DEVELOPMENT, VALIDATION AND APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF ARTEMISININ CONTENT IN *ARTEMISIA ANNUA* L. EXTRACTS.

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A thesis submitted in partial fulfillment of requirements for the award of the degree of Master of Science in Pharmacognosy and Complementary Medicine

September 2018

DECLARATION

This thesis is my original work and has not been submitted elsewhere for award of any degree.

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DEDICATION

This work is dedicated to my beloved children Lillian and Brian who are always an encouragement and inspiration.

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LIST OF ABBREVIATION

ACT	Artemisinin-based Combination Therapy
AIDS	Acquired Immune Deficiency Syndrome
AQ	Amodiaquine
^{o}C	Degrees Centigrade
C_{18}	Octadecyl silane
ACN	Acetonitrile
cm	Centimeter
CDC	Centre for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EPZ	Export processing zone
HPLC	High Performance Liquid Chromatography
ICH	International conference on harmonisation
IM	Intramascular
IPTi	Intermittent preventive treatment for infants
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spray
ITNs	Insecticide – treated nets
IV	Intravenous
KMIS	Kenya Malaria Indicator Survey
KNBS	Kenya National Bureau of Statistics
LLINs	Long-lasting insecticides nets
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
µ	Micron
mL	Milliliter

mm	Millimeter
mM	Millimolar
nm	Nanometer
PK 1	Unknown Peak 1
PK 2	Unknown Peak 2
PCR	Polymerase chain reaction
RBCs	Red Blood Cells
RDT	Rapid Diagnostic Testing
RSD	Relative standard deviation
SMC	Seasonal malaria chemoprevention
SP	Sulphadoxine – pyrimethamine
TM	Traditional medicine
v/v	Volume by volume
w/w	Weight by weight
WHO	World Health Organization

ABSTRACT

Background:

Malaria is a common and life-threatening parasitic disease in many tropical and subtropical areas especially the Africa region where about 90 % of the global malaria cases were recorded. In 2005, World Health Organization announced a switch in strategy to artemisinin combination therapy which is currently being used widely and is saving many lives. With the numerous potential uses of artemisinin and its derivatives, the future demand for the compound continues to rise. Chemical synthesis of artemisinin is an expensive and difficult multistep process that is accompanied by low yield thus *Artemisia annua* plant remains a major and economically viable commercial source of artemisinin.

Objectives:

The main objective of this study was to develop and validate a high performance liquid chromatography method that is suitable for analysis of artemisinin content in crude extracts of *A. annua* L. plant cultivated in different parts of Kenya. This is important since current methods used for analysis are only suitable for the analysis of artemisinin bulk material.

Method:

The content of artemisinin in the extracts was determined using high performance liquid chromatography method that was developed for the separation of the artemisinin from other components present in the crude extracts. The powdered leaves of the plant were subjected to various extraction conditions to determine the optimal extraction of artemisinin. The leaves of *Artemisia annua* L. were collected from various places in Kenya, namely; Malakisi, Emusaga, Kehancha, Kitengela and Kenyatta University.

Results:

The optimized chromatographic conditions for determining the content of artemisinin were a mobile phase consisting of acetonitrile-0.05 M monobasic potassium phosphate (40:60 % v/v), with 0.005 M hexanesulphonate, pH 6.0 delivered through the HPLC system at a flow rate of 1.0 mL/min. The stationary phase was a reverse phase octadecylsilane (C_{18}) column maintained at a temperature of 40 °C. The diluent used to dissolve the extracts was acetonitrile. The method was validated for specificity, precision, and linearity according to the International Conference on Harmonization guidelines where the linear regression data analysis for the calibration plots showed good linear relationship in the concentration range of 25-120 % with respect to the peak area. Highest extraction content of artemisinin was found when the plant powder was subjected to cold maceration while stirring for 12 hours. The content of artemisinin was found to range from 0.68 % to 0.89 % with highest content found in the plant grown in Emusaga.

Conclusion and recommendations:

The method developed offers the advantage of being able to quantify artemisinin content in crude extracts of *Artemisia annua* L. plant using solvents and reagents that are easily available in the market. The HPLC method developed was precise and suitable for routine analysis of crude extracts of the plant. The study also showed that artemisinin content varies from region to region. This method was being reported for the first time and can be used for routine quality control analysis of the crude extracts of the plant.

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1 Background of malaria

Malaria is a common and life-threatening parasitic disease in many tropical and subtropical areas especially the Africa region where about 90 % of the global malaria cases were recorded (World Health Organization, 2018). The malaria incidence declined steadily from 76 to 63 cases per 1000 population risk between 2010 and 2016 representing 18 % global decline (World Health Organization, 2017). The 2017 World Malaria Report estimated that 445 000 deaths had occurred globally in the year 2016, of which an estimated 91 % were in the WHO Africa Region including Kenya. In Kenya, malaria is still a major cause of morbidity and mortality with more 70 % of the population at risk of the disease (Head *et al*., 2015).

In countries with high levels of malaria transmission, the economic growth is affected due to factors like worker productivity, absenteeism, medical costs and premature mortality (Sachs and Malaney, 2002). In some African countries, malaria may reduce the potential economic growth rate by 1.3 % per year (Gallup and Sachs, 2001). The disease may account for up to 40 % of public health expenditures, up to 50 % of inpatient admissions in the hospitals, up to 60 % of outpatient visits in many countries. Malaria unjustifiably affects poor people who cannot afford treatment or have limited access to health care, which then traps families and communities in a downward spiral of poverty (World Health Organization, 2016).

1.2 Malaria transmission

There are four species of Plasmodium that cause human malaria namely; *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale. P. falciparum* and *P. vivax* are the most common with *P. falciparum* being the most deadly.

Malaria is transmitted through the bites of the female *Anopheles* mosquito (World Health Organization, 2018). The principal vectors of malaria parasites in Kenya are members of the *Anopheles gambiae* complex and *An. funestus* (Okara *et al*., 2010).

The malaria parasite has a complicated life cycle that needs both a human host and an insect vector as shown in figure 1.1. Malaria in humans takes place in two phases, an exo - erythrocytic and an erythrocytic phase. The exo – erythrocytic phase consists of infection of the hepatic system or liver, whereas the erythrocytic phase consists of infection of the erythrocytes or red blood cells (Centres for Disease Control and Prevention, 2018). The sporogenic cycle takes place in the mosquito. Humans are the intermediate host for the parasite, while the mosquito where the sexual reproduction takes place, is the definitive host (Miller *et al*., 2002).

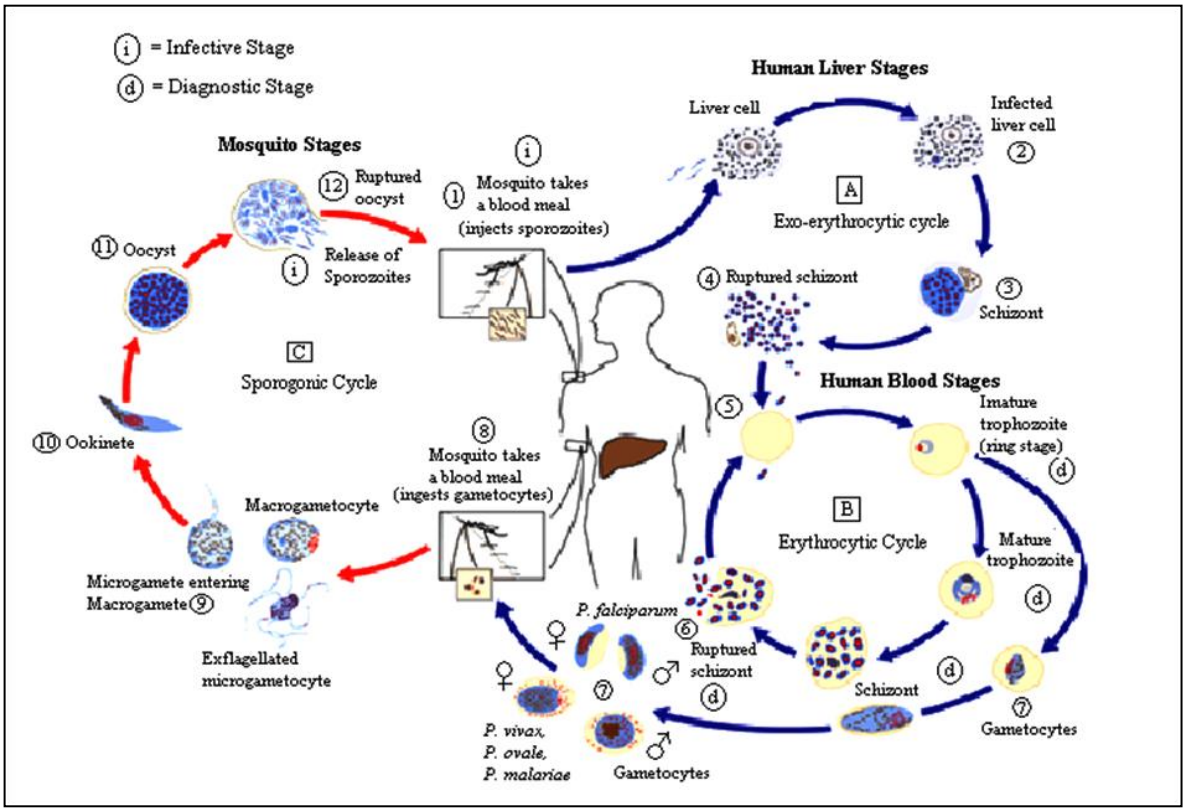


Figure 1.1: Illustration of the life cycle of malaria parasite, *Plasmodium* spp.

Source: Centre for Disease Control and Prevention, 2016.

The female *Anopheles* mosquito bites an infected patient, sucks blood infected with gametocytes. The sporozoite (1) then infect the liver cells (2) and matures into schizonts (3). The schizonts rupture and release merozoites into the bloodstream (4) (Centres for Disease Control and Prevention, 2018). Some of the *P. vivax* and *P. ovale* sporozoites may produce hypnozoites which remain dormant for periods ranging from six months to three years and are responsible for the late relapses as they get reactivated and produce merozoites (Miller *et al*., 2013). After replication in the liver; exo-erythrocytic schizogony (A), the parasites undergo asexual multiplication in the erythrocytes; erythrocytic schizogony (B). Merozoites released from the liver upon rupture of schizonts rapidly invade RBCs, where they grow by utilizing hemoglobin (5). Most of the parasites differentiate into sexual erythrocytic stages (gametocytes) (7). High fevers in infected patients may be experienced as the merozoites are released which then infect the red blood cells. The gametocytes, male (microgametocytes) and female (macrogametocytes) circulating in the person's bloodstream, are ingested by the *Anopheles* mosquito during a blood meal (8) and the parasite multiplies (sporogenic cycle – C). In the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9) which then become motile and elongated ookinetes (10) and invade the midgut wall of the mosquito where they develop into oocysts (11). These oocysts grow, rupture and release sporozoites (12) then make their way to the mosquito's salivary glands making it infective and hence are transferred to a new host when the mosquito bites again (1) (Coulibaly *et al.,* 2009; Miller *et al*., 2013)

1.3 Epidemiology of malaria

Malaria transmission is depends on the climatic factors like temperature, humidity, and rainfall patterns, hence climate will affect survival of both parasites and mosquitoes. In tropical and subtropical areas, the climate is conducive for the *Anopheles* mosquitoes to

survive and multiply hence high malaria transmission (World Health Organization, 2018). In Kenya, the malaria prevalence varies by season and also across the geographic which then divides the country into four epidemiological zones – Endemic areas, Highland epidemic – prone areas, Seasonal malaria transmission areas and Low malaria risk areas (Kenya Malaria Indicator Survey, 2015).

Nationally the malaria prevalence has reduced to less than 10 % where the prevalence among children aged 6 to 59 months has decreased from 8 % in 2010 to 5 % in 2015 (Kenya Malaria Indicator Survey, 2015). However, malaria prevalence continues to be high in the lake epidemic zone compared to other epidemiological areas even though the rate among children aged 6 months to 14 years was 27 % in 2015, which is a decrease from 38 % in 2010 (Kenya Malaria Indicator Survey, 2015).

The reduced burden of malaria in Kenya has been observed due to aggressive efforts to scale up malaria control measures, which has lessened the malaria transmission intensity in most parts of the country. Despite these efforts, moderate to high levels of transmission continue to persist in the endemic zones (Head *et al*., 2015).

1.4 Prevention of malaria

One of the methods used in prevention of malaria is the use of insecticide-treated nets (ITNs) (Head *et al*., 2015). These nets are known to reduce malaria mortality rates by approximately 55 % in children under 5 years of age in sub-Saharan Africa and when used by pregnant women, they have been shown to be effective in reducing maternal anaemia, placental infection and preventing low birth weight (World Health Organization, 2017).

In pregnant women and young children, chemoprevention has been found to be effective. The administration of sulphadoxine-pyrimethamine (SP) while on the second and third trimester,

also known as intermittent preventive treatment in pregnancy (IPTp), has lessened the incidences of severe maternal anaemia, low birth weight and perinatal mortality. In children aged 3 - 59 months living in zones of highly seasonal malaria transmission, seasonal malaria chemoprevention (SMC) with amodiaquine plus SP (AQ + SP) may prevent millions of cases and avoid thousands of deaths. SMC maintains the therapeutic antimalarial drug concentration in the blood for longer periods. Intermittent preventive treatment for infants (IPTi) with SP, which is given at the normal childhood immunization clinics, acts as protection for the first year of a child's life against clinical malaria and anaemia and also leads to decreased hospital admissions for infants with malaria (World Health Organization, 2017).

In majority of the malaria-endemic countries including Kenya, the WHO's policy has been adopted which means that all patients with suspected malaria should be tested. This means that patients in epidemiological settings with suspected malaria must be examined for evidence of infection with malaria parasites through either microscopy or Rapid Diagnostic Testing (RDT) (World Health Organization, 2015).

1.5 Pharmacotherapy of malaria

Treatment for malaria is usually guided by the infecting Plasmodium species, the clinical status of the patient and the drug susceptibility of the infecting parasite that depends on the geographic zone where the infection took place and the history on the use of the antimalarial medicine (Centres for Disease Control and Prevention, 2018).

The two main principles of malaria management are early diagnosis and prompt treatment. The diagnosis depends on the clinical symptoms and the detection of specific parasite antigens in the blood using microscopy or rapid diagnostic tests (RDTs). Other detection methods are the detection of antibodies to malaria parasite and the detection of the parasite DNA based on

the polymerase chain reaction (PCR). The duration of the infection may be reduced as well as prevention of complications and mortality may happen especially if effective treatment is administered early. Currently, it is advocated that diagnosis of malaria be confirmed in all age-groups of patients in all epidemiological settings which means that disease management should not only be viewed as a component of malaria control but also a right for all populations at risk. The prescribed first line treatment for uncomplicated malaria in Kenya is artemether-lumefantrine combination (AL) while the prescribed second line treatment is dihydroartemisinin-piperaquine combination (DHA-PPQ). In severe malaria, it is recommended that one uses parenteral quinine or parenteral artemisinins (artesunate or artemether) (World Health Organization, 2015; Kenya Malaria Treatment Guidelines, 2016).

1.6 Drug resistance in malaria

Drug resistance in malaria as defined by World Health Organization (WHO) is the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a medicine given in doses equal to or higher than those usually prescribed but are within the tolerance of the subject and as long as the drug exposure at the site of action is satisfactory (World Health Organization, 2015).

Resistance to the commonly used antimalarials like amodiaquine, mefloquine, quinine and sulfadoxine-pyrimethamine has been reported for *P. falciparum, P. vivax,* and *P. malariae* (World Health Organization, 2017). Artemisinin resistance has been reported in five countries in the Greater Mekong sub region; Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam (Miao *et al*., 2013; World Health Organization, 2015).

The most successful attempts so far to curb the spread of resistance have been in the administration of combination therapy, which is the simultaneous use of two or more blood

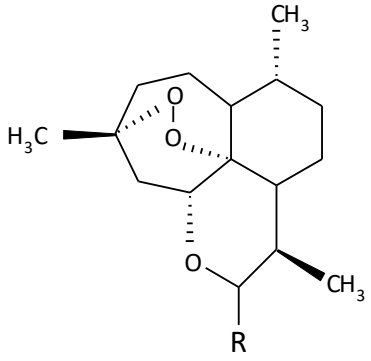
schizonticidal drugs with distinct and different modes of action and different biochemical targets in the parasite (World Health Organization, 2017). The current mainstay of antimalarial combination therapy is the artemisinin-based combination therapy (ACT).

1.7 Artemisinin and artemisinin-based combination therapy

Artemisinin, a sesquiterpenoid lactone peroxide has been shown to be adequate in killing the malarial parasites. The endoperoxide bridge is mainly responsible for the antimalarial activity and is involved in the formation of free radical intermediates resulting from interaction of the endoperoxide moiety with heme (Meshnick *et al*., 1996; Dahnum *et al*., 2012).

Artemisinin is more effective, rapid and less harmful than chloroquine in treating malaria. Chemical modifications of artemisinin have resulted in new derivatives with improved antimalarial efficacy and aqueous solubility (Njuguna, *et al*., 2012)

Different derivatives of artemisinin have been processed by reduction of the lactone carbonyl to give dihydroartemisinin followed by the formation of ether or ester derivatives as shown in figure 1.2. The methyl ether, artemether, is soluble in oil and is administered orally or through intramuscular (IM) injection while the ester salts such as sodium artesunate are water soluble and may be administered orally or by intravenous (IV) injection.



R	Name of compound
=O	Artemisinin
-OH	Dihydroartemisinin
$-OCH_3$	Artemether
$-OCH_2CH_3$	Arteether
$-OCOCH_2CH_2COOH$	Artesunate

Figure 1.2: Structures of artemisinin and its derivatives.

Artemisinin is reduced to dihydroartemisinin in the body. This is the active metabolite and is the most effective artemisinin compound, least stable and has a strong blood schizonticidal action which reduces gametocyte transmission hence used for therapeutic treatment of cases of resistant and uncomplicated falciparum malaria (World Health Organization, 2015).

Artemether, which is similar to artemisinin in mode of action, is a methyl ether derivative of dihydroartemisinin. It is has been shown that it acts greatly to decrease gametocyte carriage (World Health Organization, 2015).

Arteether is an ethyl ether derivative of dihydroartemisinin and is useful in combination therapy for cases of uncomplicated resistant *P. falciparum*. The compound is administered via intramuscular (IM) injections. No side effects have been recorded except for a few cases of neurotoxicity following parenteral administration (World Health Organization, 2015).

Artesunate which is the hemisuccinate derivative of the active metabolite dihydroartemisinin, is regularly used for all the artemisinin-type drugs. It is mainly used in combination therapy and is effective in cases of uncomplicated *P. falciparum*. Artesunate is formulated as a powder of sodium artesunate whose solution is diluted in about 5 ml of 5% dextrose and administered by intravenous injection or by intramuscular injection and should be administered freshly (Mermel *et al*., 2009; World Health Organization, 2015).

Artemisinin-based combination therapies (ACTs) are combinations in which one of the components is artemisinin or its derivatives (artesunate, artemether, arteether and dihydroartemisinin). The artemisinins accelerate the clearance of parasitaemia and resolution of symptoms through decreasing the parasite numbers by 100- to 1000-fold per asexual cycle of the parasite. The artemisinin and its derivatives are rapidly cleared from the blood stream and hence a 7-day course of treatment with an artemisinin compound is indicated. This long

duration of treatment with the artemisinins has been reduced to three days when combined with long acting drugs. The clearance of the parasites depends on the partner medicine being effective and persisting at parasiticidal concentrations until all the infecting parasites have been killed. The partner medicine may also protect the artemisinin component from resistance. Artemisinins have also been shown to reduce gametocyte carriage and hence malaria transmission, which is an added advantage from a public health perspective (World Health Organization, 2015).

Artemisinin-based combination therapies (ACTs) are advocated for treating uncomplicated malaria caused by *P. falciparum* and are thus used in preference to amodiaquine plus sulfadoxine-pyrimethamine (World Health Organization, 2017). The choice of these drugs varies from country to countr y and is dependent on the level of resistance to the constituents in the combination in that particular country. The ACTs commonly used for treatment of malaria are shown in Table 1.1.

Table 1.1: WHO-recommended ACTs medicines for the treatment of malaria (World Health Organization, 2015).

Active pharmaceutical ingredient(s)	First - Line treatment	Second - Line treatment
Artemether / lumefantrine	√	√*
Artesunate / amodiaquine	√	
Artesunate / mefloquine	√	√*
Artesunate / sulfadoxine-pyrimethamine	√	
Dihydroartemisinin / piperaquine	√	√*

* Only if not used as first-line treatment

The adoption of artemisinin-based combination therapies (ACTs) as an alternative to monotherapies against chloroquine-resistant malaria is mainly in 56 countries in Africa, Asia, and South America. The fixed dose combination ACTs, with two medicines combined in the

same tablet are most preferred due to improved patient adherence (World Health Organization, 2017).

The clinical and parasitological efficacy of ACT have not been compromised despite changes recorded in parasite sensitivity to artemisinins in the greater Mekong sub region. However, the effectiveness of both drug components of the combinations is still at risk. Using artemisinin with an ineffective partner medicine increases the risk for the development or spread of artemisinin resistance and hence if the effectiveness of the artemisinin component is lost, the effectiveness of the partner drug may be threatened (Winzeler and Manary, 2014).

1.8 The *Artemisia annua* L. plant

Artemisia annua L. (Asteraceae), also known as Sweet wormwood, Sweet annie, Sweet sagewort, Annual wormwood or *qīnghāo* in Chinese, is a type of wormwood that is native to temperate Asia, but is grown throughout the world (Hayat *et al.*, 2009).

The plant is an annual aromatic weed. It has fern-like leaves, bright yellow florets, and a camphor-like scent. It has a single, erect, ribbed and brownish or violet-brown stem, and naturally grows to 30–100 cm high but the cultivated plants may reach 200 cm high (figure 1.3). It has alternating branches and alternating leaves which range from 2.5-5 cm in length and is cross - pollinated by wind or insects (Willcox *et al*., 2004).

The plant also has tiny yellow nodding flowers or capitula of only 2 or 3 mm across that are displayed in close panicles containing numerous, greenish or yellowish, bisexual central (disc) florets containing little nectar and pistillate marginal (ray) florets (figure 1.3) The involucre is imbricated with several rows of bracts. The central flowers are perfect and can be either fertile or sterile. The ovaries are inferior and unilocular and each generates one achene of about 1 mm in length and faintly nerved. The pistillate marginal florets in the capitulum

produce numerous achenes without pappus. The pollen is tricolpate and smooth, typical of anemophilous species and has vestigial or no spines (Wetzstein *et al*., 2014).

Artemisia annua L. is a strongly fragrant, annual herbaceous plant and is the natural botanical source for artemisinin (*Qinghaosu*) and also the potential source for essential oils in the perfume industry.

(a) (b)

Figure 1.3: Photos of the *Artemisia annua* L. plant:
(a) *Artemisia annua* as grown in the field, (b) Inflorescence and leaf arrangement of *Artemisia annua*.
Source: Photo taken in Kitale farm, December 2010 by SWM

The plant was used by Chinese herbalists in ancient times to treat fever and was rediscovered in 1970s when the Chinese Handbook of Prescriptions for Emergency Treatments was found. This pharmacopeia contained recipes for a tea from dried leaves prescribed for fevers (Hayat *et al.*, 2009). In 1971, Chinese scientists isolated the sesquiterpene lactone, artemisinin and showed that it was effective against *P. falciparum*. Clinical trials later revealed that the drug was potent in treating malaria including chloroquine – resistant malaria and the fatal complication of cerebral malaria (Willcox *et al.*, 2004).

Various studies done earlier have shown that the plant has also been used traditionally to treat bacterial and parasitic infections. Artemisinin and its derivatives have demonstrated to be effective against a number of viruses, a variety of human cancer cells and several tropical diseases like schistomiasis, leishmaniasis, trypanosomiasis and some livestock diseases (Massiha *et al*., 2013; Kooy and Sullivan, 2013; Weathers *et al*., 2014).

The plant has been shown to contain other phytochemicals which include tannins, flavonoids, cardiac glycosides, alkaloids, phenols and resins especially the ethanolic extracts of the plant (Owuna *et al*., 2013). The combined use of flavonoids in treatment of malaria increases the effectiveness of artemisinin and has been shown to chelate metals such as iron and copper as part of the antioxidant activity (Ferreira *et al*., 2010).

1.9 Extraction and isolation of artemisinin

Artemisinin (Qinghaosu) was isolated and characterized in China in the early 1970s and the successful application of Qinghaosu (artemisinin) and its derivatives for treating several thousand malaria patients in China attracted worldwide attention in the 1980s (Ridder *et al*., 2008).

The artemisinin compounds are mainly found in the aerial of the *Artemisia annua* plant, where the concentration of artemisinin is known to peak just before or during full flowering. The difference in artemisinin concentration may be as a result of different climatic conditions, the plant variety, or other yet undetermined factors. Artemisinin compounds are found in plants aged between 12 to 13 weeks (Dahnum *et al*., 2012).

Industrial extraction of artemisinin in Kenya used to be carried out at East African Botanicals located at EPZ –Athi River upto 2015 when the factory shut down due price flactuations of artemisinin raw material and hence some farmers were unable to sustain production of the

plant (Botanical Extracts EPZ Limited, 2016). At the factory, the dried leaves collected from various regions of Kenya where A. annua L. is grown are milled into powder. Extraction and isolation is then carried out using an in-house method. In this method, the powder is introduced manually into a tank or chamber that is perforated at the base. Hot hexane is added and the leaves are macerated for about 15 - 30 minutes. Fresh hot solvent is then introduced while draining off the extract and ensuring that the leaves are kept submerged at all times. After about 2 hours, all the solvent was drained off. The extract is then drained into another vessel and the extract cooled to about 18 °C to remove the non-polar waxes that are present in the plant material. The solution is then filtered through industrial quartz into another vessel. This tank is heated to 45 °C and the solution passed through silica gel where adsorption of the polar waxes takes place. The extract is purified by passing it through another chromatographic column containing silica gel to separate the artemisinin from the rest of the plant components. The secondary crystals obtained are dissolved in ethanol and activated charcoal added to remove residual waxes. This mixture is transferred to chillers by passing through filters to remove the activated charcoal and is then cooled to about -2 °C. The solution is left for 24 hours to allow the artemisinin to crystallize. Analysis of the artemsinin extract is carried out using high performance liquid chromatography to determine artemisinin content. The crystals are then packed and released into the market. The whole process of extraction and purification takes about 48 hours and is summarized figure 1.4.

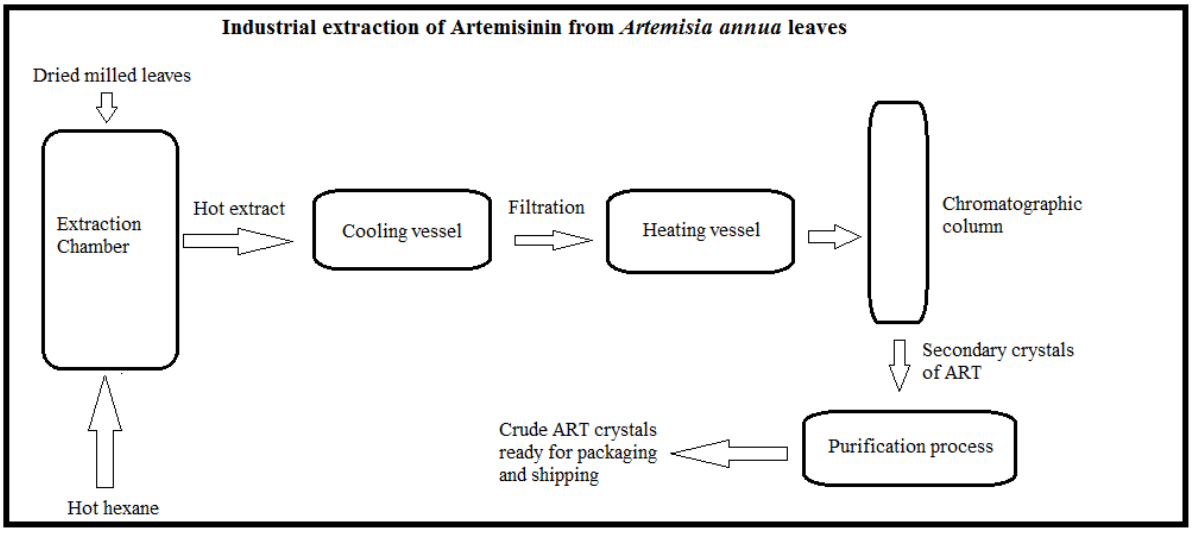


Figure 1.4: Schematic presentation of the industrial extraction of artemisinin for *Artemisia annua* L.

The above process is expensive and depends on the availability of hexane and ethanol. As at January 2018, the industrial production of artemisinin was not taking place at the factory due to supply issues of the artemisinin leaves and the global pricing of the artemisinin.

1.10 Challenges with the supply of artemisinin

The WHO recommended artemisinin-based combination therapy in 2002 and since then, the demand for artemisinin based combination drugs has grown sharply. However, artemisinin being a natural product presents a supply challenge especially due to its low content in the plant which ranges between 0.01 % to about 1.4 % (w/w) based on the weight of the dried leaves of plant grown in the wild (Damtew *et al*., 2011; Elfawal *et al*., 2012; Kooy and Sullivan, 2013).

Efforts were made to boost the production of artemisinin such as plant tissue culture and biotechnological and agronomical practices but artemisinin was found not to accumulate in callus and cell suspension cultures. Synthetic biology was used to assemble a biosynthetic pathway from the genes of the plant and other organisms into microbes to produce artemisic acid which is a precursor to artemisinin synthesis. However, a lot of work and studies still

need to be carried out for this process to be cost effective (Hale *et al*., 2007, Jelodar *et al*., 2014).

The plant is relatively easy to grow in the temperate and subtropical climates, however the relatively low yields lead to high cost of production in terms of extraction and purification which mainly rely on methods that use organic solvents like hexane and petroleum ether which are potentially unsafe and also environmentally damaging (Elfawal *et al*., 2012, Hale *et al.*, 2007).

1.11 Research justification

Artemisinin is a starting compound for the semi-synthesis of a group of highly active antimalarial drugs namely artesunate, artemether and dihydroartemisinin, which are presently used as components of artemisinin-based combination therapies (ACT). The derivatives can be obtained through chemical synthesis, recombinant DNA techniques and isolation from natural materials (Hommel, 2008). The method of isolation from natural materials has been found to be the most economical way to obtain artemisinin. Research carried out by solvent extraction using hexane obtained yields of between 0.01 % to 1.4 % (Damtew *et al*., 2011; Dahnum *et al*., 2012).

The demand for artemisinin and its derivatives is on an upward trend due to their numerous potential uses. Chemical synthesis of artemisinin is an expensive and difficult multistep process that is accompanied by low yield and thus *A. annua* plant remains the only major and economically viable commercial source of artemisinin.

The viability of cultivating the crop however depends on the costs, returns and profitability of *Artemisia* cultivation in comparison to alternative cash crops. This pricing is determined by artemisinin content in the leaves of the plant. Unfortunately, the complexity of extraction and

analysis of the accurate artemisinin content in the plant increases production costs thus hard to apply on large scale.

Current methods available for the analysis of artemisinin are only suitable for the analysis of the bulk artemisinin hence the need to develop of a High Performance Liquid Chromatography (HPLC) method that is suitable for the analysis of crude extracts of the plant.

1.12 Objectives

1.12.1 General objective

The main objective of this study was to develop and validate a High Performance Liquid Chromatography (HPLC) method that is suitable for analysis of artemisinin content in crude extracts from *Artemisia annua* leaves.

1.12.2 Specific objectives

The specific objectives of this study were to:

1. To develop and validate an analytical method for analysis of the artemisinin content in crude *Artemisia annua* L. plant.
2. To determine the artemisinin content in *Artemisia annua* L. cultivated in different regions of Kenya.

CHAPTER TWO:
DEVELOPMENT OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

2.1 Introduction

High performance liquid chromatography (HPLC), particularly reversed phase HPLC, is the most popular analytical technique in the pharmaceutical industry because of its distinctive advantages like short analysis time, high specificity, fast separation technique with wide choice of mobile phases and versatility to analyze a wide variety of samples (Siddiqui *et al*., 2017). In addition, HPLC is widely used for analysis and profiling of herbal products (Patel and Patel, 2016).

Existing methods used to analyze artemisinin require that the plant extract be purified before analytical work can be carried out (WHO GACP, 2006; WHO Ph. Int., 2006). Previous studies have also shown that artemisinin could be analyzed using either high performance liquid chromatography with electrochemical detection (HPLC-EC) or with gas chromatography, (Ferreira *et al*. 1994). These techniques are expensive and not easily available. Quantification of artemisinin from *A. annua* extraction has also been carried out using proton nuclear magnetic resonance (H-NMR) where the yields obtained were found to range from 0.77 to 1.06 % (Castilho *et al*. 2008). However, this approach is also very expensive.

The analysis of artemisinin in the crude plant material is difficult since it is present in very low concentration, is thermolabile, is acid sensitive and does not have UV chromophoric functional groups. TLC was found not be a reliable technique for quantification of artemisinin owing to the poor staining characteristics of the intact molecule and interference from other constituents of the plant (Castilho *et al*., 2008).

Analysis of *Artemisia annua* extracts by liquid chromatographic methods is also made difficult by the presence of high quantities of inteferents which often remain as solid residue in the extract (Pilkington *et al*., 2012).

The method thus developed in this study should overcome the shortcomings of existing techniques and still be accurate, reproducible, efficient, robust, cost-effective and reliable.

2.2 Experimental

2.2.1 Reagents, chemicals and solvents

Analytical grade hexane (Sigma Aldrich chemie GmbH, USA) was used for extraction of artemisinin from the powdered leaves.

Analytical grade hexanesulfonic acid sodium salt, octanesulfonic acid sodium salt (Fisher Scientific UK Ltd., Leicestershire, UK), monobasic potassium phosphate (Merck PTY Ltd., Gauteng, South Africa), dibasic potassium phosphate, potassium hydroxide (RFCL Ltd., New Delhi, India) were used during method development.

Artemisinin (ART) working standard (99.9 % w/w) was a kind donation from East African Botanicals (Nairobi, Kenya).

Acetonitrile HPLC grade (Rankem, Avantor Performance materials Ltd., Maharashtra, India) was used to as solvents for samples and standard as well as in the preparation of mobile phases. All aqueous solutions were prepared using purified water prepared in the laboratory using an Arium RO and Arium VF water system (Satorius AG, Göttingen, Germany).

2.2.2 Instrumentation

A hammer mill fabricated by Muharata food company (Nairobi, Kenya) was used to grind the dried leaves. The hexane extracts were reduced using a Rotary Vacuum Evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a cooler (Poyscience, Niles, USA), a WB2000 water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany). The reduced samples were stored in a refrigerator maintained at 2 – 8 °C.

A D – 7000 Merck-Hitachi high performance liquid chromatographic system (Hitachi High Technologies Corporation, Tokyo, Japan) was used for chromatographic experiments. It consisted of a D-7000 interface with UV detector L-7400, an L-7200 auto sampler and L-7100 low pressure gradient pump. The temperature of the column was controlled using L-7350 column oven. The LC system was controlled via computer using HSM 7000 version 2.0.

All mobile phases were degassed using a MRC DC 200H ultrasonic bath (MRC Ltd., Holon, Israel) before use.

2.2.3 Artemisinin working standard and samples

The artemisinin working standard was dissolved in acetonitrile to obtain concentrations of 1.0 mg/mL. Approximately 100 mg of the crude plant extract was also dissolved in 100 mL acetonitrile.

2.2.4 Mobile phases

The different mobile phases prepared during method development comprised variable proportions of acetonitrile, phosphate buffer at different pH values and water. The buffer was prepared by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4 under magnetic stirring.

A solution of ion-pairing agent was also incorporated into the mobile phase. The various mobile phases were prepared by mixing appropriate volumes of the stock buffer solution and adjusting the pH to the desired value using the molar equivalent solution of the parent acid or buffer salt. Thereafter, the volume of acetonitrile required to yield the desired proportions of the different mobile phase components was measured separately and then added to pH adjusted buffer solution or to a buffer solution also containing the ion-pairing agent. The resultant mixtures were degassed in an ultra-sonic water bath for about 30 minutes.

2.3 Method Development

The chromatographic parameters were fixed at the onset of method development: stationary phase, detection wavelength, flow rate and injection volume. All other chromatographic conditions were varied during method development with emphasis on the composition of the mobile phase. Chromatographic conditions that gave adequate separation of the components and minimum run time were considered optimum.

A Waters XTerra® RP18, 5 µm, 250 x 4.6 mm ID chromatography column (Waters Corp., Wexford, Ireland) was used as the stationary phase. The column brand used is claimed to be stable over an extended pH range of 1 – 12, hence giving room for mobile phase pH manipulation for the improved separation of the components present in the plant.

The UV method of detection has improved accuracy and limits of quantification in comparison to HPLC-ELSD. For this study the UV detection wavelength used was 216 nm (Lapkin *et al*., 2009).

Acetonitrile was chosen as the organic modifier following the preliminary work that favored use of the solvent. The strong eluting power of acetonitrile supported the development of an

isocratic method. Additional advantages include low column back pressures, sharp peaks, low UV absorption wavelength cut off and miscibility with water at all proportions.

The flow rate was fixed at 1.0 mL/min throughout the method development to ensure the column back pressures were maintained at below 150 bar while the injection volume was fixed at 20 µL as a compromise between peak responses and column loading.

2.3.1 Optimization of chromatographic conditions

2.3.1.1 Published methods

The monographs (WHO GACP, 2006; WHO Ph. Int., 2006) described the HPLC method using UV detection at 216 nm. However the method is only suitable for quantification and analysis of artemisinin drug substance and is not suitable for the quantification of artemisinin in extracts due to the very low absorbance of artemisinin (El-Naggar *et al*., 2013).

Based on studies carried out by Lapkin *et al*., (2009); a modified mobile phase consisting of water: acetonitrile (60:40 % v/v) was used as the starting point in method development. The column temperature was maintained at 40 °C. Under these conditions there was poor separation between artemisinin and adjacent unknown peaks.

The unknown peaks adjacent to ART were designated PK 1 and PK 2 for that eluting before and after the artemisinin peak, respectively. These three peaks were used to check the effectiveness of the mobile phase in separating the ART from other components present in the crude sample (figure 2.1).

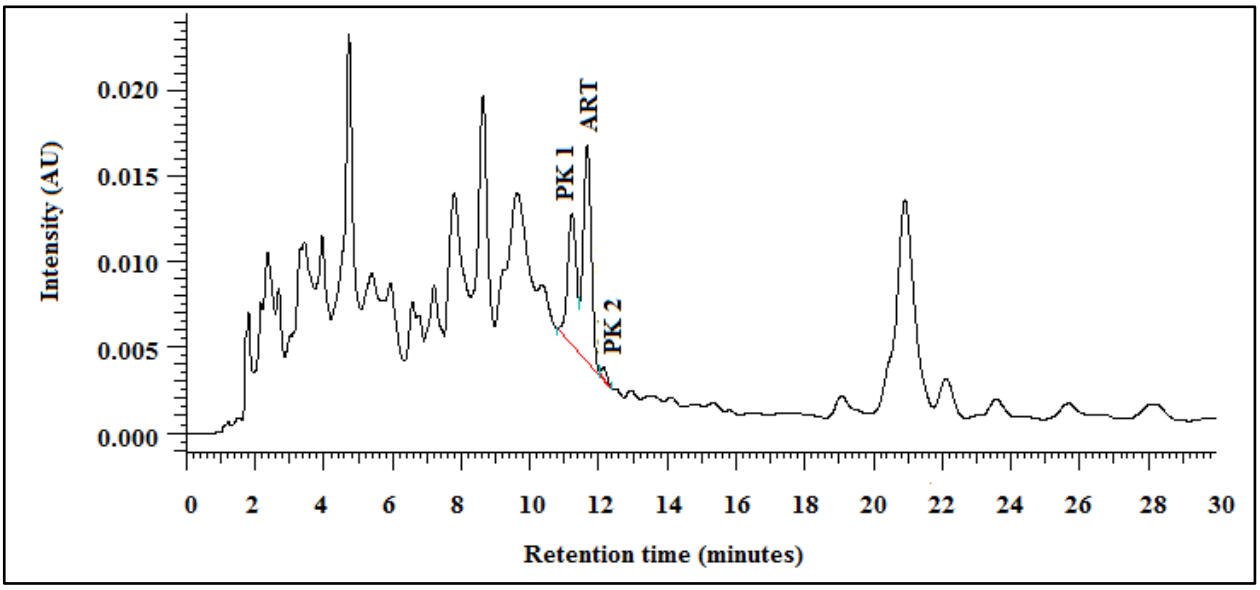


Figure 2.1: **Typical chromatogram of *A. annua* L. leaf extract obtained using unbuffered mobile phase.**
Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-water (40:60, % v/v). ART-artemisinin, PK 1 and PK 2-unknown peaks.

2.3.1.2 Effect of acetonitrile concentration

The effect of acetonitrile was investigated to observe the separation between the unknown peaks and artemisinin. This was done at concentrations of 40, 50, 60 and 70 % v/v of acetonitrile. Separation of the components present in the crude extract was not effective and thus difficult to carry out quantification of artemisinin present in the extract.

The resolution was for ART and PK 2 was calculated with reference to PK 1. Capacity factor, k' is a means of measuring the retention of the analyte in the chromatographic column. A high k' value indicates that the analyte is highly retained and has a long retention time. It was calculated based on a ratio of the retention time of the analyte on the column to the retention time of a non-retained compound which elutes with the solvent front. Table 2.1 and figure 2.2 summarize these effects. Figure 2.1 also shows the chromatogram obtained at the optimum conditions of the effects of acetonitrile concentration.

Table 2.1: Effect of acetonitrile concentration on the retention time, asymmetry, resolution and capacity factors for sample extract.

Mobile phase composition	Compound	Retention Time (min)	Resolution	k'
Acetonitrile - Water (40:60, % v/v)	PK 1	11.23	-	5.6
	ART	11.67	0.96	5.86
	PK 2	12.14	1.02	6.14
Acetonitrile - Water (50:50, % v/v)	PK 1	7.56	-	2.36
	ART	7.90	1.05	2.51
	PK 2	8.35	1.25	2.71
Acetonitrile - Water (60:40, % v/v)	PK 1	5.66	-	1.57
	ART	5.85	1.02	1.66
	PK 2	7.32	1.22	2.33
Acetonitrile - Water (70:30, % v/v)	PK 1	3.24	-	0.9
	ART	3.82	2.11	1.25
	PK 2	4.23	1.37	1.49

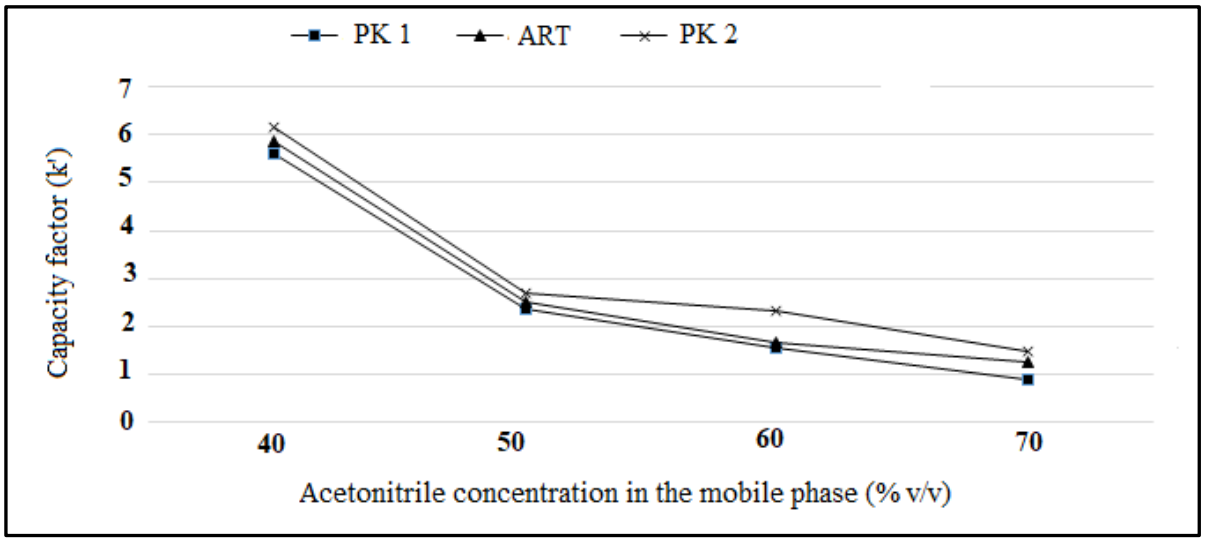


Figure 2.2: Effect of acetonitrile concentration on the capacity factors for sample extract.

Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-water. ART-artemisinin, PK 1 and PK 2-unknown peaks.

2.3.1.3 Effect of inorganic buffer concentration

The most popular buffer used is the phosphate buffer which can be used at wavelengths below 220 nm and concentration of between 10 to 50 mM (Dolan, 2012). Various ratios of the buffer to acetonitrile were investigated and effect of the retention times, asymmetry, resolution and

capacity factor of the unknown PK1 and PK 2 and ART was observed as shown in table 2.2 and figure 2.3.

Table 2.2: Effect of buffer on the retention time, asymmetry, and resolution and capacity factors for sample extract.

Mobile phase composition	Compound	Retention Time (min)	Peak Asymmetry	Resolution	k'
Acetonitrile - 0.05 M KH_2PO_4 (40:60, % v/v)	PK 1	15.23	1.03	-	5.35
	ART	15.73	1.05	0.89	5.55
	PK 2	17.28	2.0	2.36	6.20
Acetonitrile - 0.05 M KH_2PO_4 (50:50, % v/v)	PK 1	8.72	1.10	-	2.82
	ART	9.21	1.11	1.42	2.84
	PK 2	9.83	1.12	1.68	3.10
Acetonitrile - 0.05 M KH_2PO_4 (60:40, % v/v)	PK 1	5.78	-	-	1.48
	ART	5.94	-	-	1.60
	PK 2	7.17	-	2.35	2.14

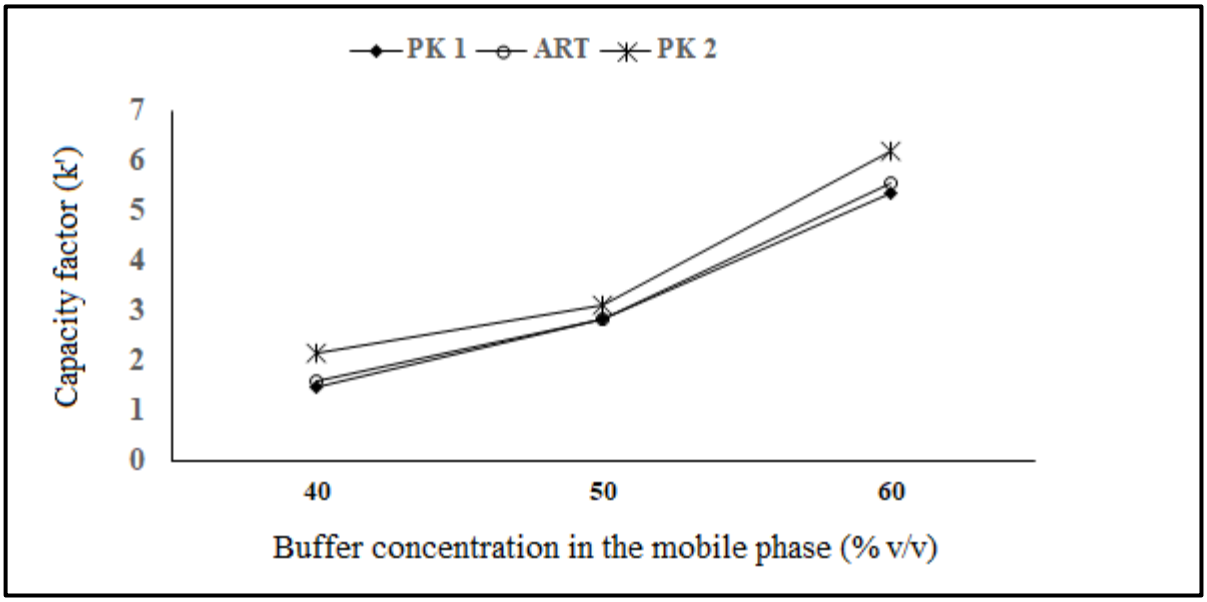


Figure 2.3: Effect of buffer concentration on the capacity factors for sample extract.

Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.05 M potassium phosphate buffer, pH 6.0. ART-artemisinin, PK 1 and PK 2-unknown peaks.

Separation of artemisinin from other compounds in the extract was best achieved at a mobile phase composition of Acetonitrile- 0.05 M KH_2PO_4 (40:60, % v/v) shown in figure 2.4. At this mobile phase composition resolution between ART and unknown PK 2 was achieved. However, resolution between PK 1 and ART was not achieved (figure 2.4).

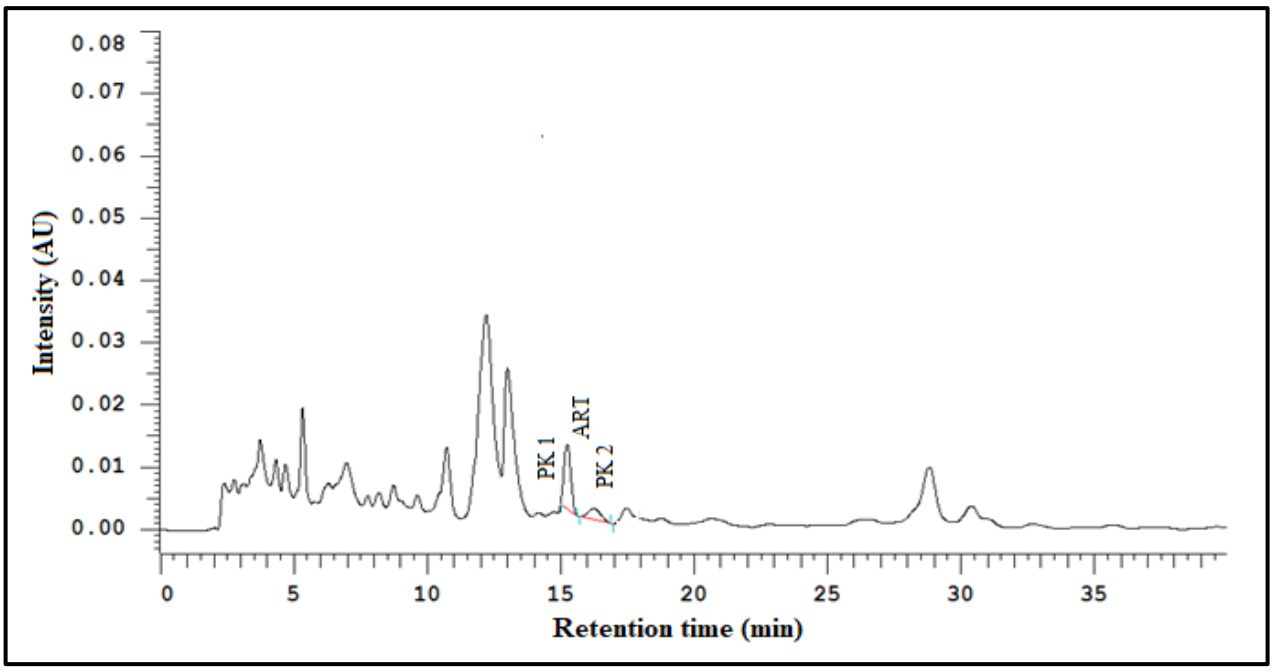


Figure 2.4: **Typical chromatogram of *A. annua* L. leaf extract obtained using buffered mobile phase.**
Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.05 M potassium phosphate buffer, pH 6.0 (40:60 % v/v). ART-artemisinin, PK 1 and PK 2-unknown peaks.

2.3.1.4 Effect of pH on separation

The pH of the mobile phase affects the retention time of ionizable analytes and hence their separation. Three different mobile phase solutions were prepared at pH 4.5, 6.0 and 7.5. The effect on the retention times, asymmetry, resolution and capacity factor of the unknown PK1 and PK 2 and ART was also observed and is summarized in table 2.3 and figure 2.5. It was found that a pH of 6.0 was most effective based of the capacity factors as shown in figure 2.4.

Table 2.3: Effect of buffer pH on the retention time, asymmetry, and resolution and capacity factors for sample extract.

Mobile phase composition	Compound	Retention Time (min)	Peak Asymmetry	Resolution	k'
Acetonitrile - 0.05 M	PK 1	16.25	-	-	4.25
KH_2PO_4 (40:60, % v/v),	ART	16.35	1.08	0.85	4.35
pH 4.5	PK 2	17.25	1.09	1.35	5.52
Acetonitrile - 0.05 M	PK 1	15.23	1.03	-	5.35
KH_2PO_4 (40:60, % v/v),	ART	15.73	1.05	0.89	5.55
pH 6.0	PK 2	17.28	2.0	2.36	6.20
Acetonitrile - 0.05 M	PK 1	14.10	-	-	2.36
KH_2PO_4 (40:60, % v/v),	ART	14.15	1.21	0.82	2.40
pH 7.5	PK 2	15.23	-	1.23	3.51

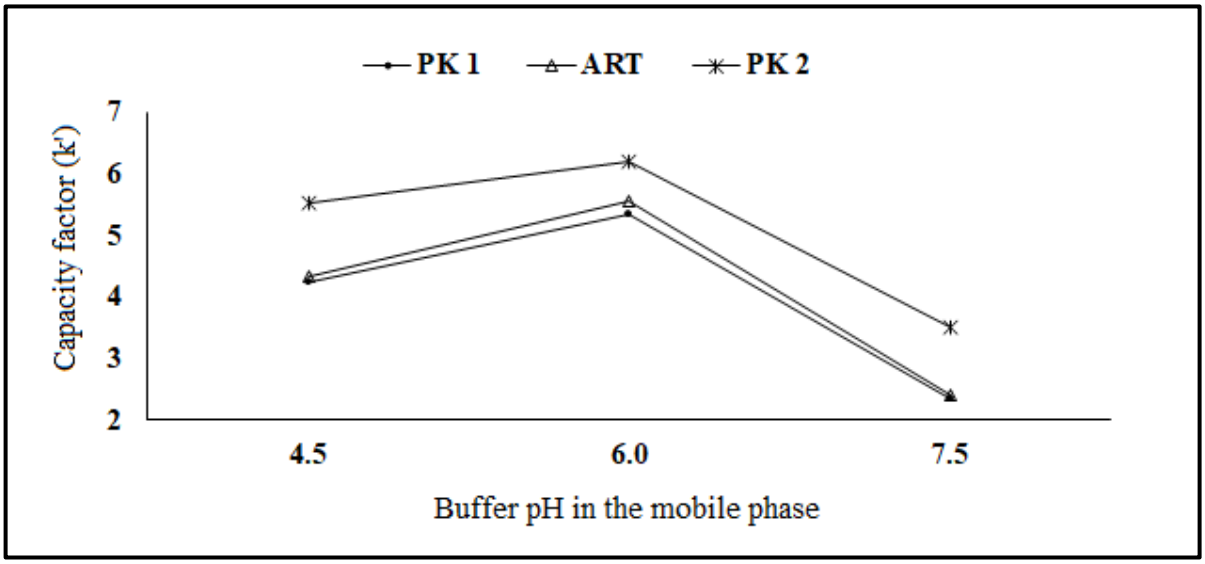


Figure 2.5: Effect of buffer pH on the capacity factors for sample extract.

Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.05 M potassium phosphate buffer (40:60 % v.v). ART-artemisinin, PK 1 and PK 2-unknown peaks.

2.3.1.5 Effect of ion pairing agent.

An ion-pairing agent is a large molecule that carries a charge opposite to that of the analyte of interest and has both a hydrophobic region to interact with the stationary phase and a charged region to interact with the analyte. Its use has been shown to enhance the peak shape and retention time when typical approaches such as modifying mobile phase ratios and buffer pH seem not to be effective. These agents also enhance the separation of ionized and non-ionized

analytes in one run and hence improved separation of the components present in the sample mixture (Meyer, 2010).

The ion-pairing agent depends upon the addition of ionic compounds to the mobile phase to promote the formation of ion pairs with the charged analytes leading to their effective separation. When choosing a suitable reagent, the alkyl chain lengths must be taken into consideration as this enables selective separation of the analyte. The longer the chain, the more hydrophobic the counter ion and hence greater retention. Retention may increase by a factor of almost 20 when going from pentyl to dodecyl (Meyer, 2010). For this study, the chain length selected was hexyl. The separation mechanism is the ion-exchange process where the target analyte undergoes ionic interaction with counter ions that were hydrophobically adsorbed by the stationary phase and is retained. This increases selectivity and the retention time is also affected.

In the present study, the effect of the ion-paring agents was aimed at improving separation of the artemisinin from the rest of the compounds present in the plant. Hexane sulphonic acid sodium salt (HSA) was selected because it is readily available in the Kenyan market.

A mobile phase consisting of acetonitrile-0.05 M KH_2PO_4 pH 6.0, 5 mM sodium hexanesulphonate (40:60 % v/v) was prepared. The effect of sodium hexanesulphonate (HSA) concentration was investigated at 5, 10 and 15m M which are commonly used in laboratory applications. The mobile phase consisting of acetonitrile - 0.05 M KH_2PO_4 pH 6.0 (40:60 % v/v) was selected for incorporation of ion-paring agents as it gave the most effective separation.

Under these conditions, the retention time for artemisinin peak was increased with improved shape and asymmetry. Improved separation of the artemisinin peak from the unknown PK 1 and PK 2 was also observed.

Table 2.4 shows the effect of concentration of sodium hexanesulphonate on the retention time, asymmetry factor and capacity factor while figure 2.6 illustrates the effect on the capacity factors of artemisinin.

Based on studies carried out by Shibue *et al*. (2005), a high concentration of the ion pairing agent has been shown to affect elution of ions present in the samples by increasing the retention time. A concentration of 5 mM was then selected as the optimum ion pairing agent concentration as it offered adequate separation of the artemisinin from the rest of the compounds in the plant and an optimum retention time as illustrated in the chromatogram figure 2.7.

Table 2.4: The effect of hexane sulphonic acid (HSA) concentration on the retention time, asymmetry and capacity factors for sample extract.

Concentration of HSA (mM)	Compound	Retention Time (min)	Peak Asymmetry	Resolution	k'
5.0	PK 1	14.98	1.35	-	5.24
	ART	15.32	0.97	0.87	5.38
	PK 2	16.25	1.25	1.37	5.55
10.0	PK 1	17.25	1.43	-	5.58
	ART	18.23	1.03	0.85	6.59
	PK 2	19.25	1.35	2.35	6.70
15.0	PK 1	17.15	1.52	-	5.25
	ART	17.99	1.19	0.88	6.46
	PK 2	18.78	1.27	1.25	6.64

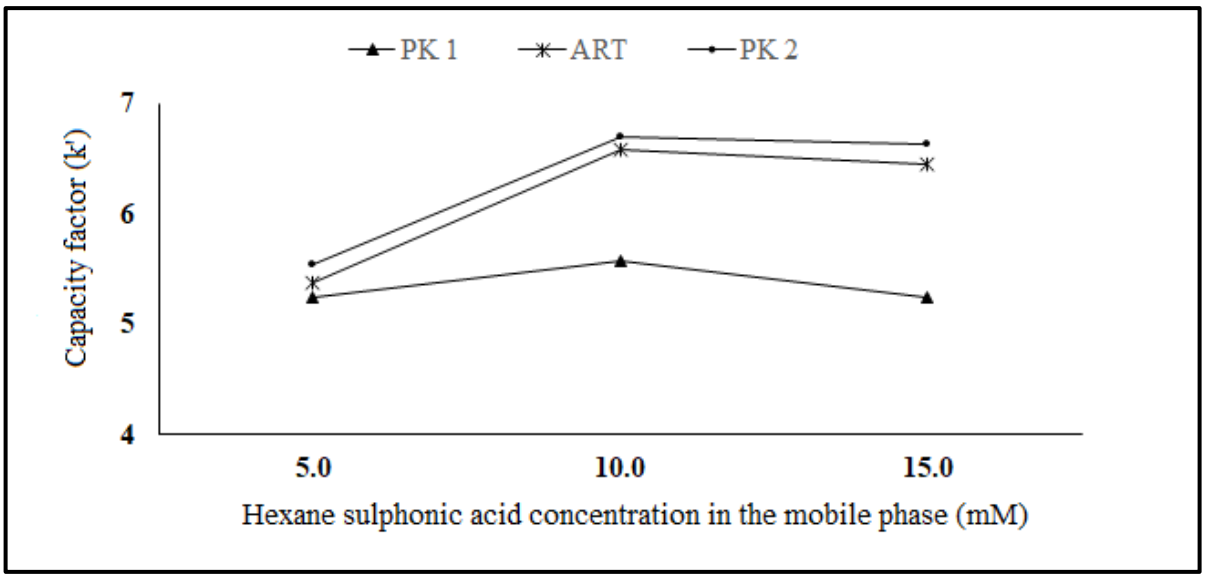


Figure 2.6: Effect of hexane sulphonic acid concentration on the capacity factors for sample extract.

Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.05 M potassium phosphate buffer with hexane sulphonic acid, pH 6.0 (40:60 % v/v). ART-artemisinin, PK 1 and PK 2-unknown peaks.

2.3.2 Optimized chromatographic conditions

Following the investigation of the influence of the various factors on effective separation of artemisinin from the rest of the components present in the crude extract, the following was established as the optimized chromatographic conditions:

A mobile phase consisting of acetonitrile - 0.05 M KH_2PO_4 pH 6.0 (40:60 % v/v), with 0.005 M hexanesulphonate delivered at a flow rate of 1.0 mL/minute; a column Octadecylsilane (C_{18}) 250 mm in length by 4.6 mm internal diameter (Waters XTerra RP18) with particle size 5 µm as the stationary phase and maintained at a temperature of 40 °C; the UV detector was set at a wavelength of 216 nm. Figure 2.7 is a typical chromatogram obtained under these conditions.

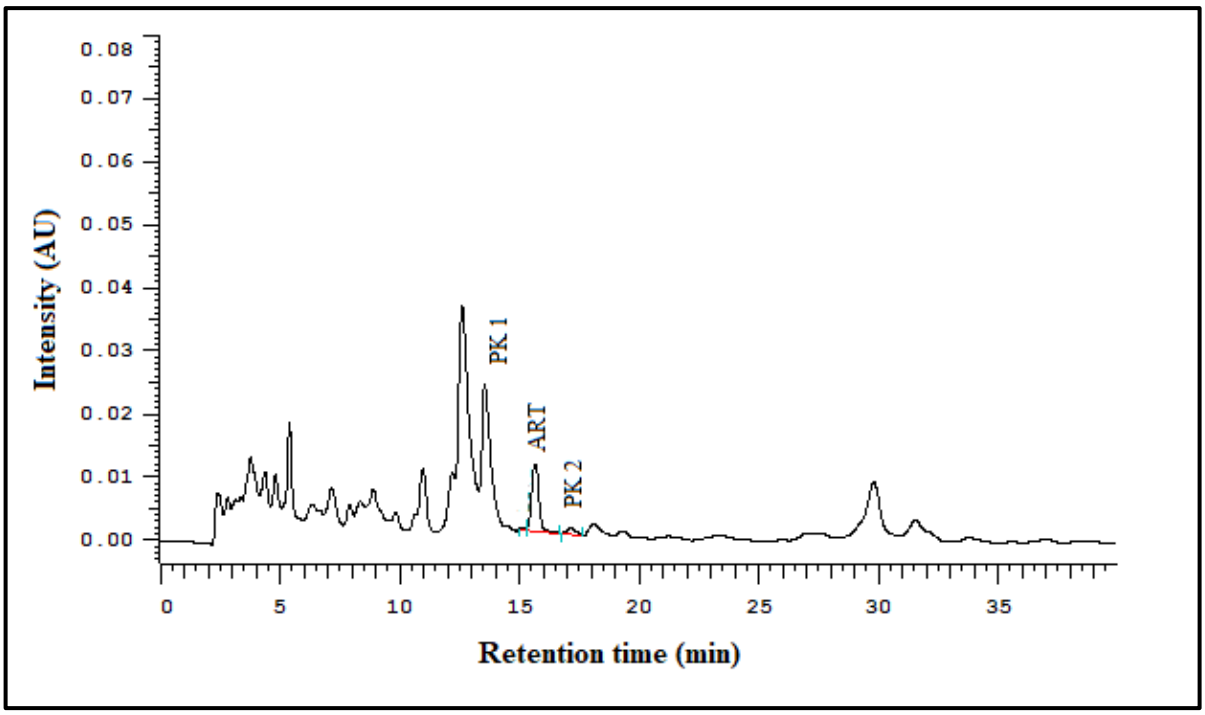


Figure 2.7: **Typical chromatogram of *A. annua* L. leaf extract obtained using optimized conditions.**

Column: XTerra® 5 µm C_{18}, 250 x 4.6 mm; Column temperature: 40 °C; Mobile phase: acetonitrile-0.05 M potassium phosphate buffer with 0.005M hexane sulphonic acid; pH: 6.0 (40:60 % v/v). ART-artemisinin, PK 1 and PK 2-unknown peaks.

CHAPTER THREE:

METHOD VALIDATION

3.1 Introduction

Validation for the analytical method is the process of confirming in the laboratory that the performance characteristics of the method under study or development meets the requirements for the intended analytical applications. The suitability of all test methods should always be verified under the actual conditions of use and should be well documented. Method validation provides the documentary evidence that the analytical method employed is suitable for its intended use. The parameters that require validation depend on the type of applications for the method (Kazusaki *et al*., 2012; Shabir, 2004; ICH, 2005).

Validation of the developed method was performed in line with the ICH requirements for new methods which includes accuracy, precision, robustness, linearity and range, limit of detection (LOD) and limit of quantification (LOQ).

3.2 Preparation of the standard solutions

A standard stock solution of artemisinin with an approximate concentration of 1.25 mg/mL was prepared by dissolving approximately 25 mg of artemisinin working standard (WS) in 20 mL acetonitrile. From this solution, five different concentrations were prepared. The concentrations corresponded to 120, 100, 75, 50 and 25 % as outlined in the ICH guidelines for validation of HPLC analytical methods with 100 % concentration corresponding to 1.0 mg/mL artemisinin in acetonitrile which was used for analysis of the extract. These were run at the optimum chromatographic conditions in triplicate and were used to study linearity and range of the method. For recovery of artemisinin, three solutions of the artemisinin spiked in plant extract at three concentrations (0.8 mg/mL, 1.0 mg/mL and 1.2 mg/mL) were prepared.

The solutions used for recovery study were also used for precision study. The LOD and LOQ determination of artemisinin was determined using solutions of low concentrations.

3.3 Linearity and range

The linearity of an analytical method is defined as its ability to elicit detector response directly proportional to the concentration of analyte in sample within a given range. The range chosen depends on the purpose of the analytical test method. The ICH guidelines on method validation recommend that linearity be demonstrated at a minimum of five concentrations levels over the range of 80 – 120 % of the analytical working concentrations (Kazusaki *et al*., 2012; ICH, 2005). This was determined by preparing a standard stock solution of artemisinin working standard from which working solutions were prepared by diluting appropriately to contain 25 % v/v, 50 % v/v, 75 % v/v, 100 % v/v and 120 % v/v of the working standard solution concentration (ART 1.2 mg/ mL).

Each of the solutions were analyzed and the normalized peak areas obtained were plotted against the concentration in mg/mL. The data obtained was subjected to linear regression analysis with the concentration of artemisinin in mg/mL injected being plotted against the peak areas obtained. The results of the analysis are depicted in figure 3.1. The method was found to be linear over the range of 25-120 % with R^2 of 0.9976.

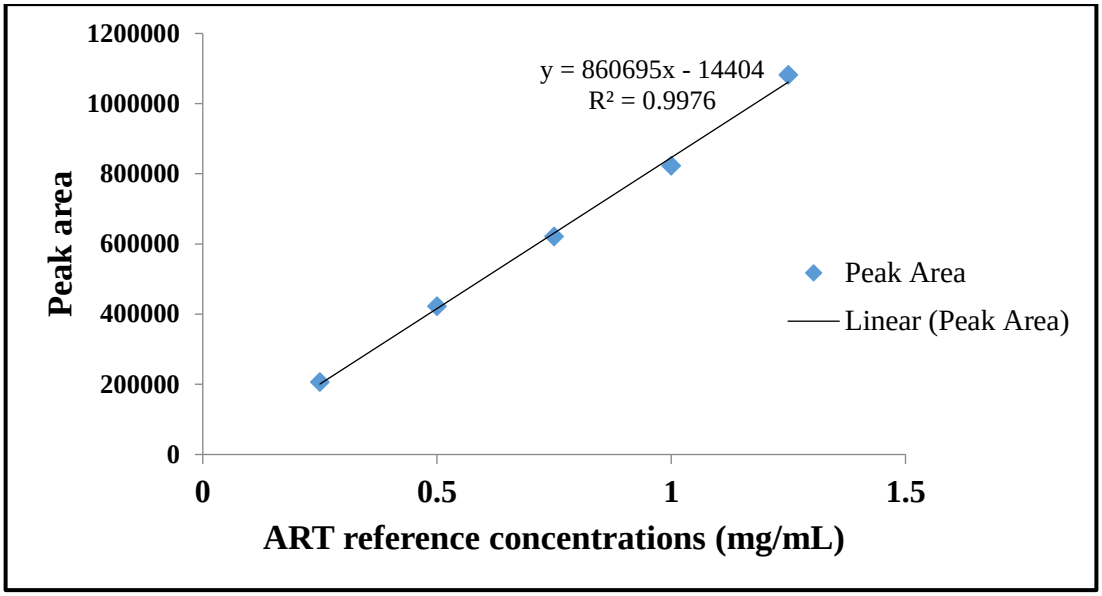


Figure 3.1: Graph showing the linearity of detector response for artemisinin (ART) working standard.

3.4 Accuracy

The accuracy of the analytical method is the closeness of test results obtained by that method to the true value or an accepted reference value. It was measured as the percentage of recovery after spiking artemisinin in an extract. Accuracy was performed at three levels of concentrations in triplicate. To the artemisinin extract three concentrations of know artemisinin working standard was added and percentage recovery was calculated for each of the three levels of concentrations (table 3.1). The relative standard deviation (RSD) of the replicates provided was determined; the mean of the replicates which was expressed as a percentage of the expected true value and this should be within 100 $\pm$ 2.0 % at each level of the concentration (Shabir, 2004; Kazusaki *et al*., 2012). Results showed that the current method had good recovery (between 98.5 % and 100.2 %) for artemisinin at the three different concentrations. The RSD of the percentage recovery calculated was less than 2.0 %. The recovery values obtained are shown in table 3.1.

Table 3.1: Percentage recovery of artemisinin

Concentration (mg/mL)	Mean	RSD (%)
0.8 (80 %)	98.5	0.9
1.0 (100 %)	99.9	0.7
1.2 (120 %)	100.2	1.1

3.5 Precision

Precision which expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under similar test conditions, was carried out at two different levels: repeatability and intermediate precision. The precision of the analytical procedure is usually expressed as the relative standard deviation of a series of measurements.

Repeatability or intra-day assay precision is the results of the method operating over a short time interval under the same conditions. This is usually assessed using a minimum of 9 determinations covering the specified range or a minimum of 6 determinations at 100 % of reference solution. This was obtained by injecting six replicate injections of the working reference solution into the LC system at the optimum chromatographic conditions and determining the relative standard deviation (RSD). This is recorded in table 3.2.

Intermediate precision or inter-day variation establishes the effects of random events on the precision of the analytical procedure which may include different days, different analysts or different equipment. For the purpose of this study, intermediate precision was carried out on three different days. Fresh mobile phase and working reference solution was prepared each day and a minimum of six replicate injections determined. The peak areas for each day were normalized to the desired concentration and the RSD of the normalized peak areas taken as the measure of intermediate precision (Table 3.2).

Table 3.2: **Repeatability and intermediate precision results for artemisinin working standard**.

Compound	Repeatability Peak areas RSD (n = 6)	Intermediate Precision Peak areas RSD (n = 18)
Artemisinin	0.72	1.96

The RSD for the repeatability and intermediate precision tests were less than 1 % and 3 % respectively, that indicates the method has adequate precision.

3.6 Robustness

Robustness measures the capacity of the analytical method to remain unaffected by small intended variations in method parameters and this provides an indication of the reliability of the analytical method during regular usage (ICH, 2005). To determine the robustness of the method, the effect of making small but deliberate adjustments in the optimized chromatographic factors was investigated. The parameters that may be adjusted included mobile phase pH, acetonitrile concentration and column temperature.

In the present study, the influence of changing each of the three chromatographic parameters was tested at three levels which are low (-1), central (0) and high (+1) as shown in table 3.3. The effect of capacity factor (k') of the components are shown in figure 3.2.

Table 3.3: Chromatographic parameter levels for robustness studies.

Chromatographic parameter	Low Level (-1)	Central Level (0)	High Level (+1)
Buffer pH	4.5	6.0	7.5
Acetonitrile concentration (%)	40	50	60
Column temperature (°C)	30	40	60

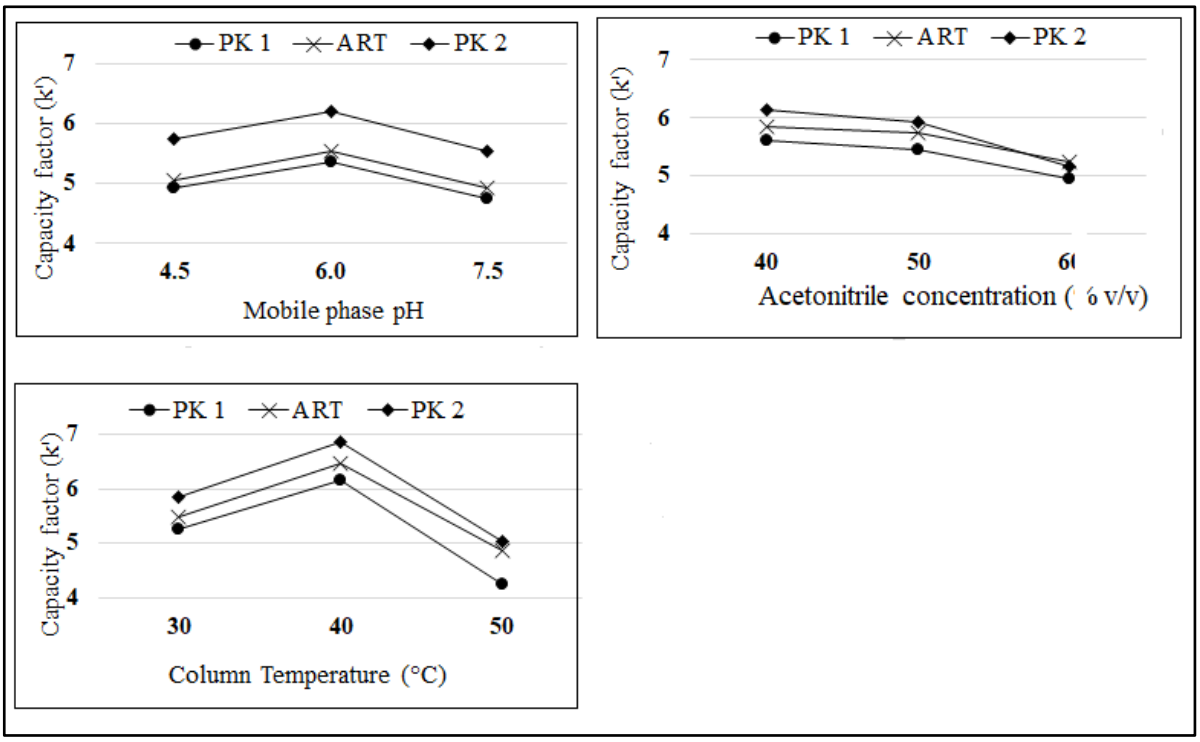


Figure 3.2: **Effect of Buffer pH, acetonitrile concentration and column temperature on the capacity factors for the sample extract.**

The separation of the components were not compromised by changes in mobile phase pH and temperature across the measured ranges while the method was not robust at higher concentrations of acetonitrile in the mobile phase since ART co-elutes with PK 2.

3.7 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) tests are mainly performed on samples containing low concentrations of the analyte. LOD is the lowest amount of analyte that gives a signal to noise ratio of 2 – 3 and is expressed as a concentration (ICH, 2005).

The LOD was determined by injecting the reference working solution in triplicate at different concentrations and calculating the signal to noise ratio. The concentration that gave signal to noise ratio of 2 - 3 was considered to be the LOD.

LOQ is the lowest amount of analyte which can be reproducibly quantified above the baseline noise and is the amount of analyte that gives a signal to noise ratio of 10 (ICH, 2005).

The results showed that the LOD and LOQ of ART are 20 ng and 30 ng. The low LOD and LOQ permits the determination of artemisinin content in the plant leaves at low concentrations.

CHAPTER FOUR:

EXTRACTION AND QUANTIFICATION OF ARTEMISININ FROM *ARTEMISIA ANNUA* L.

4.1 Extraction of artemisinin

4.1.1 Introduction

Following the increased demand for artemisinin and its derivatives, the technologies which include extraction with hexane, ethanol, tetrafluoroethane or using ionic liquids and are now being promoted. These processes were initially developed for the extraction of essential oils, fragrances and other pharmaceutical products, but after successful initial trials on *Artemisia annua* L., these methods may be used as an alternative to the existing methods for extraction. The lack of reliable data on hexane extraction, together with the emergence of these new technologies, makes it difficult for new and existing Artemisia producers to assess the productivity, financial growth, safety and environmental strain of the individual processes, and thus select the process which is best (Lapkin *et al*., 2006; Lapkin *et al*., 2009).

Several methods have been used in extraction and analysis of artemisinin from *Artemisia annua*. Previous researched methods involved the extraction of plant material with the petroleum ether followed by chromatography of the crude extract on silica gel (Klayman *et al*., 1984). Another method of extraction involved slow extraction with warm hexane by hot percolation, followed by partitioning the extract between hexane and acetonitrile followed by column chromatography of the acetonitrile phase on silica gel. Artemisinin has also been extracted using hydrocarbon extraction processes. However, because of high risk to human health and the dangers involving large volumes of combustible fluids, it was found necessary to develop an alternative processes that would be able to compete in terms of how much of

the artemisinin is extracted from the plant (yield) and cost of the extraction process (Lapkin *et al*., 2006).

Almost all of the *Artemisia annua* grown worldwide is presently processed via solvent extraction, using warm hexane or petroleum ether. However, solubility studies carried out by Nti-Gyabaah *et al*.(2010); found out that solubility of artemisinin is not only dependent on temperature but also on the polarity of the extracting solvent.

4.1.2 Optimization of extraction conditions

The extraction procedure was premised on the artemisinin content in the *Artemisia annua* leaves being approximately 1 % of the dried powdered leaves (Dahnum *et al*., 2012; Damtew *et al*., 2011; Townsend *et al*., 2013). Extraction of artemisinin from the plant was then carried out as follows: 100 g of the powdered leaves were soaked in 1000 mL of hexane and subjected to various extraction conditions to determine the optimum time and temperature at which maximum extraction of the artemisinin from the leaves took place. Sample solutions of 5 mL aliquots were taken at selected time intervals. The samples collected were dried in the oven at 30 °C for about 24 hours and stored in the refrigerator at 8 °C. These samples were then re-dissolved in an equivalent amount of acetonitrile (5 mL) and injected into the HPLC system under optimized chromatographic conditions and run against a standard solution of artemisinin of concentration 1 mg/mL. Triplicate injections were run for each of the various extraction conditions. Each run was recorded for a minimum of 40 minutes to allow sufficient elution time for other components that may be present in the crude sample extract. The concentration of artemisinin present in each sample was calculated based on the dry weight of the *Artemisia annua* L. powdered leaves.

The dried powdered leaves of *A. annua* were subjected to varying extraction conditions as follows:

Table 4.1: Extraction conditions used for the extraction of artemisinin from *A. annua* powdered leaves.

Sample	Extraction conditions	
A	Cold maceration	No stirring
B	Hot maceration	No stirring
C	Cold maceration	Stirring
D	Hot maceration	Stirring

In hot maceration, the vessel containing soaked powdered leaves were placed in water bath that was maintained at a temperature of 40 °C as shown in figure 4.2 (a). the stirring was carried out internally using a magnetic stirrer as shown in figure 4.2 (b). Stirring or agitation while extracting has been shown to increase the surface area of the powdered leaves in contact with the solvent used for extraction.

A graph was plotted showing artemisinin concentrations at the various time intervals and the effect of different extraction conditions (figure 4.1).

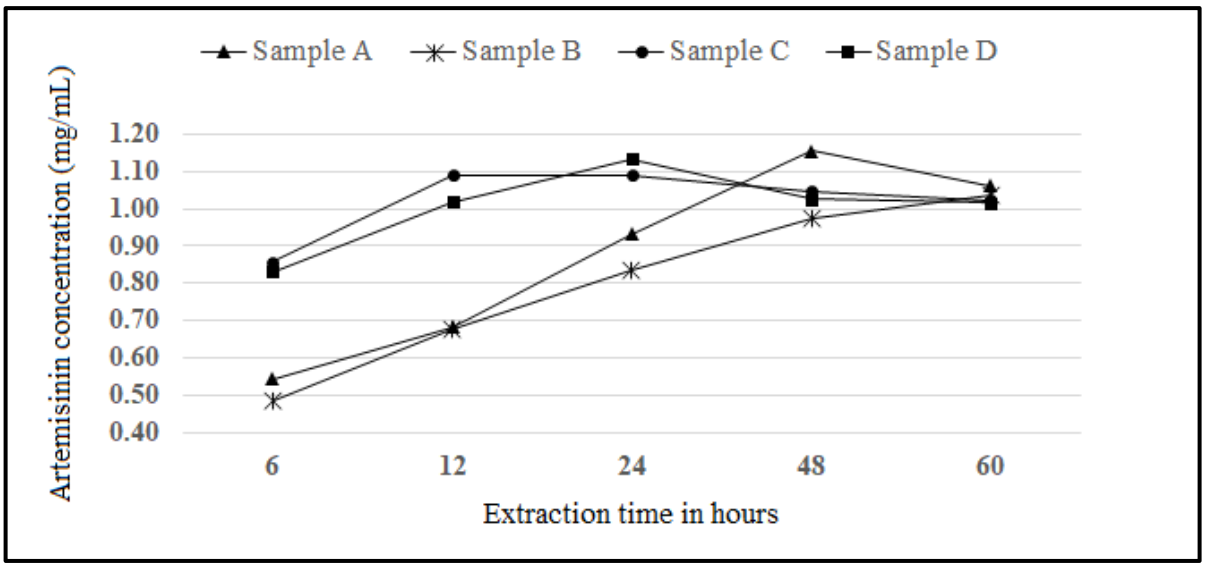


Figure 4.1: **Effect of temperature and stirring on extraction of artemisinin content.**

Sample A – powdered leaves subjected to cold maceration without stirring; Sample B – powdered leaves subjected to hot maceration without stirring; Sample C – powdered leaves subjected to cold maceration with stirring; Sample D – powdered leaves subjected to hot maceration with stirring.

Artemisinin concentration was shown to range between 0.5 mg/mL to about 1.2 mg/mL with the highest concentration obtained after 48 hours of cold maceration without stirring. A longer extraction time seemed to reduce the concentration of artemisinin, which may be caused by crystallization of the compound from solution. The waxes present in the plant have a higher solubility in hydrocarbons like hexane and therefore the suggestion to extract artemisinin with slightly polar solvents such as ethyl acetate and then recrystallize in hexane to obtain higher concentrations of artemisinin (Nti-Gybaah *et al*., 2010).

Although hot maceration with stirring gave higher yield, it was not ideal due to the complexity of the setup as shown in figure 4.2 (a). As a result the optimal extraction conditions were considered to be cold maceration with stirring for 24 hours as shown in figure 4.2 (b).

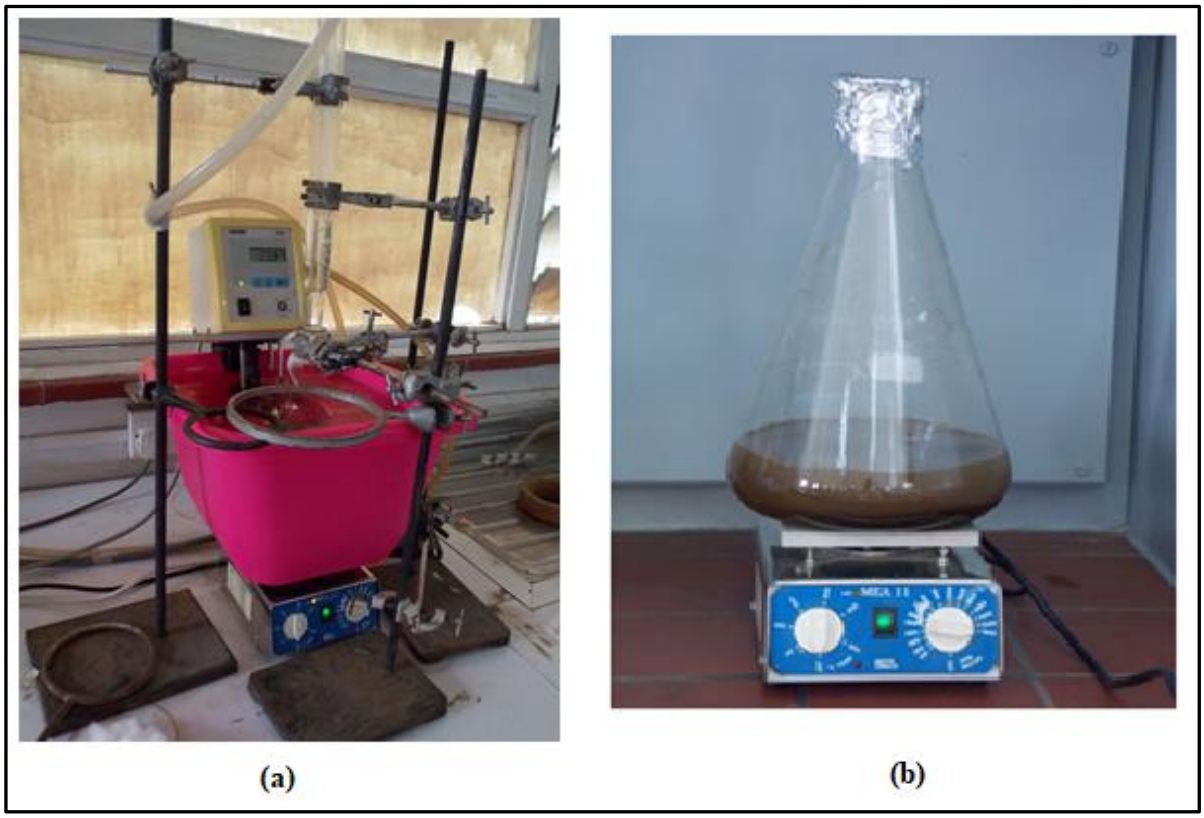


Figure 4.2: The laboratory setup showing the extraction of artemisinin from dried powdered leaves of *Artemisia annua* L. (a) Hot maceration and (b) Cold maceration

4.2 Quantification of artemisinin content from *A. annua* L.

4.2.1 Plant collection, identification and preservation

The leaves of *Artemisia annua* L. were collected from various places in Kenya, namely; Malakisi (Bungoma County), Emusaga (Kakamega County), Kehancha (Migori County), Kitengela (Kajiado County) and Kenyatta University (Nairobi County) in December 2014 to January 2015. The areas selected was based on the crop that was ready for harvesting and ready for transport to supply the dried leaves to East African Botanicals, Kenya. The plants had been planted in May 2014 and were harvested just before flowering. This period is recommended to obtain maximum extraction yield of artemisinin in the plant (El-Naggar *et al*., 2013). As with most medicinal herbs, artemisinin content and efficacy are subject to climatic, geographical and environmental conditions. The content varies from region to region depending on the quality of the soil and rainfall patterns. The regions are shown in figure 4.3.

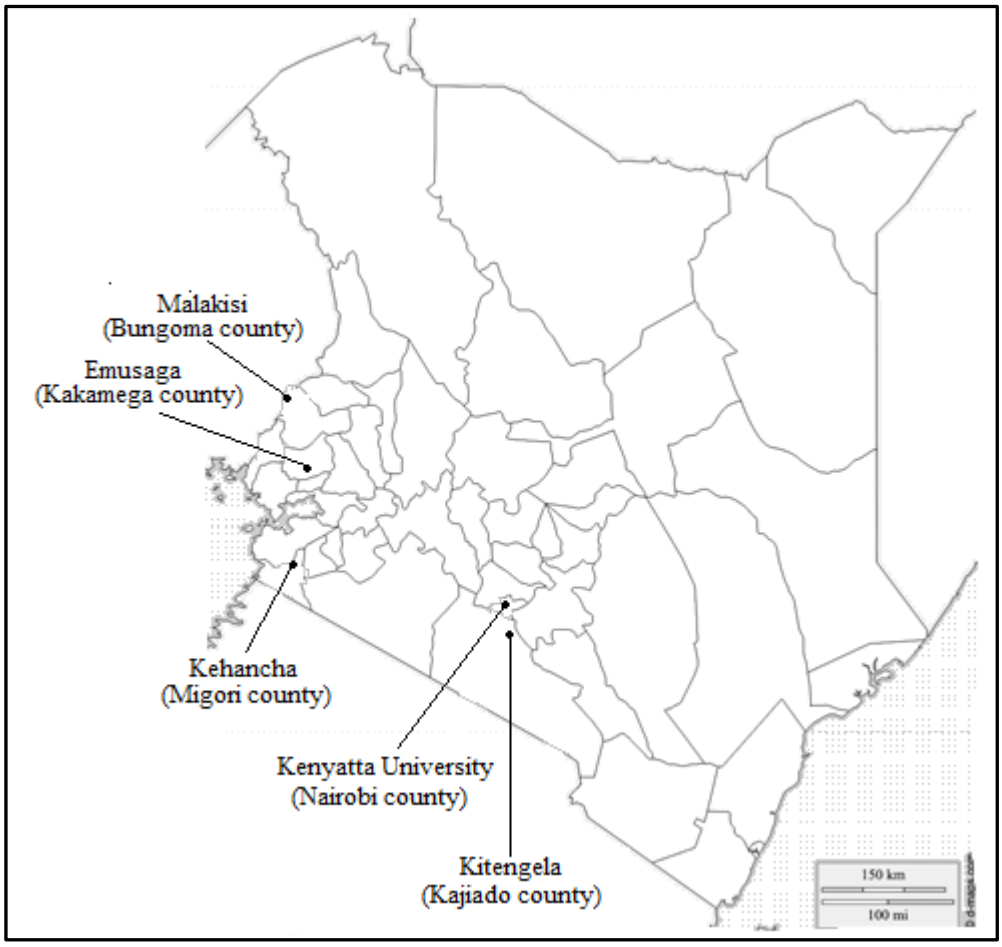


Figure 4.3: Map of Kenya showing the various regions where the *Artemisia annua* L. plant was grown and collected.

Source: https://www.onestopmap.com/product/printable-vector-map-kenya-political-83/ [Accessed: 28th November 2017].

The plants were identified in the field by Samuel Kalabata of East Africa Botanicals Limited (Kenya). The plant specimen collected from Malakisi (Bungoma County) was submitted to Botany Department, University of Nairobi (SWM 2010/01).

The leaves were evenly spread to dry at room temperature. After drying, the leaves were ground into a coarse powder and stored in separate labeled plastic containers at room temperature until use.

4.2.2 Quantification of artemisinin content from *A. annua* L.

About 100 g of the dried powdered leaves collected from the stated regions were subjected to the optimized extraction conditions using 1 L cold hexane. After extraction, the sample was concentrated using the rotary evaporator operated at about 40 °C. The reduced extract was dried in the oven to remove any excess solvent. The residue was re-dissolved in an equivalent amount of acetonitrile. A sample of this solution was subjected to HPLC analysis using the optimized chromatographic conditions and run against a standard solution of artemisinin containing an approximate concentration of 1 mg/mL. Triplicate injections were run for each of the samples collected. The percentage content of artemisinin present in each sample was calculated based on the dry weight of the *Artemisia annua* L. powdered leaves used for extraction. Table 4.2 shows the content of artemisinin in the plants collected from the five regions in Kenya.

Table 4.2: **Comparison of the artemisinin content in *Artemisia annua* L. samples.**

Collection Site (County)	Artemisinin Content (%)
Malakisi (Bungoma county)	0.87
Emusaga (Kakamega county)	0.89
Kehancha (Migori county)	0.85
Kitengela (Kajiado county)	0.75
Kenyatta University (Nairobi county)	0.68

From the results, the highest content of artemisinin was found from the plant material that was collected from Emusaga in Kakamega. Artemisinin content found in Malakisi and Kehancha also recorded high content.

Previous studies conducted by Townsend *et al*., (2013) documented the content of artemisinin to vary between 0.01 to 1.0 % of the dried powdered leaves of varieties grown in different geographical regions. This study also proved that factors such as differences in farming

practices, periods of harvesting and environmental factors such as temperature and nutrients availability contributed to artemisinin content variation in the plant (Townsend *et al*., 2013).

Other reports have also shown that artemisinin concentration varies considerably in the different plant components and not only during the vegetative growth. It was also found that the highest content of flavonoids and artemisinin were found at the blooming stage and especially in the leaves (Baraldi *et al*., 2008; Das and Sharma, 2016).

CHAPTER FIVE:

GENERAL DISCUSSION, CONCLUSION AND RECOMENDATIONS

5.1 General discussion

A new HPLC method was developed and optimized for the analysis of crude extracts of *Artemisia annua* L. Plant. This method was able to separate the artemisinin from other components found in the crude extract. An optimized extraction conditions was also developed to extract the artemisinin from the powdered leaves of the plant. From this study, it was found that the plants cultivated in Kakamega had the highest content of artemisinin. The artemisinin content variation depends on natural factors like soil type, altitude and latitude, flowering time, drying time, storage conditions and methods of cultivation (Ferreira *et al*., 2005).

5.2 Conclusion

In the present study, a simple, reliable, accurate, precise, robust and isocratic HPLC method was developed to determine the content of artemisinin in the plants collected from various regions. The HPLC method was able to almost completely separate the artemisinin peak from other compounds also present in the plant.

The method developed offers the advantage of being able to quantify artemisinin content in crude extracts of *Artemisia annua* L. plant using solvents and reagents that are easily available in the market. The method is being reported for the first time and can be used for routine quality control analysis of the plant. The HPLC method developed was precise and suitable for routine analysis of crude extracts of the plant. The study also showed that artemisinin content varies from region to region.

5.3 Recommendations

This HPLC method developed can be used to quantify and analyze the crude extracts without extensive sample preparation and also carry out routine quality control analysis during the extraction of artemisinin from *Artemisia annua* L. plant. Further work could also be carried out to determine the relation between soil types and artemisinin content present in the plant.

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