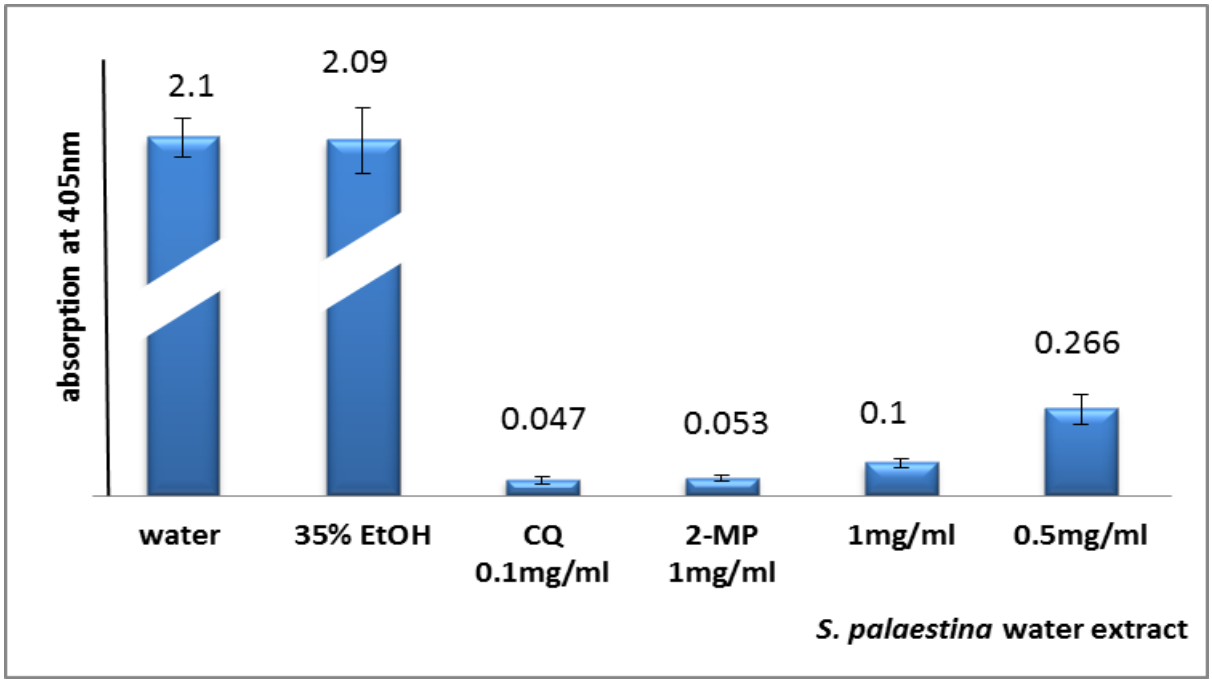

***Figure 3.4**: Column diagram representing the efficiency of S. palaestina leaf aqueous extract compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 20 individual experiments.*

Table 3.5A*: The efficiency of 35% ethanol extract of Salvia 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 E. Deharo semi-quantitative.*

	Negative control	**Positive controls**		**Crude leaf extract *of S. palaestine* in 35% EtOH**			
Test #	**Water**	**CQ. 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
1	2.296	0.058	0.085	0.044	0.044	0.166	2.093
2	2.325	0.050	0.063	0.038	0.050	0.087	2.068
3	2.259	0.044	0.102	0.035	0.045	0.105	1.915
4	2.280	0.047	0.074	0.039	0.048	0.106	2.002
5	2.279	0.058	0.063	0.037	0.040	0.102	2.038
6	2.289	0.057	0.060	0.034	0.045	0.118	2.030
7	2.244	0.054	0.059	0.037	0.052	0.115	2.086
8	2.325	0.050	0.071	0.045	0.041	0.136	2.027
9	2.301	0.040	0.098	0.038	0.053	0.167	2.051
10	2.347	0.053	0.108	0.053	0.046	0.123	2.030
11	2.140	0.062	0.058	0.041	0.064	0.136	2.069
12	2.110	0.045	0.073	0.055	0.060	0.151	2.118
13	1.978	0.042	0.059	0.050	0.043	0.173	2.123
14	2.061	0.044	0.052	0.039	0.063	0.184	2.184

***Table 3.5B**: The efficiency of 35% ethanol extract of Salvia 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 E. Deharo semi-quantitative method.*

	Negative control	**Positive controls**		**Crude extract *of S. palaestine* in 35% EtOH**			
Test #	**Water**	**CQ. 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
1	2.377	0.048	0.045	0.038	0.049	0.123	1.992
2	2.400	0.038	0.052	0.038	0.049	0.156	1.941
3	2.338	0.036	0.055	0.042	0.054	0.090	1.975
4	2.335	0.041	0.061	0.053	0.058	0.091	1.936
5	2.374	0.046	0.059	0.051	0.045	0.190	1.970
6	2.389	0.036	0.045	0.046	0.049	0.181	2.089
7	2.094	0.05	0.037	0.032	0.051	0.195	2.009
8	2.104	0.049	0.039	0.038	0.062	0.163	1.933
9	2.145	0.063	0.095	0.037	0.045	0.100	2.049
10	2.176	0.059	0.073	0.036	0.045	0.119	2.084
11	2.155	0.063	0.088	0.037	0.048	0.114	2.015
12	2.181	0.046	0.089	0.035	0.052	0.116	2.077
13	2.121	0.044	0.074	0.033	0.060	0.204	2.008
14	2.197	0.043	0.066	0.040	0.050	0.198	2.054

***Table 3.5C**: The average and standard deviation for both tables 3.5A and 3.5B. Regarding the absorption measurements of dissolved β-Hematin at 405 nm using Deharo semi quantitative method done on S. palaestina 35% crude extract dissolved in distilled water.*

	Negative control	Positive controls		*S. palaestine* 35 % crude extract			
	water	CQ 0.1mg/ml	2-MP 1mg/ml	1mg/ml	0.5mg/ml	0.3mg/ml	0.1mg/ml
Average Abs. at 405nm	2.24	0.049	0.067	0.041	0.05	0.139	2.03
STD deviation	0.113	0.008	0.02	0.006	0.007	0.037	0.063

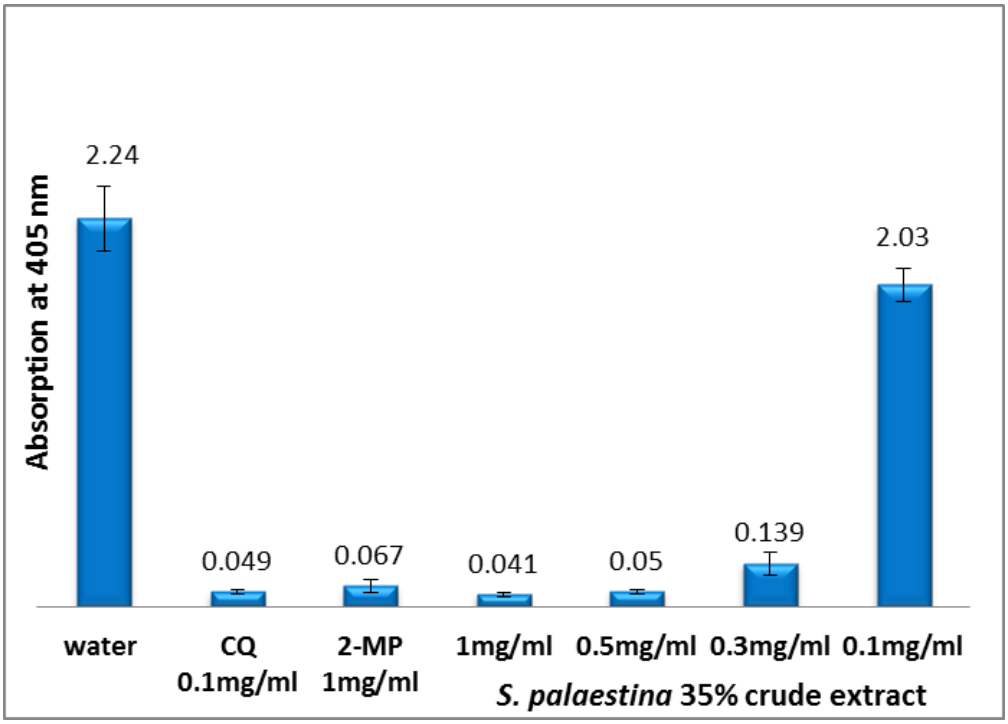

***Figure 3.5**: Column diagram representing the efficiency of S. palaestina leaf 35% ethanol extract compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 28 individual experiments.*

Table 3.6 below summarizes the results obtained testing the efficiency of *S. palaestina* stem extract in 35% ethanol in inhibiting β-hematin formation *in vitro*.

***Table 3.6A**: The efficiency of 35% ethanol extract of Salvia palaestina Stem 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 E. Deharo semi-quantitative method.*

	Negative control	**Positive control**		**Salvia stem 35% EtOH extract**			
Test #	**Water**	**CQ 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
1	2.304	0.052	0.061	0.152	0.206	1.311	2.167
2	2.254	0.043	0.053	0.121	0.09	1.404	2.247
3	2.112	0.031	0.064	0.064	0.151	1.399	2.182
4	2.310	0.066	0.046	0.07	0.124	1.432	2.184
5	2.264	0.028	0.042	0.091	0.116	1.221	2.289
6	2.186	0.033	0.055	0.109	0.146	1.549	2.206
7	1.989	0.051	0.067	0.069	0.138	1.274	2.058
8	2.045	0.035	0.071	0.055	0.183	1.66	2.107

***Table 3.6B**: The efficiency of 35% ethanol extract of Salvia palaestina Stem 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 E. Deharo semi-quantitative method.*

	Negative control	**Positive control**		**Salvia stem 35% EtOH extract**			
Test #	**water**	**CQ 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
1	2.200	0.050	0.076	0.13	0.15	1.509	2.215
2	2.135	0.064	0.059	0.082	0.26	1.638	2.208
3	2.175	0.054	0.046	0.107	0.206	1.242	2.228
4	2.057	0.053	0.061	0.125	0.173	1.549	2.164
5	2.065	0.038	0.050	0.062	0.124	1.406	2.273
6	2.110	0.062	0.055	0.079	0.118	1.428	1.924
7	2.154	0.043	0.048	0.121	0.185	1.389	2.005
8	2.026	0.044	0.042	0.061	0.203	1.501	1.968

***Table 3.6C**: The average and standard deviation for both tables 3.6A and 3.6B. Regarding the absorption measurements of dissolved β-Hematin at 405 nm using Deharo semi quantitative method done on S. palaestina stem 35% crude extract dissolved in distilled water.*

	Negative control	**Positive control**		**Salvia stem 35% EtOH extract**			
	water	**CQ 0.1mg/ml**	**2-MP 1mg/ml**	**1mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
Average	**2.149**	**0.047**	**0.056**	**0.094**	**0.161**	**1.430**	**2.151**
STDEV	**0.100**	**0.0118**	**0.010**	**0.030**	**0.044**	**0.131**	**0.110**

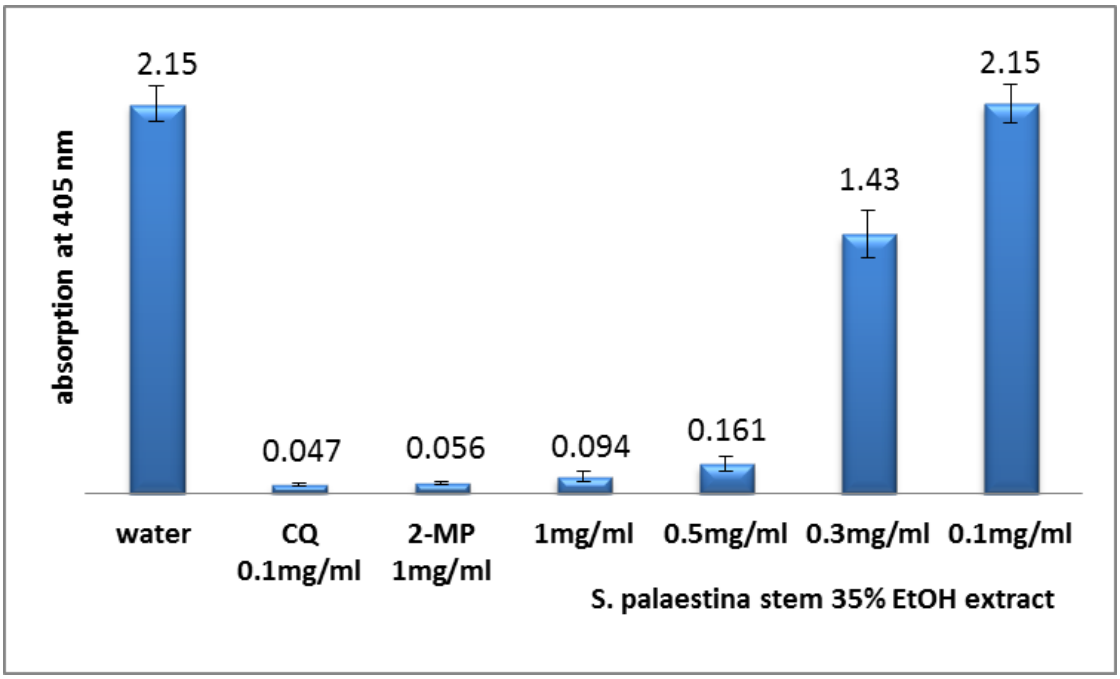

***Figure 3.6**: Column diagram representing the efficiency of S. palaestina Stem 35% ethanol extract compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 16 individual experiments.*

Table 3.7 below shows the semi quantitative test results according to E. Deharo method, done for Camphor a chemical constituent in *S. palaestina* extract, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, semi-quantitative method.

***Table 3.7**: The efficiency of camphore 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 E. Deharo semi-quantitative method.*

	Negative controls		**Positive controls**			**Tested chemical**
Test #	**Water**	**DMSO**	**CQ. 0.1mg/m**	**AQ. 0.1mg/ml**	**2-MP. 1mg/ml**	**Camphor 1mg/ml**
1	2.076	2.075	0.059	0.086	0.036	2.050
2	2.182	2.130	0.042	0.100	0.049	2.049
3	1.918	2.157	0.051	0.060	0.061	2.239
4	1.917	2.094	0.060	0.102	0.040	1.779
5	2.102	2.138	0.072	0.140	0.058	2.210
6	2.226	2.101	0.065	0.096	0.033	1.907
7	2.021	2.171	0.055	0.520	0.046	2.143
8	2.149	2.101	0.049	0.130	0.033	1.823
9	1.975	2.018	0.062	0.072	0.045	1.878
10	2.008	1.994	0.058	0.064	0.052	2.065
Average	**2.057**	**2.098**	**0.057**	**0.370**	**0.045**	**2.014**
STDEV	**0.110**	**0.057**	**0.009**	**0.140**	**0.010**	**0.160**

3.2 Preparative-HPLC-PDA separation of the active antimalarial fractions from crude *Salvia palaestina*:

A total of fourteen compounds were separated using analytical RP-HPLC at 265 nm (Figure 3.7 A, B). The overlaid UV spectra of the eluted peaks are shown in Figure 3.8.

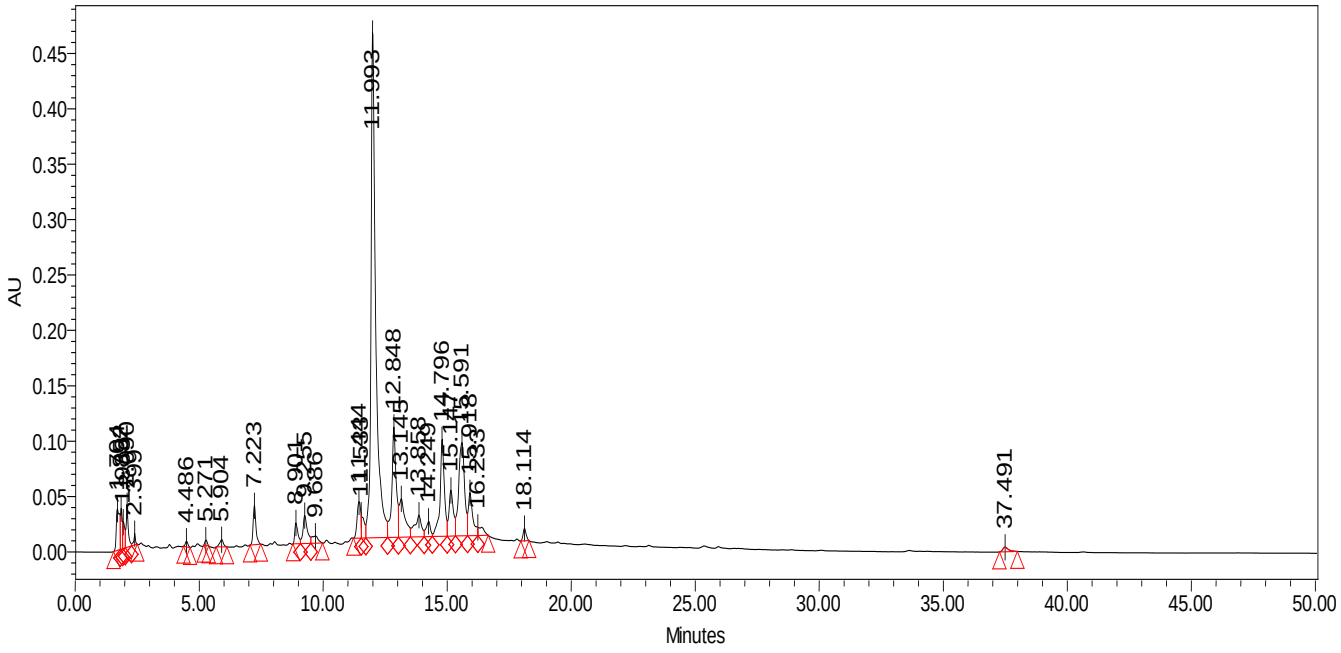

Figure 3.7A: *Typical analytical HPLC chromatogram of 35% ethanolic crude mixture of salvia palaestina. 10 µl were injected at flow rate 1 ml/min and monitoring λ at 260 nm.*

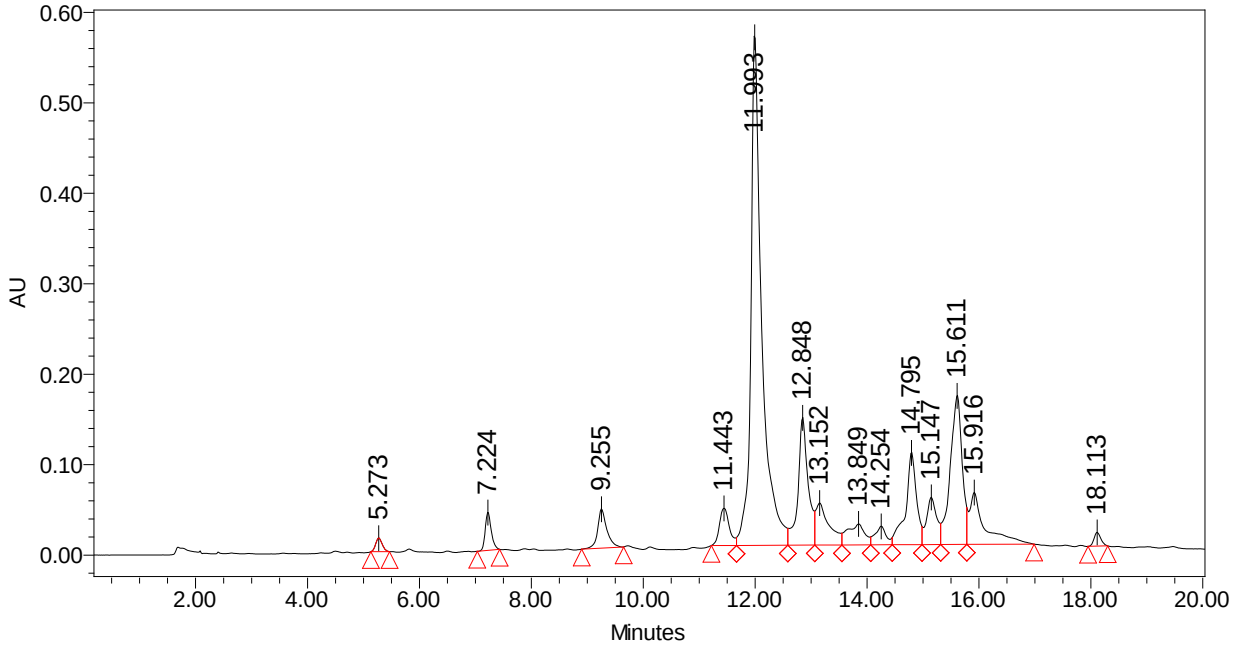

Figure 3.7B: *shows the same chromatogram as in Figure 3.7A, but zoomed, at 350 nm.*

The HPLC chromatogram revealed the following percentages of each eluted compounds at 350 nm shown in Table 3.8.

Table 3.8: *The percentages of each eluted compounds at 350 nm revealed from HPLC chromatogram.*

% Area	Peak t_R (mins)
0.68	**5.273**
1.74	**7.224**
2.70	**9.255**
2.79	**11.443**
42.12	**11.993**
9.81	**12.848**
4.25	**13.152**
2.76	**13.849**
1.57	**14.254**
7.52	**14.795**
3.72	**15.147**
13.32	**15.611**
6.30	**15.916**
0.71	**18.113**

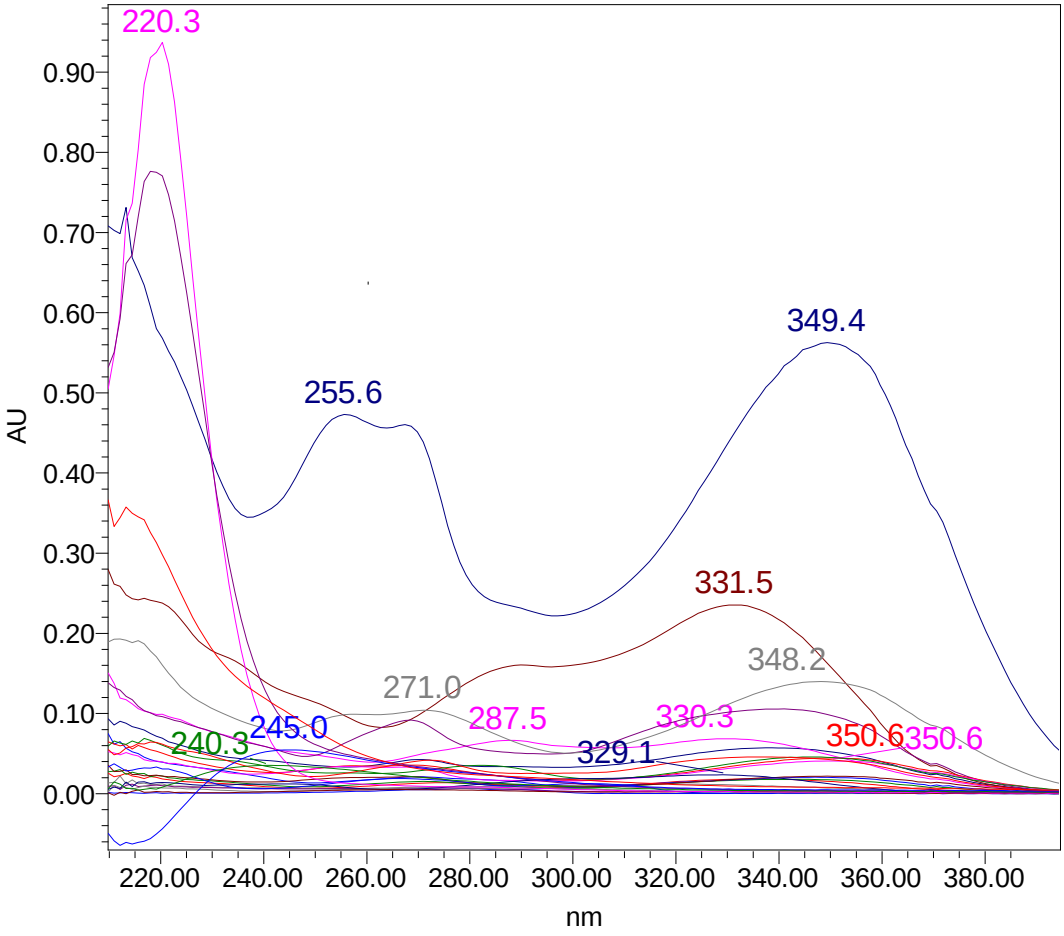

Figure 3.8: *Overlaid UV spectra of the eluted peaks in Figure 3.7A.*

Further step of purification on the 35% ethanol crude extract of *salvia palaestina* was attained by loading 1000 µl (25mg/ml) to the 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 Figure 3.8.

Only three fractions were found to exhibit anti-malarial activity when tested in-vitro. We noticed that the most pharmacological potent fraction is fraction VII which was eluted

between 14.8-19 min as shown in Figure 3.9. To acquire the active fractions in high quantity, other preparative injections were successively made and three major fractions were recollected accordingly.

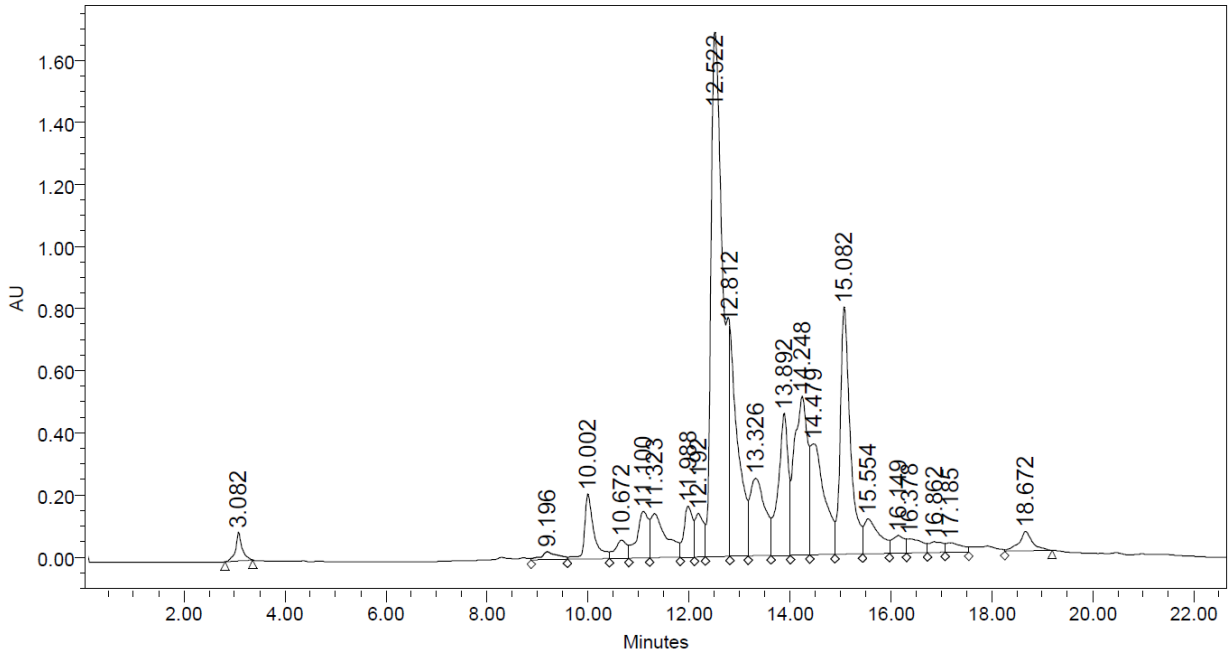

Figure 3.9*: Typical preparative HPLC chromatogram of 35% ethanol crude mixture of salvia palaestina. 1000 µl were injected at flow rate 15 ml/min and the monitoring λ of 260 nm was used. Fraction I was collected from (2-3.2 minutes), fraction II (3.3-7 minutes), fraction III (7-9.6 minutes), fraction IV (9.6-12.1 minutes), fraction V (12.2-13 minutes), fraction VI (13-14.8 minutes) and fraction VII (14.8-19 minutes).*

3.3 Salvia fractions semi-quantitative test results

Table 3.9 below shows the results of semi-quantitative method done according E. Deharo on the fractions obtained after separation of *S. palaestina* 35% EtOH crude extract, the efficiency of these extracts in inhibiting β–hematin formation *in vitro* is compared to positive and negative controls.

Table 3.9: *The efficiency of S. palaestina 35% ethanol extract fractions 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 E. Deharo semi-quantitative.*

	Negative control	**Positive control**		**Fractions obtained from *S. palaestina* 35% EtOH extract, all at concentration 0.5mg/ml**							
Test #	**water**	**CQ 0.1mg/ml**	**MP 1mg/ml**	**F. I**	**F. II**	**F. III**	**F. IV**	**F. V**	**F. VI**	**F. VII**	**F. VIII**
1	**2.045**	**0.044**	**0.032**	**1.785**	**1.954**	**0.289**	**0.322**	**0.086**	**0.058**	**0.071**	**1.281**
2	**2.062**	**0.047**	**0.035**	**1.995**	**2.007**	**0.3**	**0.211**	**0.157**	**0.065**	**0.089**	**1.184**
3	**2.065**	**0.039**	**0.064**	**1.86**	**1.928**	**0.181**	**0.19**	**0.067**	**0.075**	**0.062**	**0.856**
4	**2.153**	**0.054**	**0.045**	**1.873**	**1.768**	**0.383**	**0.25**	**0.108**	**0.082**	**0.058**	**0.819**
5	**2.144**	**0.04**	**0.052**	**1.924**	**1.823**	**0.191**	**0.256**	**0.072**	**0.099**	**0.048**	**0.758**
6	**1.98**	**0.061**	**0.027**	**1.838**	**2.056**	**0.304**	**0.312**	**0.094**	**0.062**	**0.052**	**0.971**
Average	**2.075**	**0.0475**	**0.043**	**1.879**	**1.923**	**0.275**	**0.257**	**0.097**	**0.074**	**0.063**	**0.978**
STDEV	**0.065**	**0.009**	**0.014**	**0.073**	**0.109**	**0.076**	**0.053**	**0.033**	**0.015**	**0.015**	**0.211**

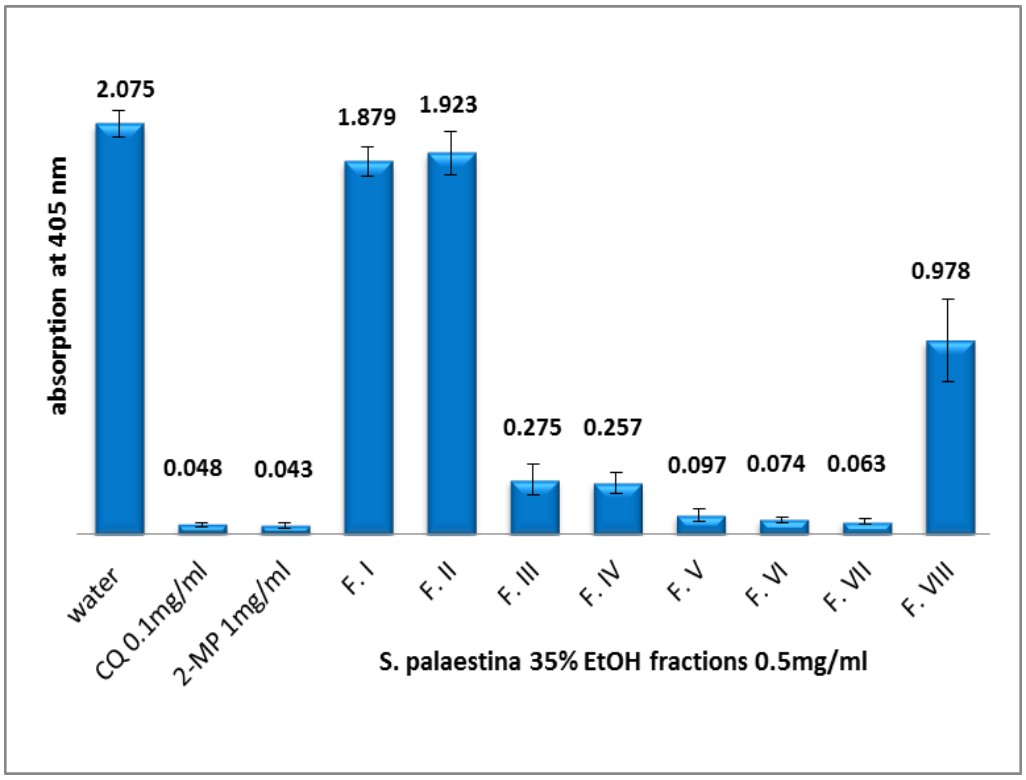

Figure 3.10: *Column diagram representing the efficiency of the eight fractions obtained from separation of S. palaestina leaf 35% ethanol extract, dissolved in water at concentration 0.5mg/ml, compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 6 individual experiments.*

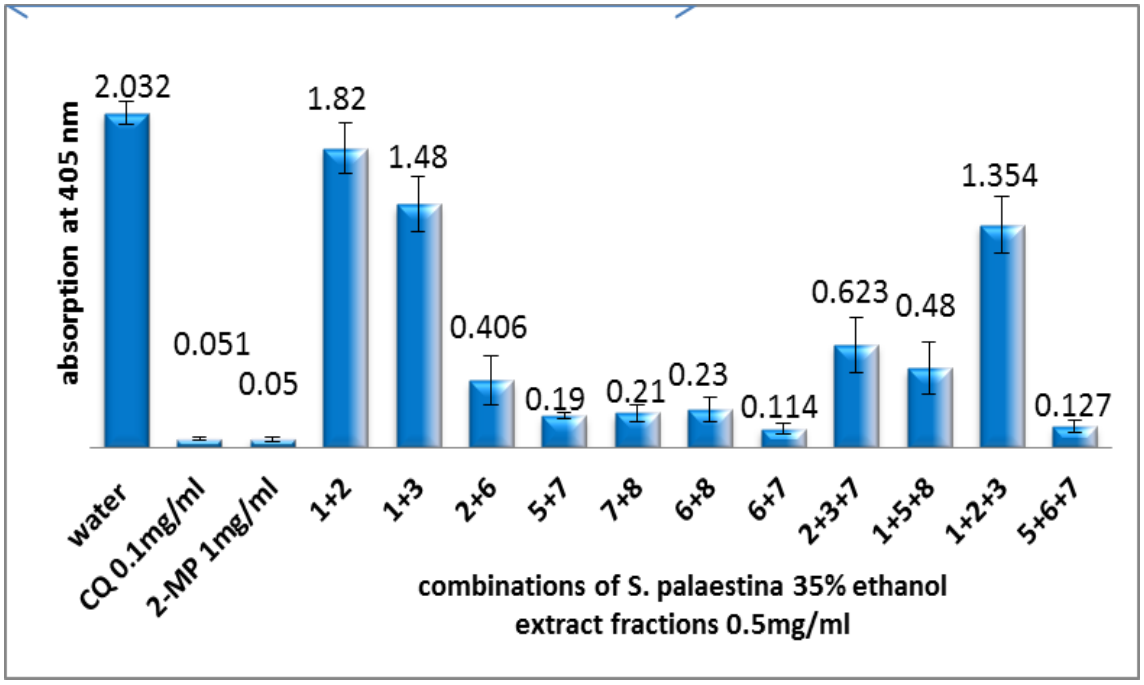

Figure 3.11: *Column diagram representing the efficiency of combinations made of the fractions obtained from separation of S. palaestina leaf 35% ethanol extract, dissolved in water, compared to the negative and positive controls, showing the absorption values of dissolved β-Hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. Each result represents the average of 5 individual experiments.*

3.4 Quantitative test results:

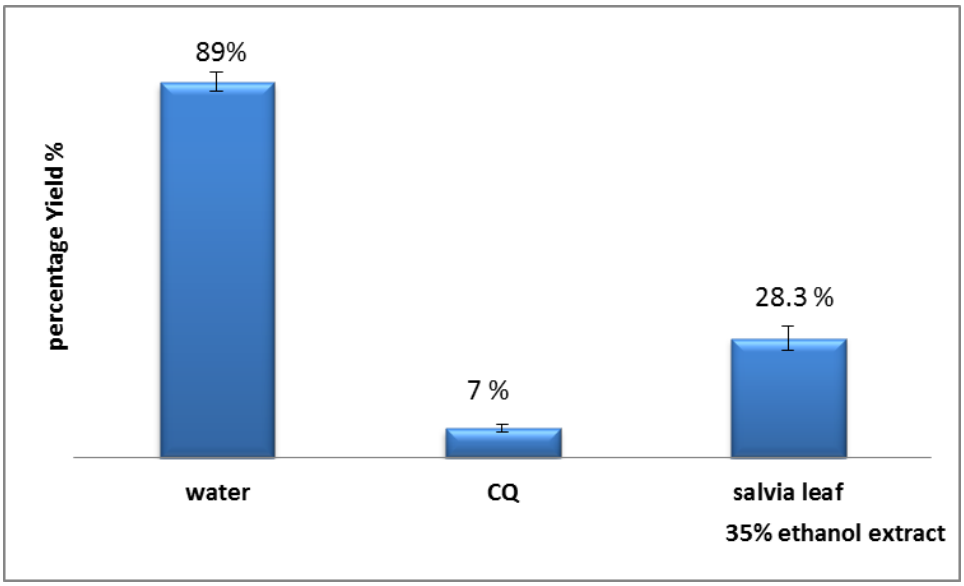

***Figure 3.12**: Column diagram representing the percentage yields of salvia leaf 35% ethanol extract as potential anti-malarial drug, compared to CQ and water. Yields are inversely proportional to drugs efficiency, the lower the yield is, the drug is considered to be more efficient.*

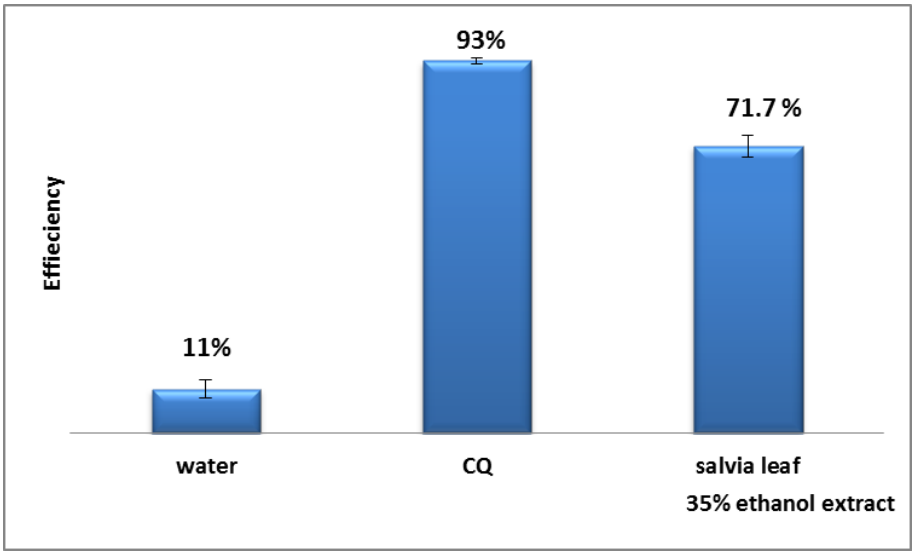

***Figure 3.13:** Column diagram representing the efficiencies of salvia leaf 35% ethanol extract as potential anti-malarial drug compared to CQ and water.*

Chapter 4

Discussion and Conclusion

4.1 Discussion

During the erythrocytic stage, massive degradation of hemoglobin generates large amounts of heme which is toxic to the parasite. Malaria parasite, nevertheless, has evolved a distinct mechanism for detoxification of heme through its conversion into an insoluble crystalline pigment, known as hemozoin. Hemozoin synthesis is an indispensable process for the parasite survival and is the target for action of numerous anti-malarials. Quinoline anti-malarials have been found to be the most consistent inhibitors of hemozoin synthesis. [Sullivan et al., 1996; 1998].

As strains of the malaria parasite *Plasmodium* emerged gaining resistant to these drugs used, the search for new anti-malarial drugs has become a must. Hemozoin detoxification pathway has, therefore, attracted significant interest for new anti-malarial drug discovery research, and is the topic of this work. A synthetic polymer structure made from Ferriprotoporphyrin (IX) named β-Hematin is believed to be structurally, chemically and spectroscopically identical to purified hemozoin, making it an outstanding alternative for *in vitro* studies [Slater et. al., 1991; Blauer and Akkawi, 1997, 2000; Pagola et. al., 2000].

In this research the potential inhibitory effect of different herbs and sea weed extracts on β-hematin (synthetic Hemozoin) formation was studied *in vitro* using a simple screening semi-quantitative method followed by a more analytical quantitative method.

A concentration of 1mg/ml of the potential drug was used in these screening tests, while the concentration of hemin chloride in all of these tests was 0.5 mg/ml. Only the alcoholic extracts of *Salvia palaestina* leaves showed promising results when compared to other plant extracts like *Achillea Fragrantissima, Ulva fascianta* and *Cladophora aegagropila* using the same positive and negative controls, as seen in Tables 3.1A/B and 3.2A/B, and Figures 3.1 and 3.2.

CQ and AQ are two universally known anti-malarial drugs that are used extensively in the fight against malaria, known to inhibit the process of β-hematin crystallization, were used as positive controls. (Mercaptopyrimidine "2-MP"), another positive control that was used as internal control was found to be highly sensitive to minor changes in the test conditions, especially temperature and thus is a good index of the test accuracy that has been used to confirm or rule out the results. Water, ethanol or DMSO were used as negative controls. According to this semi-quantitative screening method, each absorption value is inversely proportional to the efficiency of the extract to inhibit β-hematin formation; low absorption indicates higher efficiency and vice versa.

Natural compounds were used throughout as drugs. Sage is distinguished by its astonishing versatility, and considered as one of the oldest medicinal plants. We chose sage as it is known to be used against many diseases, due to the fact that salvia species are very rich in

biologically active compounds. Some researchers suggested that sage could have anti-malarial drugs [Dixon et al., 2013].

Not only the alcoholic leaf or stem extracts (35% ethanol) have anti-malarial activity, but also the aqueous leaf extract has considerable activity to prevent β-hematin polymerization. This inhibitory effect of the crude may be due to the fact that all of them contain polyphenols and thujone as evidenced by [Mokcute et al., 2003; Gürsoy et al., 2012]. Nevertheless, the most distinctive effect was that of *S. palaestina* leaf extract in 35% ethanol.

"Hydroalcoholic mixtures of ethanol are possibly the most suitable solvent system for the extraction of sage polyphenols due to the different polarities of the bioactive constituents, and the acceptability of this solvent system for human consumption" [Dent et al., 2013], also aqueous sage extract does contain polyphenols [Walch et al., 2011].

Polyphenols are known to be anti-oxidants, 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.

The mechanism of action of these plant extracts as anti-malarials is thought to be by inhibition of β-hematin formation which is probably through formation of a complex between the active compounds in these extracts and ferri-heme. Different non-covalent interactions may be

responsible for this inhibition. For example we can talk about the possibility of forming hydrogen bonding between propionic acid in ferri-heme and the active compounds in the extract, containing a carbonyl group as an example, can terminate β-hematin extension, see Figure 4.1 below, this may explain the inhibitory effect of the crude extract and HPLC fractions on the formation of synthetic Hemozoin.

In the case of the crude extract, the inhibitory effect may be due to at least to the presence of different chemical constituents in the extracts, such as different polyphenols and thujone. Thujone is the main constituent of sage essential oil, which may bind to the free hemin and thus prevents β-hematin extension.

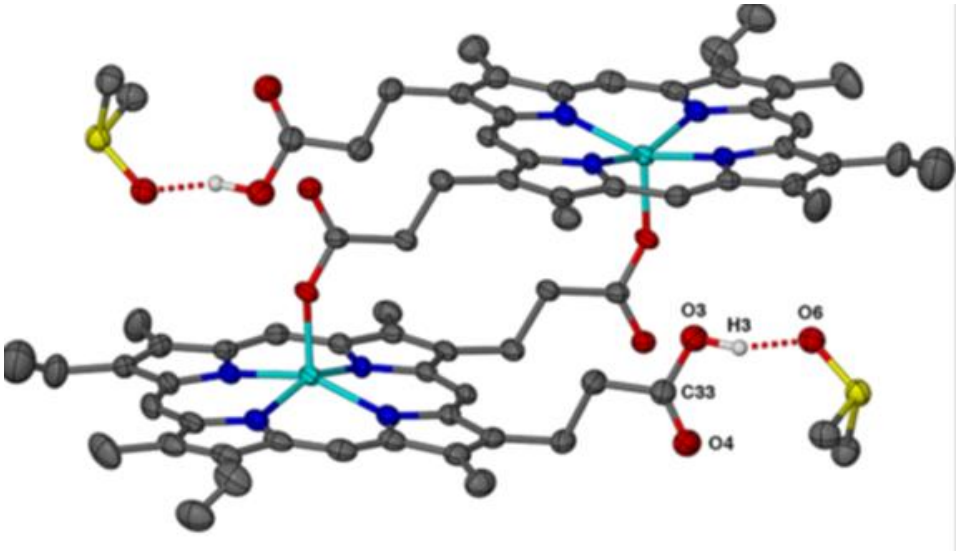

Figure 4.1: *The dimer of Fe (III) PPIX observed in the β-hematin DMSO solvate structure. The hydrogen bond (O3−H3···O6) between the propionic acid group of Fe (III) PPIX and an included DMSO molecule is indicated as a dashed red line. Atom colors are: gray, C; white, H; blue, N; red, O; cyan, Fe; and yellow, S. [Gildenhuys et al., 2012]*

The basic structural of flavonoid compounds is three rings; two aromatic rings linked by a three-carbon bridge [C6-C3-C6] [Pedro et al., 2012]; Apigenin and Luteolin are good examples of flavonoids.

Luteolin (see Figure 4.2) is widely distributed in many types of plants including fruits, vegetables, and medicinal herbs. Its anti-cancer activity has been reviewed by [Seelinger et al., 2008].

The effect of different flavonoids on the growth of *Plasmodium falciparum* has been studied by [Lehane and saliba, 2008]; luteolin was found to be the most active with IC50 values around 11 µM. The flavonoid luteolin is found in Artemisia species [Brown G., 2010; Seddik et al., 2010] presenting an anti-malarial activity; luteolin is also present in salvia species according to [Đorđević et al., 2000]. Another study showed that when luteolin is administered to rodents in high doses it has no toxic effect [Mukinda J., 2005].

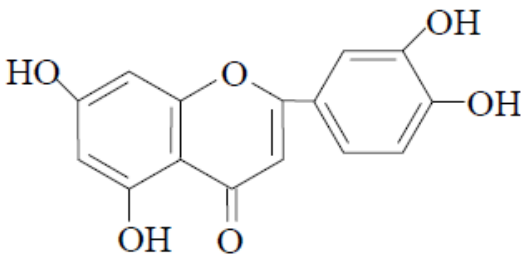

Figure 4.2: *The structure of luteolin.*

It is known that flavonoids chelate metals such as iron and copper as part of their antioxidant effects and that iron chelating therapies have been recommended for malaria patients.

The essential oil composition of Salvia showed the dominance of monoterpenes and sesquiterpenes, like thujone, 1,8-cineole and camphor [Gürsoy et al., 2012]. One of these, camphor, was tested using the semi-quantitative method and was found to have no effect on beta hematin polymerization as shown in Table 3.7.

Thujone is a ketone and a monoterpene, found in many different plants including sage, as well as many other herbs and spices. Thujone occurs naturally in two diastereomeric forms: α-thujone and β-thujone see Figure 4.3. Thujone is considered potential anti-malarial agent according to [Ramazani et al., 2010]. It is also present in Artimisia species [Abad et al., 2012], that are used to treat malaria.

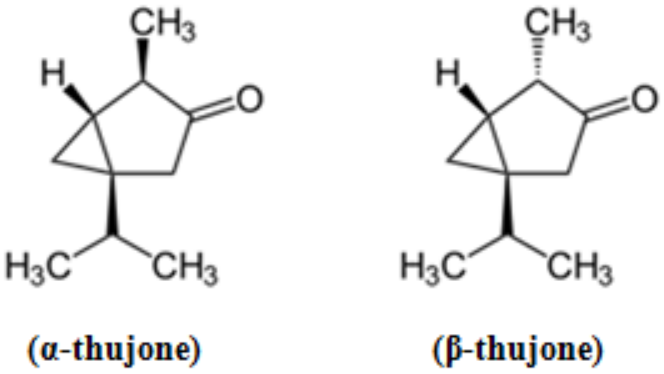

Figure 4.3: *The structure of α and β-thujone.*

Evidence that thujone is the active anti-malarial molecule in many artemisia species is confirmed by the fact that *Artemisia dracunculus* which contains no thujone has no noticeable antimalarial activity [Lutgen, 2012]**.** Many recent studies also found that the anti-cancer properties of several plants, including *Artemisia absinthium,* are proportional to the concentration of thujone [Biswas et al., 2011; Russo et al., 2013]. Other plants like sage contain large amounts of thujone and for that are considered as potential anti-malarial drugs.

Quantitative test done on the crude alcohol extract confirmed the previous results obtained with efficiency 71.1% when compared to CQ 93% water 11%, see Figures 3.12 and 2.13.

Differences in the efficiency of leaf and stem ethanol extracts may be attributed to the fact that the amount of thujone present in the leaf is double that in the stem [Velickovi et al.,2003].

High performance liquid chromatography (HPLC) is the best technique to separate phenolic and triterpenoid compounds. Only three fractions (V, VI and VII) out of the eight fractions obtained using the preparative HPLC were found to exhibit anti-malarial activity when assayed *in-vitro*.

We noticed that the most pharmacological potent fraction is fraction VII which was eluted between 14.8-19 minutes. As shown in Figure 3.9, this fraction contains a major peak at 15 minutes and to a lesser extent another few peaks that lack chromophore which strengthens our believe that this compound is the one responsible for anti-malarial activity. Previous studies

on other salvia leaves species have proved the presence of phenolic compounds which possess strong UV absorption. [Lu and Foo, 2002].

Different combinations of the HPLC fractions obtained were tested using the semi-quantitative method, to study if there is any synergistic effect of these random combinations, as shown in Figure 3.11. Any combination that contains one of the three active fractions enhanced the inhibitory effect on β-hematin formation, as shown in Table 4.1. For example a combination between fraction II and VI, showed increased effectiveness in inhibiting β-hematin formation when compared to the calculated values. Experimental evidence suggests that some flavonoids may enhance the action of artemisinin against P. falciparum [Ferreira et al., 2010]. This also can be seen in other combinations as seen in the Table 4.1 below.

***Table 4.1**: Result of different HPLC fraction combinations tests compared to the average calculated value of β-hematin dissolved in (0.1N) NaOH absorption at 405 nm.*

Fractions Combination	**Theoretical value**	**Experimental value**
1+2	**1.901**	**1.820**
1+3	**1.077**	**1.480**
2+6	**0.999**	**0.410**
5+7	**0.080**	**0.190**
7+8	**0.521**	**0.210**
6+8	**0.526**	**0.230**
6+7	**0.069**	**0.114**
2+3+7	**0.748**	**0.620**
1+5+8	**0.985**	**0.480**
1+2+3	**1.359**	**1.350**
5+6+7	**0.078**	**0.130**

The identity of the active compound responsible for anti-malarial activity of *S. palaestina* ethanol extract noted is not determined in this study. However, such determination requires further studies using elemental analysis, Nuclear magnetic resonance NMR and Gas chromatography–mass spectrometry GC-MS. But the quantity of the fractions left after we finished the experiments was not enough to do all these identification tests. Currently we are planning to repeat the extraction in order to identify the active compounds.

What we have done in this regard is FTIR measurement of the fractions. Although, FTIR measurement alone can't be used to identify the active ingredient, the FTIR absorption of the HPLC fractions were measured, see Appendix.

The FTIR spectral analysis of the ethanolic extract's revealed bands in the region between 1500 – 500 cm^{-1}. Absorption bands at 3360 cm^{-1} is due to OH stretching , while 1654.98 - 1525.74 cm^{-1} on the other hand is due to either C = C or C = O stretching. The intensive band at 1606 cm^{-1} is most probably the result of vibrations of C=O group.

This is in correlation with the functional groups constituting flavonoids in the extract, which basically have C = O, C-OH as contributing functional groups and are correlated in the spectral peaks.

4.2 Conclusions

In conclusion malaria is one of the oldest diseases known to man and considered as a global health problem. It is stoppable, yet more than 600,000 people die of it every year, due to the rise of resistant strains of Plasmodium, there is an urgent need to find new cheap and easily available anti-malarial drugs preferably from natural products.

Salvia palaestina a safe and widely available herb, this herb has many traditional uses in the folk medicine. The extract is a natural product and has been used in folk medicine with no reported toxicity. It is clear from our work that sage 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 and *in vivo* tests must be done to study the antimalarial activity of this plant.

Further Nuclear magnetic resonance NMR and Gas chromatography–mass spectrometry GC-MS ought to be done to identify the active compound(s).

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Appendix

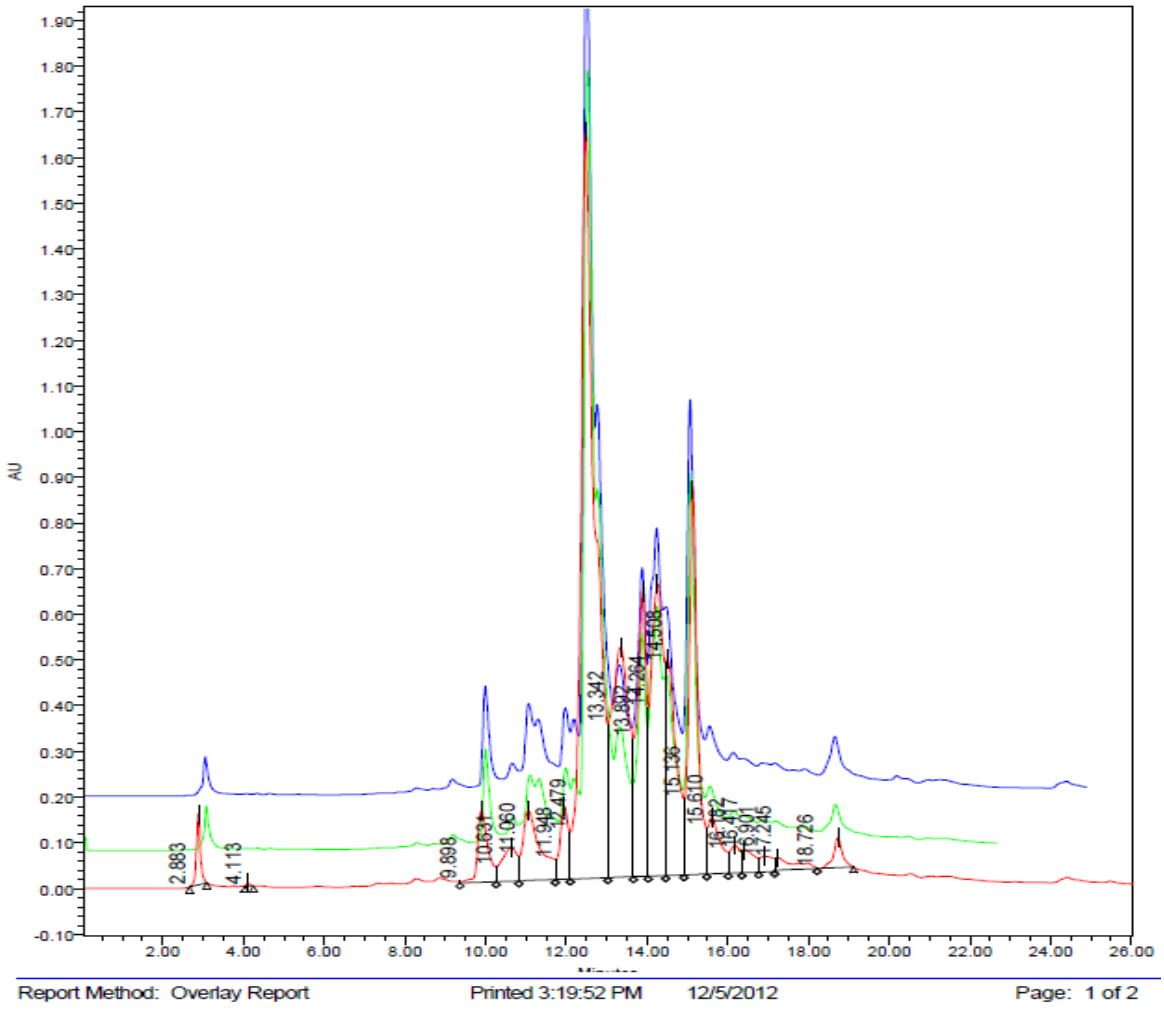

Figure 1: *Overlaid preparative HPLC chromatograms of 35% ethanol crude mixture of salvia palaestina. 1000 µl were injected for each at flow rate 15 ml/min and the monitoring λ of 260 nm was used*

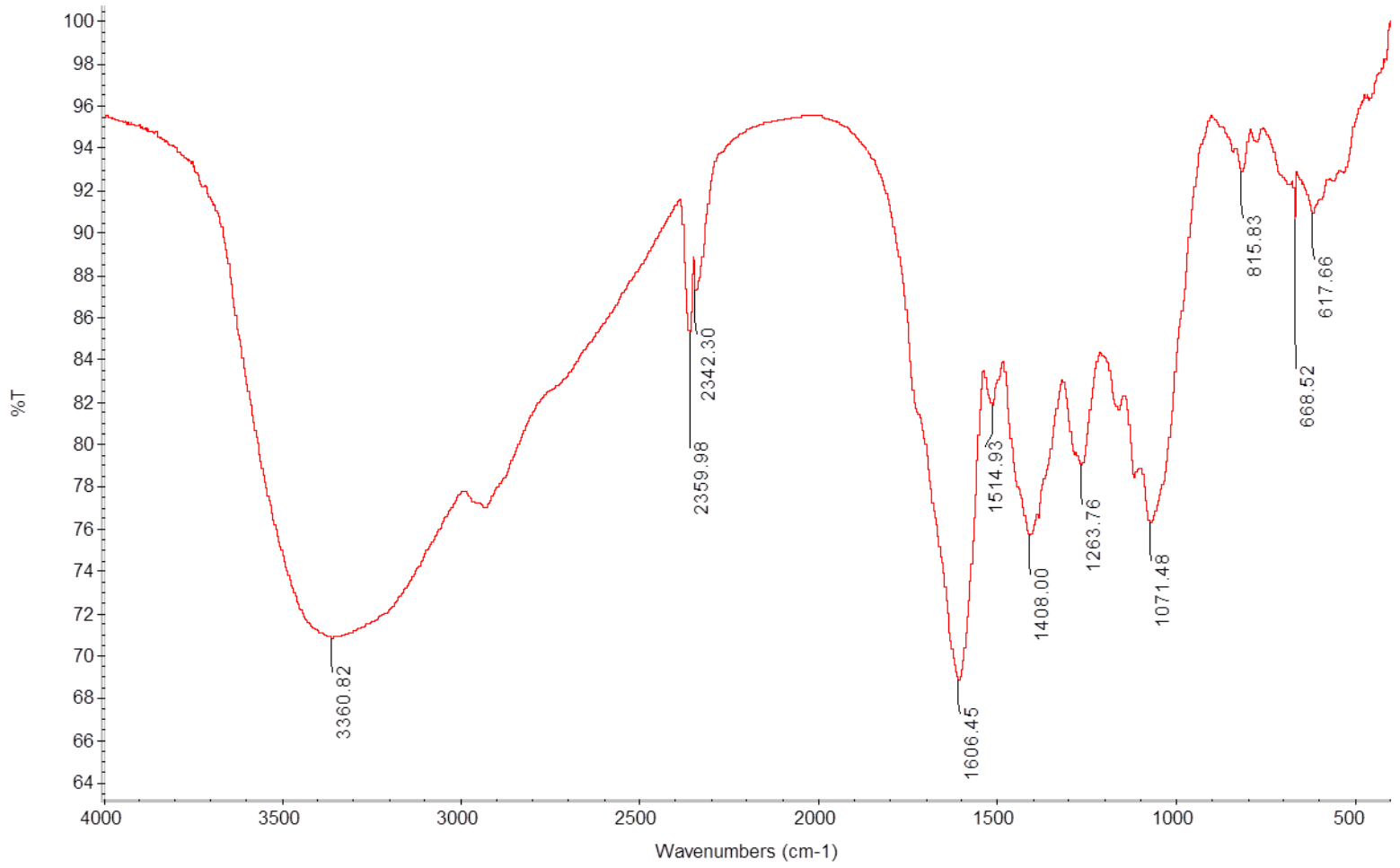

***Figure 2**: FTIR spectrum of salvia palaestina 35% ethanol extract crude.*

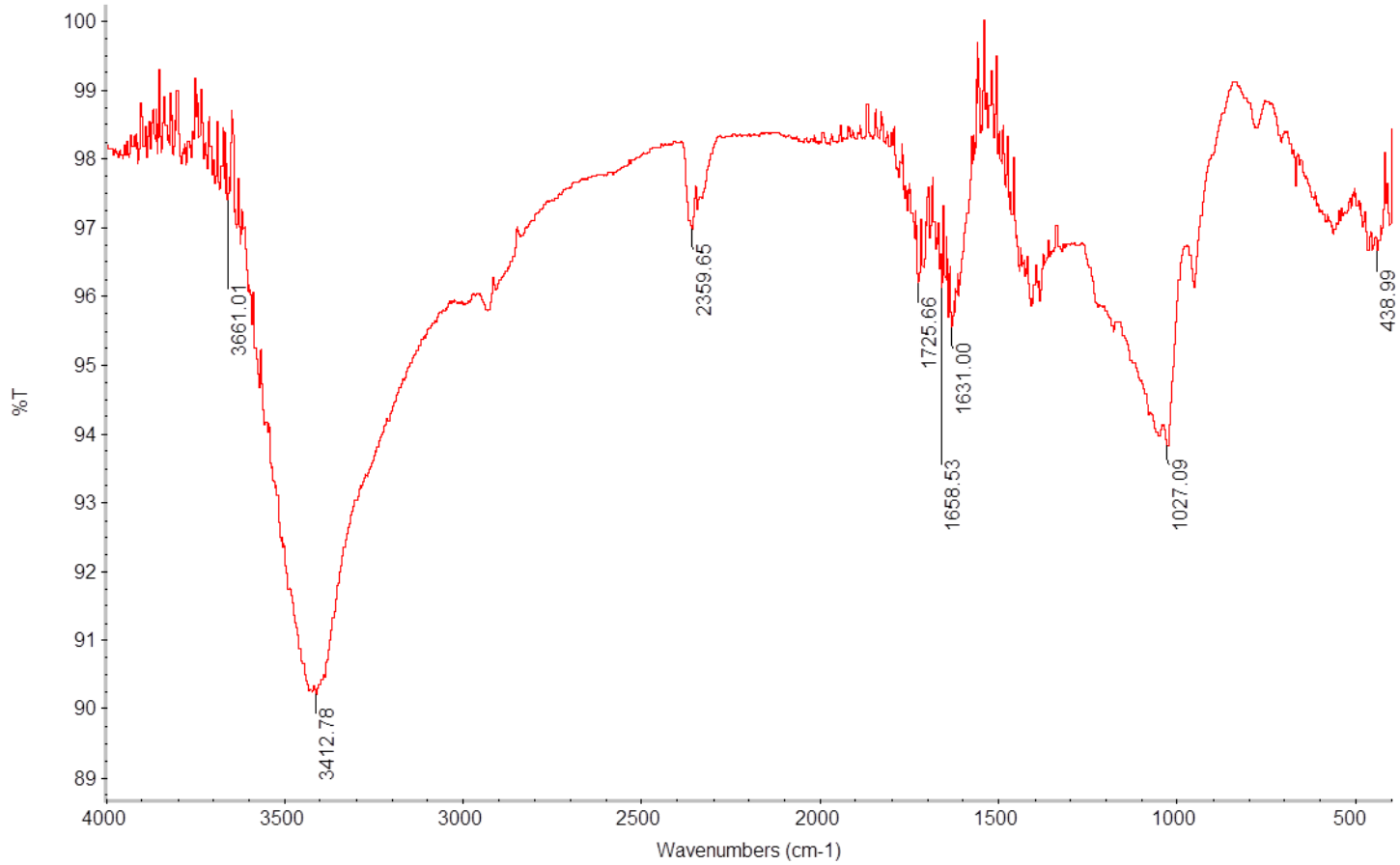

***Figure 3**: FTIR spectrum of salvia palaestina 35% ethanol extract Fraction I.*

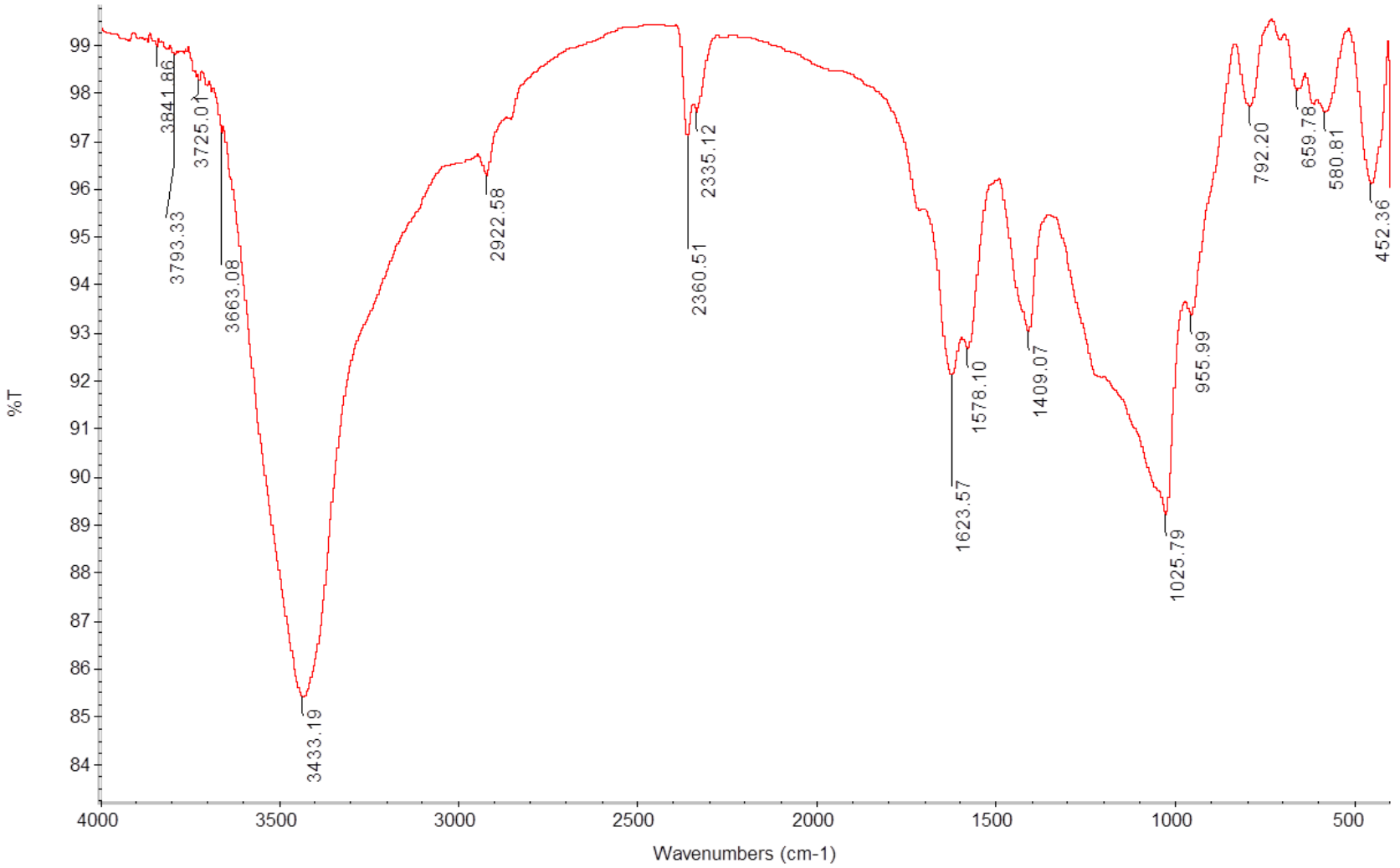

***Figure 4**: FTIR spectrum of salvia palaestina 35% ethanol extract Fraction II.*

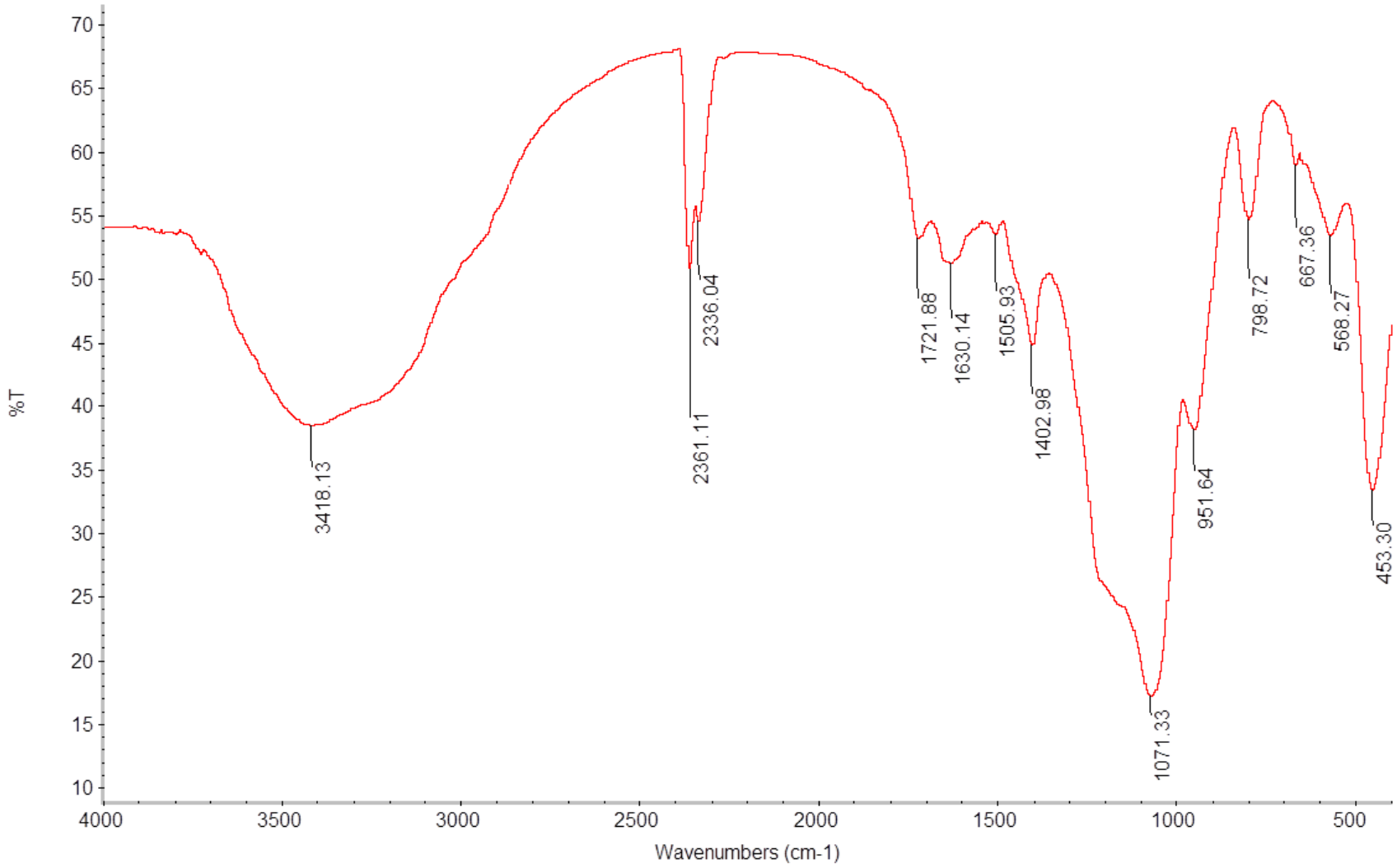

***Figure 5**: FTIR spectrum of salvia palaestina 35% ethanol extract Fraction III.*

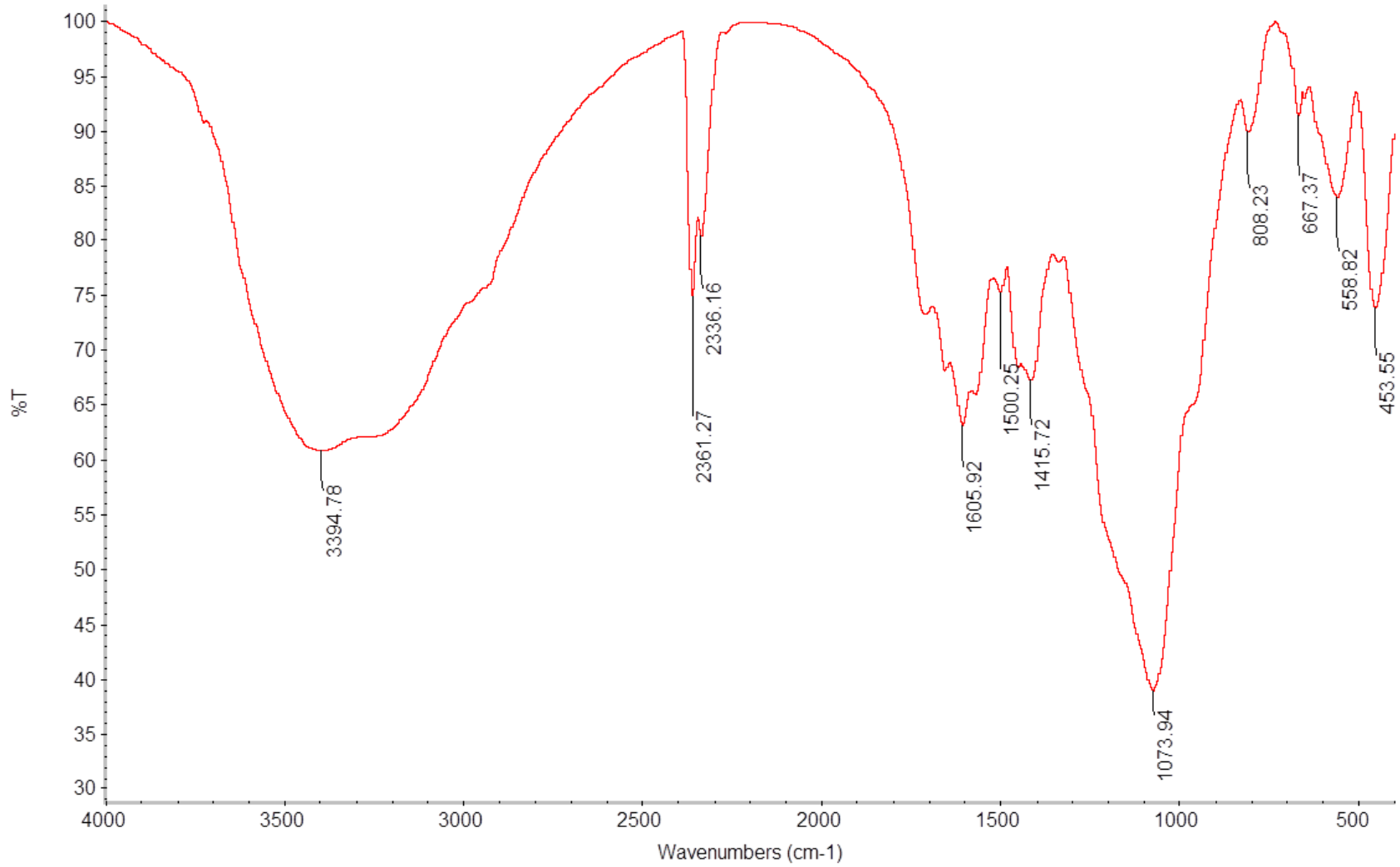

Figure 6: *FTIR spectrum of salvia palaestina 35% ethanol extract Fraction IV.*

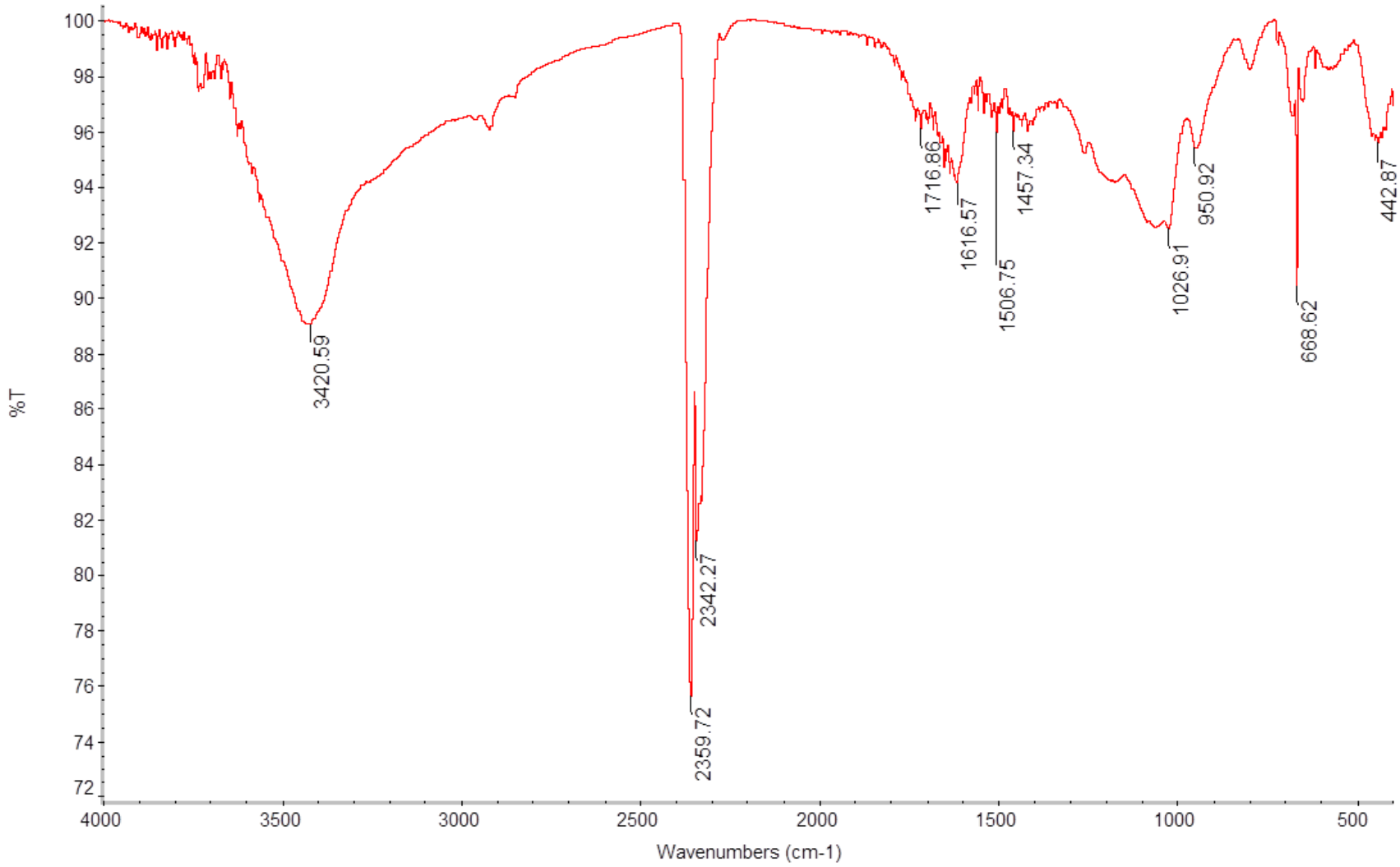

Figure 7: *FTIR spectrum of salvia palaestina 35% ethanol extract Fraction V.*

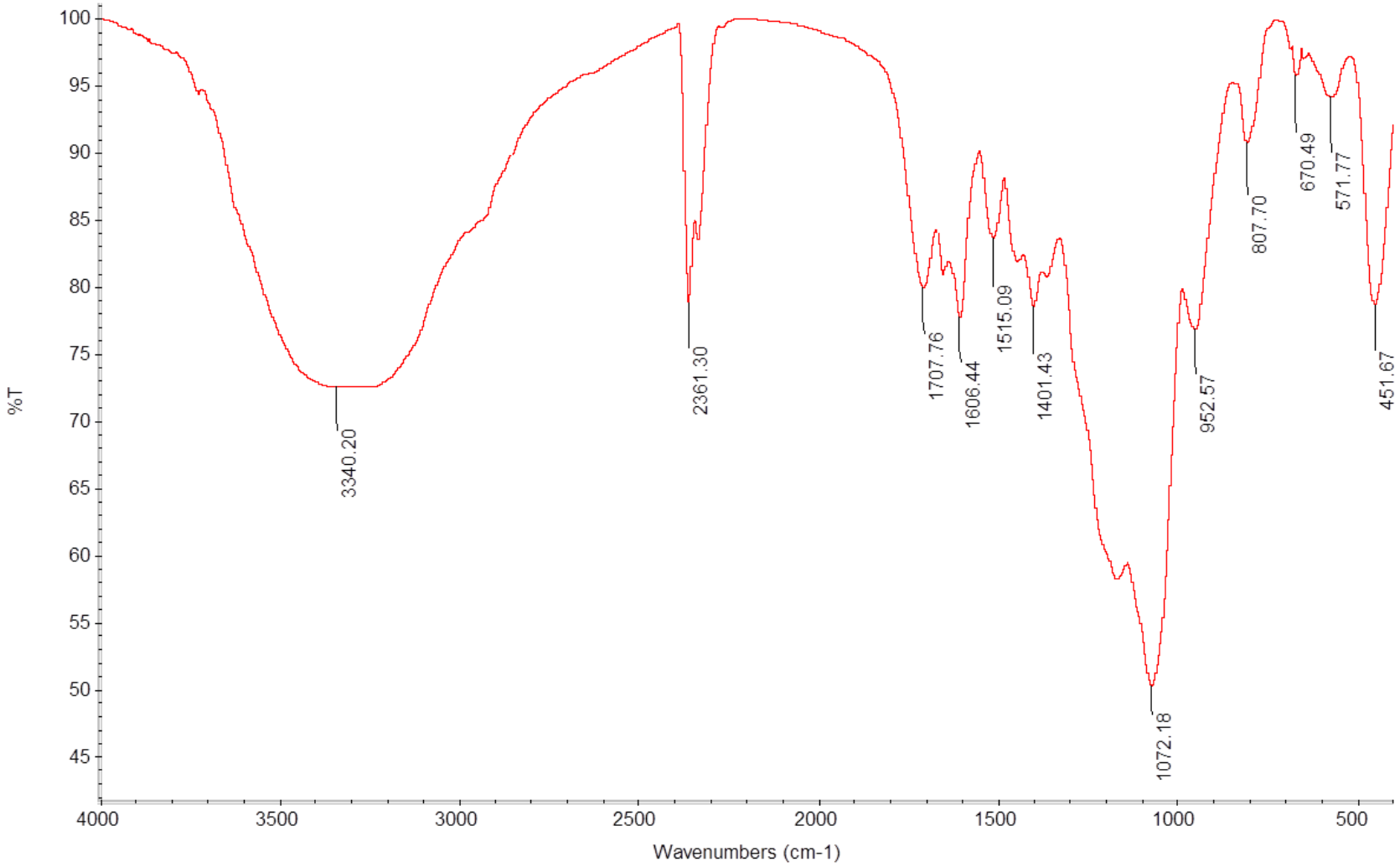

***Figure 8**: FTIR spectrum of salvia palaestina 35% ethanol extract Fraction VI.*

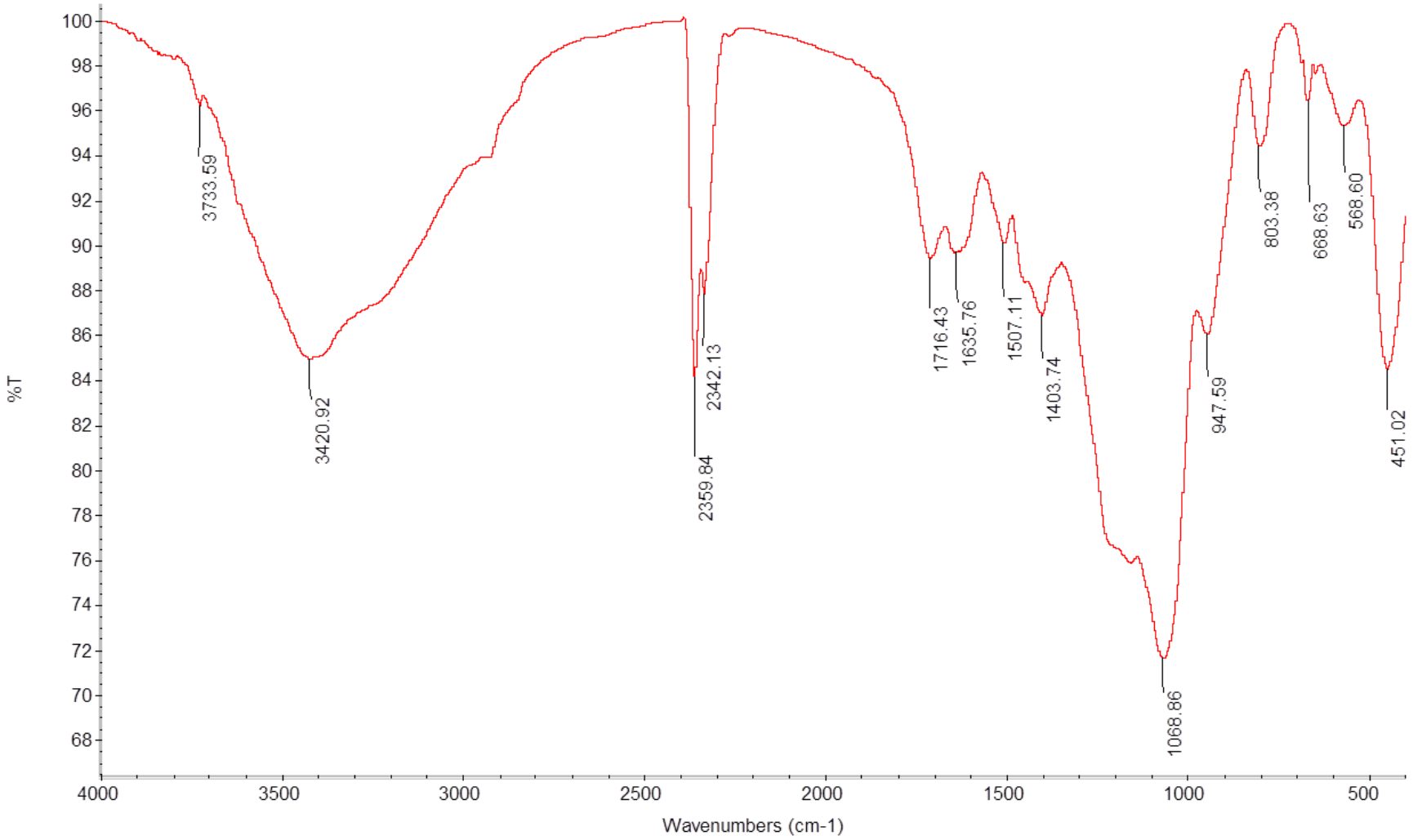

***Figure 9**: FTIR spectrum of salvia palaestina 35% ethanol extract Fraction VII.*

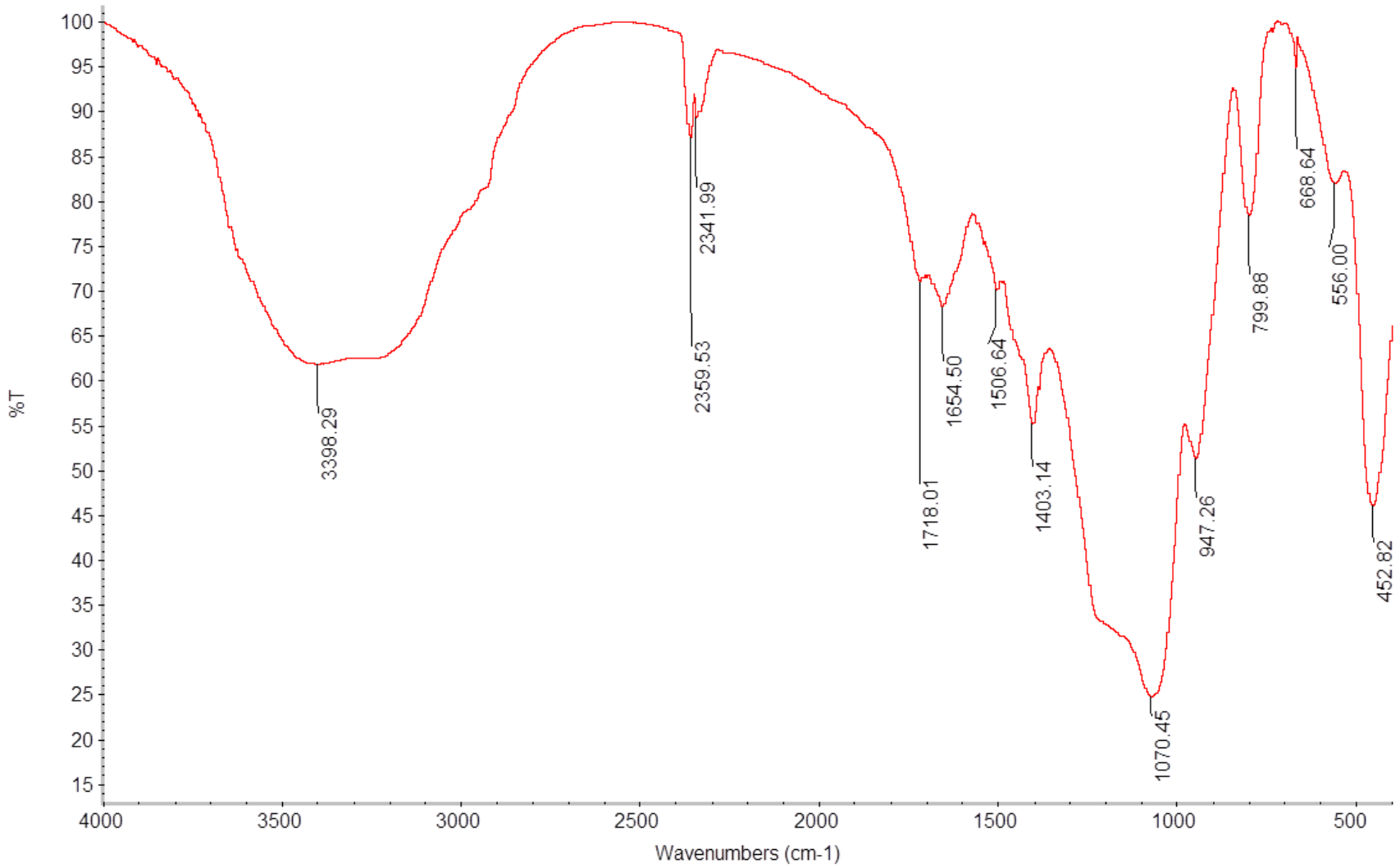

Figure 10: *FTIR spectrum of salvia palaestina 35% ethanol extract Fraction VIII.*