Determination of Artemisinin and essential oil contents of *Artemisia annua* L. grown in Ethiopia and *In vivo* Antimalarial activity of its crude extracts against *Plasmodium berghei* in mice

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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrum</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median inhibitory concentration</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Medium lethal dose</td>
</tr>
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<td>m/z</td>
<td>Mass to charge ratio</td>
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<td>Q292</td>
<td>Derivatized artemisinin possessing UV absorption at 292 nm</td>
</tr>
<tr>
<td>Q260</td>
<td>Derivatized artemisinin possessing UV absorption at 260 nm</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violate light</td>
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Abstract
Malaria is a major public health problem in the world in general and developing country in particular. It causes about 1.5-3 millions deaths per year, an annual incidence of 300-500 million clinical cases. Chloroquine has been the drug of choice for chemoprophylaxis and treatment of *P. falciparum* for several decades, but its clinical utility greatly reduced in many malaria endemic regions due to the spread of chloroquine resistance. Chinese investigators introduced a new compound, artemisinin, a sesquiterpene lactone, from *Artemisia annua* a few years back. It possesses a potent antimalarial activity with different mechanism of action from that of the conventional drugs in use. Currently the plant is introduced to many African countries including Ethiopia. Reports at different countries showed the variability of artemisinin content for the same plant grown at different geographical regions.

Seventy percent ethanol and hot water crude extracts from the dried leaves of *A. annua* grown at Wondogenet (Ethiopia) gave 20.2 and 14.00 % yields per dry weight respectively. Preliminary phytochemical screening using standard procedures showed the presence of steroidal compounds, phenolics, flavonoids and trace amount of tannins. Quantification of artemisinin from leaf of crude ethanol extract of *A. annua* was carried out using HPLC-UV with an attempt to show the plant is biosynthesizing artemisinin. The limiting step in sample preparation was the presence of large amounts of chlorophyll in the crude extract. The standard calibration curve for the quantification was linear with correlation coefficient of 0.96150. In this study the average content of artemisinin (w/w) was determined to be low in concentration (0.014% ± 0.001). Analysis of essential oil (0.32%) from *A. annua* by GC/MS gave a total of 38 compounds comprised of 99.72 % of the total volatile constituents and of these twenty-two monoterpenes (57.89%), fourteen sesquiterpenes (36.84%) and two phenols (5.55%) were identified. Among the volatile constituents camphor was identified to be the major component (43.84%).

An *in vivo* experiment was performed to know whether *A. annua* grown in Ethiopia has retained its antimalarial activity. The two crude extracts at three different doses
were tested on mice infected with *Plasmodium berghei*, in a four day test procedure. The results of the *in vivo* experiment showed that the parasite multiplication was inhibited by 77.97 % and 64.75 % for 70 % ethanol and hot water extracts respectively at dose level of 450 mg/kg. The study revealed that *A. annua* grown in Ethiopia retained the desired antiparasodial activity, as seen from its suppressive effects against *P. berghei*.

**Key words:** Malaria, *Plasmodium falciparum*, *Plasmodium berghei*, *Artemisia annua*, artemisinin
1. INTRODUCTION

1.1. Epidemiology of malaria and its life cycle

Malaria is a disease that is widespread in many parts of the world, mainly in tropical and subtropical regions. It is caused by protozoan parasites belonging to the genus *Plasmodium* (Goodman and Gilman, 2001) and represented by over 120 species. It is infectious to man, simians, rodents, birds and reptiles (Krettli et al., 2001). However, only four species: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* cause human malaria (MFS, 2004). At the moment among these, *P. falciparum* is responsible for the majority of malaria related deaths worldwide (Worrall et al., 2004). It has developed resistance to most of the available commercial drugs used to treat the disease.

Malaria causes about 350-500 million infections and 1.5-3 million deaths every year (Ali et al., 2004). One child dies every 40 seconds resulting in a daily loss of more than 2000 young children worldwide. This makes it among the ten leading causes of disability adjusted life years in the world and it is ranked eighth with a share of 2.8% of the global disease burden (Bisoe et al., 1995; WHO, 2003; MFS, 2004; Ali et al., 2004). The epidemiological situation of *falciparum* malaria worsened over the last decade in Africa and accounts for 85-90% of malaria fatalities (MCT, 2005, and MCL, 2006). It has spread to previously malaria free areas because of changes in the climate (Deresa et al., 2003). Studies in Sub-Saharan Africa have shown that it constitutes 20-60% of all outpatient consultations and about 10% of hospital admissions (MCL, 2006).

In Ethiopia malaria is the number one health problem. More than 65% of the people are exposed to the parasite and more than 5 million cases occur each year (Deresa et al., 2000; MoH, 2002). Over the last 50 years the country had experienced severe episodes of malaria epidemics. The worst was in 1958 with an estimated 3 million cases and 150,000 deaths (MoH, 2003). In 2003, the other epidemics occurred between April and December, resulting in 2 million clinical cases and 3000 deaths, affecting 3368 localities in 211 districts (MoH, 2005). In 2002-2003, malaria was the primary cause of reported morbidity and mortality, accounting for 16% of outpatient visits, 20% of hospital admissions and 27% of hospital deaths. The dominant species of malaria in Ethiopia are *P. falciparum* (60%) and *P. vivax* (40%) where as *P. malariae* and *P. ovale* are rare accounting for less than 1% of the cases (HIPD, 2005/06). The major vector of malaria is *Anopheles arabiensis* (MoH, 2000).

The malaria parasite life cycle involves 3 cycles: pre erythrocyte, erythrocyte and exo erythrocyte (figure 1). During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. *P. vivax* and *P. ovale* are a dormant stage that can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the
erythrocytes (erythrocytic schizogony). Merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinical manifestations of the disease.

![Life cycle of malaria parasite](image)

Figure 1. Life cycle of malaria parasite

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal. The parasites' multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (CDC, 2006).

1.2. Antimalarial drug resistance

Resistance to antimalarial drugs has been observed in *P. falciparum* and *P. vivax*. *P. falciparum* has developed resistance to nearly all antimalarial drugs in current use (Nallan *et al.*, 2006). *P.*
vivax infection acquired in some areas has been shown to be resistant to chloroquine and/or primaquine. Chloroquine resistant P. falciparum has been described everywhere except few malarious areas. Chloroquine has been the mainstay of antimalarial treatment for decades. Resistance against chloroquine lead a shift to sulfadoxine-pyrimethamine but sulfadoxine-pyrimethamine resistance is also becoming more prevalent in Africa (Fidock et al., 2004; Wang et al., 2005).

In Ethiopia, chloroquine has been the first-line drug for the treatment of uncomplicated malaria over the last forty years (WHO, 2003). The first report on the emergence of chloroquine-resistant P. falciparum was made in 1986 after isolation of chloroquine-resistant P. falciparum from patients in areas bordering the neighboring countries (Sudan, Somalia, and Kenya) (MoH, 1999). Since then, studies conducted in different parts of the country showed that chloroquine-resistant P. falciparum has spread to all malarious areas of the country (Mengesha et al., 1997; Mengesha et al., 1999; Desta and Gebrat, 1999).

Development of resistant by P. falciparum towards chloroquine has led treatment policy change to sulfadoxine-pyrimethamine. The use of sulfadoxine-pyrimethamine as first line for the treatment of falciparum malaria was then adopted in 1998. However, a nationwide study conducted in 2003 on the therapeutic efficacy of the drug revealed high treatment failure. Accordingly, sulfadoxine-pyrimethamine was replaced by artemether-lumfantrine for the treatment of falciparum malaria in 2004. Mefloquine replaced chloroquine-proguanil for chemoprophylaxis of malaria (MoH, 2004). The increased resistance development of P. falciparum to almost all the available drugs necessitated the need for the search of new antimalarial drugs.

1.3. Traditional medicinal plants as sources of antimalarial drugs

Medicinal plants, since times immemorial, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations, obtained from commonly used medicinal plants, has been traced to the occurrence of natural products with medicinal properties (Hoareau and DaSilva, 1999; Weiss, 2006). At least 80% of the population in most developing countries relies for their primary health care on traditional forms of health care (Zhang, 1998; Gedif and Hahn, 2002; Machroja et al., 2005). Traditional medicine has maintained its popularity in all regions of the developing world and its use is rapidly spreading in the industrialized countries (Craker and Simon, 2002; Fang et al., 2005).

In Ethiopia looking to the cultural acceptability of healers and local pharmacopeias and the relatively low cost of traditional medicine and less access of modern health facilities up to 80% of the population uses traditional medicine (Abebe and Ayehu, 1993; Gedif and Hahn, 2002; Kassaye et al., 2006). The vast majority of Ethiopia's population lives in rural areas where the
health care coverage is low and where existing public sector resources are being stretched to the limits, hence, these necessitated a need to look for an alternative source of medicine, such as herbal drugs, which could be produced locally.

Medicinal plants can serve as a resource for antimalarial drug discovery. Quinine formed the basis for the synthesis of the commonly used antimalarial drugs, chloroquine and mefloquine (Kassa et al., 1998). In line with the emergence of resistance to these drugs in many tropical regions, another medicinal plant long used in the treatment of fevers in traditional Chinese medicine, Artemisia annua (Quinhaosu), has yielded the agents, artemisinin (I) and its derivatives, arteether (II) and arteether (III) (structures are presented in the figure 2 below). These have been found to be effective against resistant Plasmodium strains (Kassa et al., 1998; Krettli et al., 2001; Machroja et al., 2005).

![Chemical structure of artemisinin and its derivatives](image)

Currently several research groups are working to screen plant extracts for their antimalarial activity. As a result, many medicinal plants have been reported from several countries to have a significant antimalarial activity against human and rodent malaria parasites. Most of the studies were conducted on P. berghei in vivo and P. falciparum in vitro, mostly on the basis of ethinobotanical information (Lopes et al., 1999). Ethinobotanical investigation had been conducted in various parts of Ethiopia and the traditional use of many plant species for the treatment of malaria has been documented (Abebe and Ayehu, 1993). However very few of the plants were scientifically studied for their antimalarial activity.

1.4. The Genus Artemisia

The genus Artemisia is the most widely distributed of the nearly 100 genera in the tribe Anthemideae of the Asteraceae (Compositae) family (Torrell and Valles, 2001). Asteraceae are a natural family represented by members that possess characters such as aggregation of flowers into capitula and production of achenes as a typical fruit of the family. It occupies a large range of habitat types and is found abundantly in every continent except Antarctica. The family has a total of 23,000 species (Simon et al., 1990). Terpenoids and certain phenolic compounds are responsible for the value of many species of Asteraceae in pharmacy and medicine (Wright, 2002).
Artemisia species are herbs or small shrubs, frequently aromatic, and alternate leaves. They are mostly perennial herbs and shrubs dominating vast steppe communities of Asia. It is a highly evolved genus with a wide range of life forms, from tall shrubs to dwarf herbaceous alpine plants, occurring in a variety of habitats between Arctic alpine and mountain environments to the dry deserts. Many of artemisia species have been screened for the presence of artemisinin but only \textit{A. annua} and to a lower extent \textit{A. apiacea} were found to produce artemisinin (Namdeo \textit{et al.}, 2006).

1.4.1. \textit{Artemisia annua} L.

\textit{Artemisia annua} L. (figure 3) is a highly aromatic annual herb traditionally grown in China as a medicinal plant and more recently in Europe and India for its aromatic leaves, which are used as a source of aroma chemicals for the fragrance industry (Sangwan \textit{et al.}, 1998; Bhakuni \textit{et al.}, 2001). Earlier research activities conducted in China brought \textit{A. annua} into attention because of the discovery of artemisinin that proved to be a potent anti-malarial agent with little or no side effects. Further more, the use of combined artemisinin therapy against malaria is widely recommended to confront drug-resistant \textit{P. falciparum} by WHO in different countries including Ethiopia. Currently many countries have therefore approved the use of artemisinin or aqueous extracts of \textit{A. annua} to treat malaria (MEE, 2003).

![Figure 3: The plant Artemisia annua (adapted from Picture Encarta)](image)

1.4.2. \textit{Artemisia annua} L. in Ethiopia

Recently \textit{A. annua} has been successfully introduced in to many African countries including Ethiopia. The altitude at which \textit{A. annua} grows varies from country to country, for example, 50-500 m in Viet Nam, 600-800 m in China and 1000-1500 m in Kenya and
the United Republic of Tanzania (WHO, 2006). Here in Ethiopia attempts were made to
grow the plant both in low and high land areas. The plant has been cultivated in north
Shoa, Wondogenet and Gamugofa; northern and eastern parts of Gojam, northern and
southern parts of Gonder, southern parts of Wollo and in Enderta district of Tigray. The
yield of \textit{A. annua} has been found to be twenty quintal per hectare.

The plant \textit{A. annua} was first introduced to Ethiopia in early 2001, by a German catholic church
around “chencha’ area of Gamugofa (MCL, 2006). Since then the dried herb is locally prescribed
as tea against malaria clinical cases. At the same time, essential oil research center of Ethiopia
introduced the species from Austria and performed several agricultural research activities across
Ethiopia. Local investors are also engaged with the production and promotion of Artemisia
technology on more than 150 hectare of land in different parts of the country. However, since the
plant is new to the country there is a need to investigate if there is any change in the content of
artemisinin, the active ingredient against \textit{P. falciparum}. Different reports from different countries
showed the variability of artemisinin content for the same plant grown at different geographical
region (Wright, 2002).

1.4.3. Chemical constituents of \textit{A. annua} L.

Several secondary metabolites characterize the chemical composition of the genus Artemisia.
According to surveyed literature, almost all classes of compounds are observed to be present in
the genus with particular reference to terpenoids and flavonoids. A large number of
monoterpenoid compounds have been characterized from the essential oil of \textit{A. annua} (Bhakuni
\textit{et al}., 2001). The yield of the oil generally varies between 0.3\% and 0.4\% (v/w). The chemical
constituents of \textit{A. annua} volatile oil varied from country to country for example principal
constituents of Chinese oil were 63.9\% Artemisia Ketone, 7.5\% Artemisia alcohol 5.1\% myrcene, 4.7\% \textalpha-guaiine and 3.3\% comphor. The Vietnamese oil contained camphor (21.8\%),
germacerene D (18.3\%) and 1.8-cineole (3.1\%) etc (Bhakuni \textit{et al}., 2001; Wright, 2002).
Sesquiterpenes, in this plant were given much attention and artemisin, which is responsible for
the antimalarial activity of \textit{A. annua} belongs to this group of compounds. Artemisinin is
characterized by lack of nitrogen containing heterocyclic ring system which is found in most
other antimalarial drugs. A large number of flavonoids have been reported from the plant \textit{A.
anhua} but a few compounds like triterpenoids and steroids have been reported (Wright, 2002).

A large variation in artemisinin content has been observed in the leaves of different samples of \textit{A.
anhua}. Contents varying from 0.01 to 1.38\% in the dry leaves have been reported (table1). The
variation in the content of artemisinin might be because of several reasons. In addition to the use
of different methods for extraction and analysis, the time of collection and preparation of the
samples, contributes a lot to the variation among different samples. Moreover, an environmental
factor such as temperature and availability of nutrient has also much to do with the variations
(Wright, 2002).
Table 1. Artemisinin content in different samples of *A. annua* (% dry weight) *

<table>
<thead>
<tr>
<th>Origin of the plant</th>
<th>Artemisinin content (%)</th>
<th>Year</th>
</tr>
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<tbody>
<tr>
<td>Europe (Connecticut) USA</td>
<td>0.01</td>
<td>1998</td>
</tr>
<tr>
<td>Argentina</td>
<td>0.1</td>
<td>1986</td>
</tr>
<tr>
<td>China</td>
<td>0.14</td>
<td>1990</td>
</tr>
<tr>
<td>Dakota (USA)</td>
<td>0.21</td>
<td>1990</td>
</tr>
<tr>
<td>Spain</td>
<td>0.24</td>
<td>1993</td>
</tr>
<tr>
<td>China</td>
<td>0.79</td>
<td>1980</td>
</tr>
<tr>
<td>Vietnam</td>
<td>0.86</td>
<td>1994</td>
</tr>
<tr>
<td>China</td>
<td>1.07</td>
<td>1993</td>
</tr>
<tr>
<td>Hybrid (Switzerland)</td>
<td>1.38</td>
<td>1996</td>
</tr>
</tbody>
</table>

*(Adapted from Wright, 2002)*

1.4.4. Pharmacological activities of constituents of *A. annua* L.

Among the different pharmacologically active compounds isolated from *A. annua*, the most interesting one is artemisinin, a potent antimalarial against chloroquine and quinine-resistant *P. falciparum* and other malaria-causing parasites. Artemisinin and its derivatives have shown cytotoxic activity against different types of tumor cells. Artemisinic acid, a well-known precursor for semi synthesis of artemisinin has shown antibacterial activity. Scopoletin, a coumarin and the flavonoid fisetin and patuletin-3, 7-dirhamnoside isolated from *A. annua* have been reported to possess anti-inflammatory and non-peptide angiotensin converting enzyme inhibitory activity respectively (Bhakuni *et al.*, 2001).

1.4.5. Mechanism of action of artemisinin

The manner in which artemisinin kills the malarial parasite is a complex matter and several different mechanisms are at work. The main action seems to be: disruption of haemoglobin catabolism in the *plasmodial* parasite; damage to the haem detoxification system of the parasite; generation of free radicals from the sesquiterpene lactone which attack the membranes of the parasite; and alkylation of intracellular proteins in the parasite either by free radicals or by the haem-artemisinin complex (Lee, 2002).
The ability of the endoperoxide to generate free radicals is not a mechanism shared with the quinolone antimalarials and this might explain why artemisinin has proved superior to chloroquine. It has also stimulated the search for other plant derived (or semi-synthetic) terpenes that could attack the plasmodia in a similar way. It is now very clear that artemisinin is the most exciting compound to be developed against malaria since the second world war and that it is, on the whole, very safe (Lee, 2002).

2. Objectives

2.1. General objective
This study aims in evaluating the content of artemisinin and essential oil composition of the leaf of *Artemisia annua* grown in Ethiopia and the *in vivo* antimalarial activity of its crude extracts against *P. berghei*.

2.2 Specific Objectives
- To extract *A. annua* leaves using ethanol and hot water
- To perform preliminary phytochemical screening test on the study plant
- To determine the amount of artemisinin in crude ethanol extract using HPLC
- To perform GC/MS analysis of essential oil of *A. annua*
- To perform *in vivo* antimalarial screening on ethanol, and hot water extracts of the leaves of *A. annua* for their antiplasmodial activity and to check on scientific basis if the study plant grown in Ethiopia still contain sufficient amount of artemisinin to retain the desired antimalarial activity
3. Materials and Methods

3.1. Plant materials
The leaf of *A. annua* (growth stage five month) was collected before the flowering of the plant in December 2006, from Wondogenet (270 km south west of Addis Ababa) where it is cultivated for different research purposes. The leaf was authenticated by Mr. Melaku Wandafrash and voucher specimen (AA-01) was deposited at the national herbarium, biology department, science faculty, Addis Ababa University.

3.2. Chemicals
The following chemicals and solvents were purchased and used as received: Ethanol 96%, Absolute methanol (ACS, Wardle Chemicals Ltd, USA), Methanol HPLC grade (Techno Pharmchem, Bahadurgarm, India), Water HPLC grade (Superchem Product, England), Artemisinin reference standard (Sigma Chemicals Company, USA), Chloroform (ACS, ISO, Merck), Petroleum ether 60-80°C (Labmerk Chemicals LTD India), n-Hexane (Rathburn Chemicals Ltd, England), Ethyl acetate (ACS, Merck), Sulfuric acid (Farm Italia Carrloerba, Italy), Trisodium citrate (The General Chemical Ltd, England), Giemsa’s (BDH Ltd, England), Tween 80 (BDH Laboratory, England), Coartem (Ageca, Switzerland) and Chloroquine (Addis Pharmaceutical Factory SC, Adigrat, Ethiopia) were purchased from Kenema no. 4 pharmacy, Arat kilo, Membrane filter paper, filter type, 0.45μm (Whatman International Ltd, England).

3.3. Test animals
Swiss albino mice of both sex, weight 22-35 g and age 6-8 weeks were purchased from Ethiopian Health and Nutrition Institute. The animals were housed in standard cages and acclimatized for a period of 7 days before use. They were maintained on standard pelleted diet and water.

3.4. The parasite
The rodent malaria parasite, *P. berghei* ANKA strain, obtained from Aklilu Lema Institute of Pathobiology was used to infect the mice for a four-day suppressive test.

3.5. Preparation of crude extracts of *A. annua* L.
The air-dried and powdered plant material (100 gm) was extracted using soxhlet apparatus with 70% ethanol till the last drops of the extract became colorless. The extract was then concentrated in vacuo and the heavy viscous liquid obtained after concentration was dried in a microwave oven under controlled temperature (80°C). The extract was placed in a well-closed bottle and kept in a refrigerator until used for anti-malarial activity testing.

The water extraction was done by adding (100 gm) powdered *A. annua* into 1000 mL hot distilled water, with continued gentle steering for about 15 minute. The mixture was filtered using Whatmann number one filter paper. Lypholizer (freeze dry) (type: Heto power dry LL3000 Wag tech) was used to dry the aqueous extract. The freeze-dried extract was kept in a refrigerator until used for the anti-malarial testing.

3.6. Solubility test
One mg of ethanol extract of *A. annua* was added to one ml of the following solutions: normal saline, 1% ethanol, 2% ethanol, 2% tween 80, 5% tween 80 and in the case where solubility did not occur one ml of additional solvent was added until solubilaization took place. The solvent that solubilize the specified amount of extracts in small volume was then selected as a vehicle of the extract.

3.7. Hydro distillation of the essential oil
Hundred grams of powdered *A. annua* was distilled in a Clevenger type apparatus fitted to a 1000 mL flask, with 500 mL of water as distillation liquid. The distillate was collected in a calibrated tube. The yield of essential oil was calculated after determining the volume of the oil as % v/w. The oil sample was kept at -20°C until analyzed for its chemical composition using GC/MS.

3.8. Preliminary phytochemical screening
The crude extract was screened for the presence/absence of alkaloids, steroidal
compounds, phenolic compounds, flavonoids, saponins and tannins using standard procedures (Hymete, 1986)

i. Test for alkaloids

**a) Preliminary test:** A portion of an alcoholic extract was dissolved in dilute hydrochloric acid. The solution was clarified by filtration. The filtrate was tested with Dragendorff’s and Mayer’s reagents. The treated solutions were observed for any precipitation.

**b) Confirmatory test:** 0.5 g of the alcoholic extract was treated with 40% calcium chloride solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10 mL portions of chloroform. The chloroform extracts were combined and concentrated in vacuo to about 5 mL. The chloroform extract was then spotted on thin layer plates. Solvent system (n-hexane-ethyl acetate, 4:1) was used to develop the chromatogram and detected by spraying the chromatograms with freshly prepared Dragendorff’s spray reagent. An orange or dark colored spots against a pale yellow background was confirmatory evidence for alkaloid.

ii. Test for steroidal compounds

**a) Salkowski’s test:** 0.5 g of the alcoholic extract was dissolved in 2 mL chloroform. Concentrated sulphuric acid was carefully added to form a lower layer. A reddish brown color at the interface indicated the presence of a steroid ring (i.e. the aglycone portion of the glycoside).

**b) Lieberman’s test:** 0.5 g of the alcoholic extract was dissolved in 2 mL of acetic anhydride and cooled well in an ice-bath. Concentrated sulfuric acid was then carefully added. A color change from purple to blue to green indicated the presence of a steroid nucleus (i.e. aglycone portion of’ the cardiac glycoside).

iii. Test for phenolic compounds

To 2 mL of filtered solution of aqueous macerate of the plant material, 3 drops of a freshly prepared mixture of 1ml of 1% ferric chloride and 1ml of potassium ferrocyanide was added to
detect phenolic compounds. Formation of bluish-green color was taken as positive. The dried alcoholic extract (100 mg) was dissolved in water. Few crystals of ferric sulfate were added to the mixture. Formation of dark-violet color indicated the presence of phenolic compounds.

iv) Test for flavonoids

a) Test for free flavonoids: Five milliliter ethylacetate was added to a solution of 0.5 g of alcoholic extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow color in the organic layer which is taken as positive for free flavonoids.

b) Lead acetate test: To a solution of 0.5 g of alcoholic the extract in 5 mL water about 1 mL of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids.

c) Reaction with NaOH: Dilute sodium hydroxide solution was added to a solution of 0.5 g of the extract in water. The mixture was inspected for the production of yellow color which considered as positive for flavonoids.

v) Test for saponins

Froth test: 0.5 g of the alcoholic extract was dissolved in 10 mL of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 minute period of time. If a “honey comb” froth above the surface of liquid persists after 30min. the sample is suspected to contain saponins.

vi) Test for tannins

a) Ferric chloride test: A portion of the alcoholic extract was dissolved in water. The solution was clarified by filtration. To the clear filtrate was added 10% ferric chloride solution. This was observed for a change in color to bluish black.

b) Formaldehyde test: to a solution of about 0.5 g of the extract in 5 mL water, 3 drops of formaldehyde and 6 drops of dilute hydrochloric acid was added. The resulting mixture was heated to boiling for 1 minute and cooled. The precipitate formed (if any) was washed with hot water, warm alcohol and warm 5% potassium hydroxide
successively. A bulky precipitate, which leaves a colored residue after the washings indicates phlobatannins.

c) **Test for phlobatannins:** deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken evidence for the presence of phlobatannins.

d) **Modified iron complex test:** To a solution of 0.5 g of the plant extract in five milliliter of water a drop of 33% acetic acid and one gram sodium potassium tartarate was added. The mixture was warmed and filtered to remove any precipitate. A 0.25% solution of ferric ammonium citrate was added to the filtrate until no further intensification of color is obtained and then boiled. Purple or blackish precipitates which is insoluble in hot water; alcohol or dilute ammonia denotes pyrogallol tannin present.

### 3.9. High performance liquid chromatography (HPLC)

HPLC analysis of *A. annua* crude 70% ethanol extract was carried out to quantify artemisinin found in the leaves of *A. annua* in comparison with reference standard. It is a method described by Woerdenbag *et al.*, 1992. Crude extract of *A. annua* was treated with alkali and converted into Q292, possessing UV absorption at 292 nm, upon acidification, Q292 yields Q260, which has a strong UV absorption at 260 nm and more stable than Q292 (Wright, 2002). Q260 was separated by means of a reversed phase Agilent technologies HPLC system comprised of quaternary pump, a column oven, sample freeze and UV detector. A ZORBAX Eclipse XDB-C$_{18}$ column (4.6x150mm, 5µm) was employed, at 35°C. Separation was made in isocratic mode, using methanol: water (60:40 v/v) at a flow rate of 1ml/min with 20 µl injection volume; detector and column temperature were set at 35°C. The detection wave length was 260 nm.

**Sample preparation**

The clean up was done using the method developed by Celeghini *et al.*, 2006. Silica (25 g) was added to a glass column. The column was conditioned with hexane (80 mL) and not allowed to dry. The crude extract (250 mg) dissolved in methanol (20 mL), was mixed with (6 g) silica and dried under vacuum. The silica adsorbed concentrated extract was then applied to the column. The analytes were eluted with hexane-ethyl acetate (4:1, 100 mL), filtered and concentrated almost to dryness under vacuum. The residue was re-dissolved in 20 mL methanol and subjected to further clean up by solid phase extraction (cartridge fitted with Gilson peristaltic pump, column: C$_{18}$, flow rate1.5 mL/min).
The cleaned sample was pre-column derivatised for HPLC. The extract dissolved in methanol (200 µl) was mixed with 800 µl 0.05 M NaOH, and then the mixture was vortexed and heated at 50ºC for 30 min. After cooling 200 µl methanol and 800 µl 0.08 M acetic acid was added for quantification using HPLC (Wright, 2002). Determination of the content of artemisinin in plant material was performed by external standard method. A stock solution of Sigma standard artemisinin was pre column derivatized (figure 4) and solutions with concentrations of 0.125, 0.25, 0.5, and 1mg/mL were used to draw calibration curve. Triplicate determinations were carried out.

![Derivatization of artemisinin](image)

Figure 4. Derivatization of artemisinin

### 3.10. GC-MS conditions

One micro liter of the volatile sample was injected into the GC using an automatic injector in a split less condition. Chromo pack Cp – SiL5 (fused silica, 30m x 0.32mm i.d. with a film thickness of 0.25µm) capillary column was used during the investigation. The carrier gas was helium (5psi) at 50 ml/min through the injector and a 30cm/sec through the column. The injector temperature was 220ºC for the analysis. The column temperature program was 40-220ºC at a rate of 3ºC/min and a hold up at 220ºC for 3 min.

A Varian star 3400cx gas chromatograph coupled with a Varian Saturn 3 mass spectrometer was used for the analysis. The MS detector was set at 175ºC and a mass range of 40-400 (m/z) was recorded. All mass spectra were acquired in EI mode. The compounds in volatile fractions were identified by the use of a combination of mass spectrum data base search (Software: IMS Terepene Library, 1989; NIST MS Data base, 1992 and 1998), the relative retention index (Software: ESO Database of essential oils, 1999) and comparison of mass spectra in the literature (Adams, 1995). Quantitative analysis (in %) was performed by peak area normalization measurements (TIC=Total ion count).
3.11. Acute toxicity test of the crude extracts

Toxicity test was done on five groups of mice, each having four mice. Each group contained two male and two female mice. The mice in each group were fasted over night. The weight of each mouse was measured. Mice in the first group were treated with the vehicle (control group) while group two, three, and four were given, 1000, 2000, 3000 mg/kg of the extract respectively and the fifth group was given the highest dose 5000 mg/kg. Toxicity signs were determined every four hours post treatment in the first day and then every twelve hours for the next 72 hrs (experimental period) and continued for seven days. Acute toxicity signs such as death, gross behavioral and physical observations like, lacrimation, muscle weakness, sedation, urination and convulsion were observed (Dikasso et al., 2006).

3.12. In vivo antimalarial activity test of crude extracts of *A. annua* L.

Antimalarial activity tests were performed using a 4-day standard suppressive test following the methods of Fidock *et al.*, (2004). *P. berghei* ANKA strain (chloroquine sensitive strain), and Swiss albino mice purchased from the Ethiopian Health and Nutrition Research Institute were used for the study. *P. berghei* was maintained in the laboratory by blood transfer from mouse to mouse. The mice were allowed to acclimatize to the laboratory environment under a controlled temperature of 20°C and at optimum humidity for seven days before being used for the experiments (Malagon *et al.*, 1997; Dikasso *et al.*, 2006).

A donor mouse with rising parasitemia level of approximately 20-30% (i.e. 20-30% of *P. berghei*) parasitized erythrocytes was used for infection. Blood from the auxiliary vessels of donor mouse was collected using a syringe containing trisodium citrate and diluted in physiological saline to $10^7$ parasitized erythrocytes per ml. Each experimental animal received inoculums of 0.2ml (About $2\times10^7$ Million *P. berghei* parasites) on day zero, which was expected to produce a steadily rising infection.

The inoculum’s was injected intraperitonialy to ensure the direct delivery of parasitized blood into the circulatory system. The infected mice were weighed & then randomly divided into six groups of five mice per cage. Group 1 received the vehicle (5% tween 80 in distilled water) and served as a negative control. Groups 2 & 3 took 20 mg/kg/day of the standard drugs chloroquine and coartem respectively and served as positive controls. Groups 4, 5 and 6 were treated with *A. annua* crude extracts at dose level of 150, 300 and 450 mg/kg/day respectively. The dose levels of the extracts were selected based on information obtained from literature on traditional methods of usage of species of this plant (Malagon *et al.*, 1997).

Treatment started three-hour post infection on day zero. The treatment continued on day one (24 hr), day two (48 hr) and day three (72 hr) post-infection, with the same dose and by the same
route as on day 0. Twenty four hours after the last treatment (i.e. 96 hr post-infection, 5th day), blood smears were made from the tail of all mice, air dried, fixed with absolute methanol & stained with Giemsa. Parasitemia was then determined microscopically by counting 4 fields of approximately 100 erythrocytes per field according to the method of David et al, 2004.

4. Data analysis

Results of the study were expressed as mean ± standard deviation. Statistical significance for suppressive test was determined by one-way ANOVA. Data on body weight and survival time were analyzed using Microsoft office excel 2003. All data were analyzed at 95% confidence limits (p=0.05).

Formula for calculating percent parastemia:

\[
\% \text{Parastemia} = \frac{\text{Number of infected RBC}}{\text{No. of total RBC}} \times 100
\]

Formula for calculating percent suppression:

\[
\% \text{Suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in study group}}{\text{Parasitemia in negative control}} \times 100
\]
5. Results and Discussion

The extraction of natural products is an essential tool for evaluation of raw plant materials as well as for the quality control of natural products. In plant screening process it is essential to have simple, rapid and specific extraction procedure, which allows the quantitative determination of the analysts (Celeghini et al 2006).

In this work seventy percent ethanol and hot water were used in the preparation of crude extracts from the dried leaves of *A. annua*. The extracts, as obtained, were dark green in color. The yields obtained by extraction of the dried leaves were 20.2 and 14.00 % for 70 % ethanol and water respectively.

Among the two extracts obtained, the 70 % ethanol extract was found to be a viscous mass. Therefore solubility test was done to find solvent that solublize the highly viscous crude extract and make it suitable for administration to mice and easy passage through the gavages. For solubility test the choice of the solvent and its concentration was made considering its safety and potential health risk to the mice.

Better solubility of the crude 70% ethanol extract was observed in 5 % tween 80 in distilled water as compared to the other solvents. Medium solubility was observed for 2 % tween 80 in distilled water and no solubility was observed while using 2 % ethanol, 1 % ethanol and normal saline. Following this result 5% tween 80 was selected as a vehicle for the crude 70% ethanol extract throughout the experiment. The hot water extract was found to be freely soluble in water at room temperature.
5.1. Preliminary phytochemical screening

In the present study phytochemical screening was done using precipitation and color tests, following the method compiled by Hymete, 1986. The tests revealed the presence or absence of the main secondary metabolites in the extracts. As can be seen in table 2 the presence of steroidal compounds, phenolics, flavonoids, and trace amounts of tannins were detected in crude plant extracts.

In this work the presence of flavonoids in crude extract was observed which may contribute for antimalarial activity of the plant in addition to the already isolated active constituent, artemisinin. Previous study indicated the presence of secondary metabolites like flavonoids, and steroidal compounds (Wright, 2002). Among these, a large number of flavonoids have been reported from *A. annua*, namely artemetin (I) and casticin (II) (structures are presented in the figure 5 below) which have a marked and selective potentiating effect on the antiplasmodial activity of artemisinin (Wright, 2002).

According to Chiung-Sheue *et al.*, (2004) the chloroform fraction of methoxylated flavonoids such as artemetin, chrysoplenetin, chrysosplenol-D and cirsilineol were tested for their antimalarial activity. The chloroform fraction of these flavonoids was found to have IC\(_{50}\) values at 2.4-6.5 x 10\(^{-5}\) M against *P. falciparum in vitro*.

![Artemetin](structure1.png)  
![Casticin](structure2.png)

Figure 5: Chemical structure of flavonoids identified from *A. annua*
Table 2: Results of phytochemical screening (color test) of dried leaf extracts of *A. annua*

<table>
<thead>
<tr>
<th>Test</th>
<th>Reagents</th>
<th>Inferences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for alkaloids</td>
<td>Dragendorff’s</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>Mayer’s</td>
<td>_</td>
</tr>
<tr>
<td>Test for steroidal compounds</td>
<td>Acetic anhydride and conc. sulfuric acid</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Chloroform and conc. sulfuric acid</td>
<td>++</td>
</tr>
<tr>
<td>Test for Phenolic compounds</td>
<td>Ferric chloride and potassium ferrocyanide</td>
<td>+++</td>
</tr>
<tr>
<td>Test for flavonoids</td>
<td>10% Lead acetate</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Sodium hydroxide</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>+++</td>
</tr>
<tr>
<td>Test for saponins</td>
<td>Froth test</td>
<td>_</td>
</tr>
<tr>
<td>Test for tannins</td>
<td>Ferric chloride</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>hydrochloric acid</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Modified iron complex</td>
<td>±</td>
</tr>
</tbody>
</table>

Key:  +++ , Very strong positive
+++, Strong positive
±, Trace
-, Negative

5.2. Analysis of artemisinin in A. annua L.
The analysis of artemisinin is possible for HPLC with UV detector only after derivatization with alkali. Sample preparation is a limiting step in the analysis of crude extracts of A. annua with HPLC. This is due to the presence of large amounts of chlorophyll in the crude extract. In this work the sample preparation and clean up was done utilizing column chromatography and solid phase extraction. The crude extract was purified on a column containing silica gel that was preconditioned with hexane. Purification of crude A. annua extract with silica gel retains more of the polar interfering materials, trap larger amounts of impurities at the top of the column (Thakur and Vishwakarama, 1990).

Determination of artemisinin content in plant material was performed by external standard method. The system was calibrated by quantifying the qinghaosu 260 produced from basic hydrolysis of authentic artemisinin standard solutions (0.125 -1.0 mg/L). As can be seen in the chromatogram (figure 6) the artemisinin peak was observed to have a retention time of 2.069 minutes. The standard calibration curve was linear with correlation coefficient 0.96150. The average artemisinin content (w/w) in A. annua leaf extract was determined to be 0.014% ± 0.001.
Figure 6. HPLC chromatogram of A. annua crude ethanol extract

There is a global interest in growing A. annua as a commercial crop for artemisinin production. However, the artemisinin content of the leaves has been found to vary widely (table 1.1) (Wright, 2002, Zhenghao et al., 2007). For example, one study of 18 accessions reported a range of artemisinin levels of 0.003 to 0.21% dry weights (Charles et al., 1990), whereas another study of 10 accessions reported a range of 0 to 0.1% dry weights (Liersch et al., 1986). The reported levels of artemisinin in leaves of accessions originating from the Washington, DC area were 0.04 to 0.11% dry weights (Liersch et al., 1986; Charles et al., 1990). Thus, the artemisinin level of the A. annua leaf investigated in this study was low (0.014%) and this makes the leaf similar in terms of concentration with that of the previous study (Liersch et al., 1986; Charles et al., 1990; Wright, 2002; Zhenghao et al., 2007).

The variation in the content of artemisinin obviously has several causes. Besides the utilization of diverse methods for extraction and analysis the collection and the preparation of samples, especially the separation of the leaves from the stems, are very variable too. Also, it is well known that the artemisinin content of the plant varies during the season; obviously a part of variation reported is due to harvesting at different stages of growth and at different times. Moreover, an environmental factor such as temperature or availability of nutrients and geographical distribution of the plant has also much to do with the variations (Zhenghao et al., 2007).

5.3. GC-MS analysis of volatile oil of A. annua L.

Essential oils are complex mixture comprising of many chemical constituents. Each of these chemical constituents may contribute to the different activities exhibited by the oils. Data on essential oil constituents can be obtained by analyzing the oil with GC-MS. The identification of the constituents was carried out by comparing their mass spectra with Wiley spectral library (Adams, 1995).
Apart from artemisinin from *A. annua*, essential oils are another active research interest as it could be potentially used in perfumery, cosmetics and aromatherapy. The oil has been also reported to possess antimycotic and antimicrobial activities (Woerdenbag *et al.*, 1993). Depending on its geographical origin, the oil yield in *A. annua* ranges between 0.02-0.49% on fresh weight basis and 0.04-1.9% on dry weight basis (Namdeo *et al.*, 2006). In this study *A. annua* grown in Ethiopia was analyzed for its volatile oil contents. The yield of the oil (V/W) obtained from the dried leaves of *A. annua* was 0.32 % ± 0.04. Accordingly, the yield of the essential oil obtained in this study is in agreement with the previous report (Bhakuni *et al.*, 2001; Namdeo *et al.*, 2006).

As can be seen in the table 3 a total of 38 compounds which comprises 99.72 % of the total volatile constituents were identified. Majority of the compounds identified were monoterpenes accounting for 57.89 % of the constituents. Sesquiterpenes and phenols comprised 36.84 and 5.55 % of the identified compounds respectively. In this analysis camphor was identified as the major component (43.84%). This is in agreement with Vietnamese oil that contained camphor as a major constituent (Bhakuni *et al.*, 2001). Among the identified monoterpenes, 52.17 % were monoterpane alcohols, 30.43 % were monoterpane hydrocarbons, 13.04 % were monoterpane aldehydes and 4.35 % were monoterpane oxides.

Among the identified sesquiterpenes, 57.14 % were sesquiterpane hydrocarbons, 35.71 % were sesquiterpane alcohols and the remaining 7.14 % were sesquiterpane oxides. In this work phenolic compound were found to be among the minor volatile components of the oil. According to Namdeo *et al.*, (2006) major components in the oil were reported to be artemisia ketone, isoartemisia ketone, artemisia alcohol, 1, 8-cineole and camphor. Artemisia ketone, isoartemisia ketone and artemisia alcohol were not identified in this study, however the volatile constituents obtained from *A. annua* studied showed similarity in constituents with that obtained in previous study (Bhakuni *et al.*, 2001).
Table 3: Volatile constituents of *A. annua* obtained by hydrodistillation and analyzed by GC-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>KI</th>
<th>Name of compound</th>
<th>Peak area %</th>
<th>Compound type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>939</td>
<td>α-Pinene</td>
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<td>Monoterpene hydrocarbon</td>
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<td>2</td>
<td>954</td>
<td>Camphene</td>
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<tr>
<td>3</td>
<td>975</td>
<td>Sabinene</td>
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<td>4</td>
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<td>α-Terpinene</td>
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<td>“</td>
</tr>
<tr>
<td>5</td>
<td>1025</td>
<td>p-Cymene</td>
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</tr>
<tr>
<td>6</td>
<td>1031</td>
<td>Cineol(1,8)</td>
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<td>Sabinene hydrate (cis)</td>
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<td>9</td>
<td>1122</td>
<td>Menth-2-en-1-ol (cis)</td>
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<td>1126</td>
<td>α-Campholenal</td>
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<td>11</td>
<td>1141</td>
<td>Menth-2-en-1-ol(trans)</td>
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<tr>
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<td>1217</td>
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<td>1229</td>
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<td>Carvone</td>
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<td>Monoterpenes aldehyde</td>
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<tr>
<td>20</td>
<td>1290</td>
<td>Thymol</td>
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<td>Phenolic</td>
</tr>
</tbody>
</table>

Table 3. Volatile constituents of *A. annua* obtained by hydrodistillation and analyzed by GC-MS (continued)

<p>| | | | | |</p>
<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>1299</td>
<td>Carvacrol</td>
<td>0.25</td>
<td>Phenolic</td>
</tr>
<tr>
<td>22</td>
<td>1342</td>
<td>Carvyl acetate (trans)</td>
<td>1.05</td>
<td>Monoterpenes alcohol</td>
</tr>
<tr>
<td>23</td>
<td>1359</td>
<td>Eugenol</td>
<td>tr</td>
<td>“</td>
</tr>
<tr>
<td>24</td>
<td>1368</td>
<td>Carvyl acetate (cis)</td>
<td>0.57</td>
<td>“</td>
</tr>
<tr>
<td>25</td>
<td>1377</td>
<td>α-Copaene</td>
<td>0.19</td>
<td>Sesquiterpene hydrocarbon</td>
</tr>
<tr>
<td>26</td>
<td>1419</td>
<td>β-Caryophyllene</td>
<td>0.95</td>
<td>“</td>
</tr>
<tr>
<td>27</td>
<td>1451</td>
<td>α-Himachalene</td>
<td>3.97</td>
<td>“</td>
</tr>
<tr>
<td>28</td>
<td>1478</td>
<td>β-Chamigrene</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>29</td>
<td>1485</td>
<td>Germacrene D</td>
<td>2.61</td>
<td>“</td>
</tr>
<tr>
<td>30</td>
<td>1490</td>
<td>β-Selinene</td>
<td>1.35</td>
<td>“</td>
</tr>
<tr>
<td>31</td>
<td>1514</td>
<td>γ-Cadinene</td>
<td>0.15</td>
<td>“</td>
</tr>
<tr>
<td>32</td>
<td>1523</td>
<td>δ-Cadinene</td>
<td>0.63</td>
<td>“</td>
</tr>
<tr>
<td>33</td>
<td>1563</td>
<td>Nerolidol (trans)</td>
<td>2.89</td>
<td>Sesquiterpene alcohol</td>
</tr>
<tr>
<td>34</td>
<td>1578</td>
<td>Spathulenol</td>
<td>8.46</td>
<td>“</td>
</tr>
<tr>
<td>35</td>
<td>1583</td>
<td>Caryophyllene Oxide</td>
<td>7.55</td>
<td>Sesquiterpene oxide</td>
</tr>
</tbody>
</table>
5.4. Acute toxicity study

A preliminary toxicity test was conducted to assess the acute lethal, physical and behavioral effects of crude extracts after oral administration to mice. The toxicity study was designed to demonstrate the approximate safe dose that could be used for subsequent experiments rather than to provide complete toxicity data on the extract. In this study, 70% ethanol and aqueous crude leaf extracts of *A. annua* were tested for their acute toxic effect against laboratory breed mice.

Oral administration of the extracts in doses from 1000 to 5000 mg/kg did not produce significant acute toxic effects on the experimental mice. Gross behavioral and physical observation revealed the absence of acute toxicity effects like lacrimation, hair erection, urination, muscle weakness, sedation and convulsion, reduction in motor and feeding activity in the test mice. Animals were observed during the experimental period (72 hours). During seventy-two hours of the experiment no death occurred in any of the test groups.

The mice were monitored for over a week but no sign of toxicity was observed. It was not possible to evaluate the toxicity of *A. annua* crude leaf extracts above 5000 mg/kg because of high viscosity of the suspension, which made it difficult to administer orally using the narrow gavages. The results of the study in single doses showed no adverse
effects for the crude extracts of *A. annua*, indicating that the median lethal dose (LD$_{50}$) of the extract could be higher than 5000 mg/kg/day for mice through oral route.

The results showed that 70 % ethanol and hot water extracts of *A. annua* were safe in oral administration in mice. This could explain the safe use of the plant by the local people, in traditional treatment of malaria, in “Chencha” area of Gamogofa in Southern parts of Ethiopia (MCL, 2006).

5.5. Antimalarial activity of the extracts against *P. berghei*

In this study the in vivo antimalarial activity test was done using the 4-day suppressive test procedure. It is a standard procedure, which has been used for early investigations of the *in vivo* efficacy of candidate antimalarial compounds, in the rodent parasite *P. berghei* (David *et al.*, 2004).

Both intraperitoneal and intravenous routes could be used for inoculation of infected erythrocytes to mice. The intraperitoneal route was used in this study due to its simplicity and ease of use. In four day suppressive test study, an extract was considered active if it suppressed the parasite level by 10 % or more (Peters, 1980).

Table 4 shows the effect of 70% ethanol extract of leaf of *A. annua* against *P. berghei* in mice. The extract showed significant suppression against the parasite in experimental mice at all dose levels tested compared to the negative control group. After 4 days treatment with the extract (150, 300 and 450 mg/kg), the mean parastemia of the test groups were found to be 18.35±1.00%, 14.60% and 6.30±0.99% respectively. The mean parastemia of the negative control was 28.60±0.98 %. The mice treated with chloroquine and coartem were completely free of the parasite on day 4.
Table 4: Antiplasmodial activities of 70% ethanol extracts of *A. annua* leaves in male Swiss albino mice infected with *P. berghei* (n=5).

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Dose mg/k</th>
<th>Activity against <em>P. berghei</em> in mice (%)</th>
<th>Mean survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/day</td>
<td>% Parasitaemia %</td>
<td>Suppression</td>
</tr>
<tr>
<td>A. annua leaf extract</td>
<td>150</td>
<td>18.35 ± 1.00</td>
<td>35.84</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>14.60 ± 1.00</td>
<td>48.95</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>6.30 ± 0.99</td>
<td>77.97</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Coartem</td>
<td>25</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>NC</td>
<td>1ml</td>
<td>28.60 ± 0.98</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Key: Values are M ± SD, P<0.05

NC: Negative control (1ml 5% tween 80 in distilled water, Vehicle)
ND: not done
The ethanol extract showed lowest (35.84 %) suppression against *P. berghei* at dose level of 150 mg/kg. Medium suppression (48.95%) was shown in mice treated with 300 mg/kg dose of the extract. The highest suppression of parastemia (77.97 %) was observed at the dose of 450 mg/kg. The suppressive activity of the extract was found to be dose dependent. As the dose increased the suppressive activity of the extract increased.

In this work the presence of artemisinin in crude plant material was determined to be low in concentration. However, the high suppression obtained in all dose levels could be partly due to the presence of artemisinin as well as other secondary metabolites like flavonoids which are claimed to have synergetic effects to the antimalarial activity of crude *A. annua* extracts.

The crude 70% ethanol extract has exerted its effect on the mean survival times of the treatment groups compared to the untreated control group. The mean survival time of the mice treated with dose level 150, 300, 450 mg/kg were 9.75 ± 1.10, 10.00 ± 0.00, 10.60 ± 1.34 days respectively. The mean survival times of the control (untreated group)
was 9.00 ± 1.00 days. All mice treated with the extract lived longer as compared to untreated mice. The mean survival time was observed to be dose dependant.

The water extract of the leaves of *A. annua* showed significant suppressive effect on day four of parastemia compared to the negative control (table 5). The average parastemia of the mice treated with the lowest dose was 26.45 ± 0.86 %. The parastemia of the mice treated with the highest dose was 11.35 ± 1.14 % while the mice in the control group were observed to have average parastemia level of 32.20 ± 1.60 %. This extract showed 17.86 % suppression against *P. berghei* at the lowest dose. Medium suppression was shown with mice treated with 300 mg/kg dose of the extract. The highest suppression of parastemia (64.75 %) was observed at the dose of 450 mg/kg. The suppressive activity was found to be dose dependent.

The survival time of mice treated with *A. annua* crude water extract showed significant changes compared to the untreated negative control. All mice treated with extract lived longer in comparison to untreated mice. It was also observed that as the dose of the extract increased the survival time was also increased.
Table 5: Antiplasmodial activities of hot water extracts of *A. annua* leaves in male Swiss albino mice infected with *P. berghei* (n=5)

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Dose (mg/kg/day)</th>
<th>Activity against <em>P. berghei</em> in mice (%)</th>
<th>Mean survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. annua</em> leaf extract</td>
<td>150</td>
<td>26.45 ±0.86</td>
<td>8.40 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>20.45 ±1.14</td>
<td>9.20 ± 1.10</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>11.35 ±1.14</td>
<td>9.40 ± 1.89</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Coartem</td>
<td>25</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td><em>NC</em></td>
<td>1ml</td>
<td>32.20 ±1.60</td>
<td>7.80 ± 0.84</td>
</tr>
</tbody>
</table>

Key: Values are M ± SD,  P<0.05

*NC*: Negative control (1ml distilled water, Vehicle)

*ND*: not done
As can be seen in figure 7 the hot water extract of leaves of *A. annua* showed lower antimalarial activity compared to ethanolic extract at all dose levels tested. However, it depicted good *in vivo* activity against the parasite. The observed antiplasmodial activities of the two extracts may indicate the presence of artemisinin and other compound(s) responsible for the antimalarial activity of the test plant.

Figure 7: Suppression level of 70 % ethanol and hot water extracts against *P. berghei*
In this study crude 70% ethanol extract was shown to have higher antimalarial effect than water. Artemisinin is claimed to have higher solubility in ethanol. However, because of health and other religious concerns towards alcohol the local people in chencha area of Gamogofa prepare *A. annua* extract in the form of green tea as an antimalarial remedy. Therefore the observed antimalarial activity of the two extracts particularly that of hot water is in agreement with the traditional use of the plant as herbal medication against the disease in Ethiopia (MCL, 2006).

Although the two solvents used in this study are polar in nature they could have different power in extracting the active ingredient from the plant material. Hence, the variation between the antimalarial activity of the water and ethanol extract of *A. annua* may indicate the difference in the presence of active compound(s) responsible for the observed activity in each of the extracts. It is clear from the results that in *P. berghei* infected mice treated with the extracts, the percentage suppression of parastemia measured changed significantly compared with the control animals. This significant suppression of parastemia on day four was also in agreement with that shown for ethanol extracts of the same plant against *P. berghei* in vivo in mice (Yao *et al.*, 1992).

In the course of the study it was observed that the plant extracts tested were less active than chloroquine and coartem, the standard antimalarial drugs used in the study. The standard antimalarial drugs used during this study suppressed parastemia to non detectable level, which is in agreement with previous study (Kiseko *et al.*, 2000). The mice treated with the extracts were not free from the parasites. The possibility of increased doses of the extracts producing maximum level of suppression cannot be ruled out as this observation is based on the tested doses.

The 70% ethanol and water extracts of leaves of *A. annua* prevented loss of body weight which was expected to be shown by all groups of mice as a result of infection with the parasite. The comparison among the groups indicated that the extracts prevented weight loses at all dose levels compared to the control. However, there is no significant loss or gaining of weight because all of the results are within the standard
deviation of the determination. The percent weight change indicated that increase in body weight was not found to be dependent on dose levels (table 6 and 7).

Table 6: Body weight of *P. berghei* infected mice on day zero and four after treatment with crude 70% ethanol extract of leaves of *A. annua* (n=5)

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose (mg/kg/day)</th>
<th>Wt. Day 0 (g)</th>
<th>Wt. Day 4 (g)</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. annua leaves</td>
<td>150</td>
<td>33.00±0.66</td>
<td>33.30±1.95</td>
<td>0.91</td>
</tr>
<tr>
<td>A. annua extract</td>
<td>300</td>
<td>31.30±1.48</td>
<td>32.30±0.45</td>
<td>3.14</td>
</tr>
<tr>
<td>A. annua extract</td>
<td>450</td>
<td>34.06±0.82</td>
<td>34.90±1.20</td>
<td>2.44</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>28.80±0.57</td>
<td>29.00±0.71</td>
<td>0.69</td>
</tr>
<tr>
<td>Coartem</td>
<td>25</td>
<td>30.38±0.41</td>
<td>30.50±0.61</td>
<td>0.39</td>
</tr>
<tr>
<td>NC</td>
<td>1ml</td>
<td>31.40±1.64</td>
<td>30.90±1.95</td>
<td>-1.61</td>
</tr>
</tbody>
</table>

Key: Values are M ± SD,  P<0.05
NC: Negative control (1ml 5% tween 80 in distilled water, Vehicle)
Day 0 = day infection was initiated
Day 4 = 5th day of infection

Table 7: Body weight of *P. berghei* infected mice on day zero and four after treatment with crude hot water extract of leaves of *A. annua* (n=5)

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose (mg/kg/da)</th>
<th>Wt. Day 0 (g ± SD)</th>
<th>Wt. Day 4 (g ± SD)</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. annua leaves extract</td>
<td>150</td>
<td>26.78±1.34</td>
<td>28.40±1.29</td>
<td>5.87</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>29.30±0.97</td>
<td>29.60±0.94</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>29.90±0.89</td>
<td>30.80±0.91</td>
<td>2.97</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>32.80±1.30</td>
<td>33.96±0.59</td>
<td>3.48</td>
</tr>
<tr>
<td>Coartem</td>
<td>25</td>
<td>33.40±1.10</td>
<td>33.56±0.59</td>
<td>0.48</td>
</tr>
<tr>
<td>NC</td>
<td>1ml</td>
<td>31.88±1.78</td>
<td>31.30±1.20</td>
<td>-1.84</td>
</tr>
</tbody>
</table>

Key: Values are M ± SD,  P<0.05
6. Conclusion

In this work seventy percent ethanol and hot water were used in the preparation of crude extracts from the dried leaves of *A. annua*. The yields obtained on dry weight bases were 20.2 and 14.00 % for 70 % ethanol and water respectively. Results from this study showed that oral administration of the aqueous and 70% ethanol extracts of *A. annua* in single doses ranging from 1000 to 5000 mg/kg showed no adverse effects, indicating that the extracts are safe in mice by oral route.
In this study, *in vivo* testing was used for assessing the antimalarial activity of *A. annua* leaf extracts. Theoretically an extract is considered active if it inhibits the growth of the parasite by more than 10%. Both extracts showed parasite suppressive effects on *P. berghei* infected mice in a dose related fashion and the 70% ethanol extract was observed to have a stronger activity compared to the water extract. The highest suppressive effect observed at 450 mg/kg of the 70% ethanol and water extracts were 77.97% and 64.75% respectively. Although the active ingredient responsible for antimalarial activity of these extracts is claimed to be artemisinin, phytochemical investigations also revealed the presence of groups of compounds like flavonoids which are reported to synergize the antiplasmodial activity of the test plant. This work clearly demonstrated the antimalarial effects of the 70% ethanol and water extracts for *A. annua* grown in Ethiopia, as seen in their ability to suppress *P. berghei* in Swiss albino mice.

The plant was also observed to continue producing the active component artemisinin, although in lower concentration (0.014%) when grown at Wondogenet. This concentration is within the reported range in previous studies. However, there could be a possibility to maximize its production by the plant by developing proper agricultural conditions. Factor such as altitude, rainfall, types of soil and seasonal variation with respect to artemisinin content need to be considered.

The essential oil yield of the study plant was 0.32% (v/w). From the GC-MS analysis data, it can be seen that the essential oil of *A. annua* is dominated by monoterpenes and sesquiterpenes. In this study, camphor was identified as the major component (43.84%) making the oil closely similar in terms of constituents to the oil from the Vietnamese plant. Artemisia ketone, isoartemisia ketone and artemisia alcohol were not detected during this study. The volatile constituent of the study plant except for the ones mentioned above was found to have similar constituents as the plants investigated elsewhere.
7. Recommendations and Suggestion

- In this work lower concentration of artemisinin was determined. Reports in different country also showed a large variation in artemisinin content in the leaves of different samples of *A. annua*. Further research work is thus recommended to be undertaken to find out the geographical location within Ethiopia for a better artemisinin yield.

- Essential oils obtained from *A. annua* are known for their flavoring agent in food and liquor industry and in pharmaceutics. A total of 38 compounds have been analyzed. A
large variation in essential oil contents has been reported. Therefore, further investigation of the volatile constituents need to be carried out to see if the plant can be applied for such purpose.

8. Reference


MCT (Malaria control today, 2005). Current WHO recommendations.

(http://www.who.int/malaria/docs/MCT_workingpaper.pdf) pp1-75 (Accessed on 10.05.07)

MEE (Malaria epidemic in Ethiopia, 2003). Effective treatment needed now.


MFS (Malaria fact sheet, 2004). Campaign for access to essential medicines

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