IN VIVO ANTI-MALARIAL ACTIVITY OF CRUDE EXTRACTS OF *MORINGA STENOPETALA* AND *WITHANIA SOMNIFERA* IN MICE

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ANOVA = Analysis of Variance.

CQ. = Chloroquine.

D4 = Day 4.

D6 = Day 6.

Dist. H_2O = Distilled water.

EOH = Ethanol.

M $\pm$ SEM = Mean plus or minus Standard Error of the Mean.

MOH = Ministry Of Health.

MSF = Medecins Sans Frotieres

NRBM = National Roll Back Malaria.

Param. = Parameters.

RITAM = Research Initiative on Traditional Anti-malarial Methods.

% Para. = % of Parasitemia.

% Supp. = % of Suppression.

SPSS = Statistical Package for Social Sciences.

WHO = World Health Organization.

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1. INTRODUCTION

Malaria is the world's most important tropical vector born, most prevalent parasitic disease, killing many people than any other communicable disease except tuberculosis and HIV/AIDS (Letvin *et al.*, 2001). It is a major public health problem in more than 100 countries, inhabited by a total of some 2.4 billion people (Angulo and Freson, 2002). Each year 300-500 million people contract malaria and it is the cause of an estimated 1.5-2.7 million deaths (WHO, 2000). In many developing countries malaria is a huge disease burden in medical costs, days of labor lost, and is also a cause for school absenteeism (WHO, 1996).

Ninety percent of malaria cases and deaths occur in Africa south of the Sahara, mostly among young children under five years old (Lindsay and Martens, 1998). It is also a major public health problem in Ethiopia; prevalent in about 75% of the total area of the country and about 65% of the total population is at risk with about 4 to 6 million cases annually (MOH, 2002). *Plasmodium falciparum* (60%) and *Plasmodium vivax* (40%) are the dominant parasites in the country (Deressa *et al.*, 2003).

The global malaria situation is worsening progressively; particularly in Africa, where most of the severe malaria epidemics have taken place, but in recent years large-scale malaria epidemics have occurred almost in all continents (Baird, 2000). The dramatic consequences of malaria epidemics in many areas of the world stem in large part from the economic and human factors. Major malaria epidemics coincide with periods of famine, economic crisis, war, or civil disturbances involving impoverished or displaced populations that are often affected by other diseases (WHO, 2000).

The risk caused by malaria in a population varies based on transmission, age, pregnancy and occupation. In areas where transmission is high, such as in wet savanna areas of tropical Africa and Papua New Guinea, children under the age of five and women in their first pregnancy are most vulnerable to the disease (WHO, 1997a). The risk associated with malaria infections in pregnant women includes, low birth weight, spontaneous abortion, and maternal mortality. In areas of low transmission, where epidemics may be common and the immunity of the population is low, all age groups are at risk (Kondrachine and Trigg, 1997).

Malaria species known to infect man are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. The parasite is transmitted mostly by mosquito vectors, and by blood transfusion. No reservoirs were known, except that chimpanzees are suspected to serve as reservoirs in central Africa (Gilman, 1997). The sporozoites inoculated to man by infected female *Anopheles* mosquito during blood meal, then infect liver cells, mature into schizonts, and release merozoites. The merozoites infect red blood cells and transform to trophozoites that mature and release merozoites, which infect more red blood cells. Some of the merozoites differentiate into male and female gametes. The gametes ingested by female *Anopheles* mosquito during blood meal are fertilized in the gut of mosquito and form zygotes. The zygotes elongate and become motile ookinetes, which invade the mosquito's midgut wall and develop into oocysts, in which the sporozoites develop. The sporozoites make their way to the mosquito's salivary glands. The inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

The four malaria species are more or less similar in the early clinical symptoms they cause. The patient will complain of headache, fever and pains all over the body, diarrhea and abdominal pain are sometimes present (Miller *et al.*, 1994). A patient with severe and complicated malaria will often present with impaired consciousness, weakness, and jaundice. Other complications are cerebral malaria, anemia, splenomegaly, hepatomegaly, fluid, electrolyte and acid-base disturbances and hypoglycemia (Crlark and Schofield, 2000).

1.1 MALARIA CONTROL AND DRUG RESISTANCE

Different control methods that target the vector and parasite were used to overcome economic, and health problems associated with malaria.

In door residual spraying using insecticides, such as DDT was responsible for the success of malaria control in the 1950s and 1960s (Teklehaimanot and Bosman, 1999). However, because of financial and operational constraints, applications carried on with time intervals caused insecticide resistant varieties and environmental pollution. Personal protection using insecticide treated materials as bed nets, curtains, mats, repellents, help as barriers to reduce human mosquito contact. However, due to economic constraints and behavioral changes of the vector, personal protection is not as effective as expected (Rozendaal, 1997). Biological control, using larvivorous fishes (WHO, 1995) and the bacteria *Bacillus thuringiensis var israelensis* H-14 (Bti) (Lengeler *et al.*, 1996) have been effective in eradication of the vector in confined environment but are not effective on large scale application where the breeding sites are not manageable. Environmental management approaches by modifying the environment to deprive sites for breeding, resting, and feeding of the vector can reduce human

vector contact, and reduce disease transmission but the method is not effective enough at community level (WHO, 1995).

Chemotherapy is the principal control method where different anti-malarial drugs have been widely in use and have great role in protecting malaria than all other methods. However, the worsening problem of drug resistance in many parts of the world and the limited number of anti-malarial drugs available have made it more difficult to develop effective anti-malarial drug policies and provide adequate disease management (White, 1998).

The other best, safe and cost effective method in malaria control is vaccine. Research activities have been in progress for several years on this important issue, and currently trials are seeing for three stages of the parasite using cocktail or DNA vaccine and promising results were obtained (Richie and Saul, 2002). However, since malaria parasites exist within red blood cells for much of its life cycle and due to antigenic variation, the parasite is not exposed to the immune system, and hence candidate vaccines are not as effective enough as expected (Holder, 1999).

In the early 1960s, chloroquine resistance arose almost simultaneously among non-immune migrants working in the areas of hyperendemic malaria along the Thailand-Cambodia border (Young and Moore, 1961). In 1977 the ineffective chloroquine was replaced by sulfadoxine pyrimethamine in these areas, but resistance to it also developed. As a result, the first line treatment was changed to quinine in 1980 and to quinine and tetracycline in 1981 (Meek, 1988).

Plasmodium falciparum to both mefloquine and halofantrine have been detected *in vitro* and *in vivo* in Africa (Brasseur *et al.*, 1992, Basco *et al.*, 1991).

Resistance of *Plasmodium vivax* to chloroquine was first documented in 1989 in infections acquired in Papua New Guinea (Rieckmann *et al.*, 1989). It has then been confirmed in Indonesia, and Burma (Baird *et al.*, 1991; Myat-phone-kyaw *et al.*, 1993). In some areas of Papua New Guinea, 20 to 30% of patients infected with *Plasmodium vivax* have recurrences of parasitemia 1 to 3 weeks after a full treatment dose of chloroquine 25 mg /kg (Schurkamp *et al.*, 1992).

In Ethiopia 25 mg/kg of chloroquine administered for three days has been the first line drug for the treatment of uncomplicated malaria. The emergence of chloroquine resistant *Plasmodium falciparum* was first reported by Teklehaymanot (1986) in areas bordering with neighboring countries. Further study carried by the Ministry of Health confirmed that treatment with chloroquine failed to produce acceptable clinical improvement in the majority of *Plasmodium falciparum* patients in most parts of the country (MOH, 1999).

Then after, sulfadoxine pyrimethamine was used in place of chloroquine for laboratory confirmed resistant cases of *Plasmodium falciparum*. To treat confirmed cases of *Plasmodium vivax* chloroquine is recommended, relapse and gametocyte stages are treated by primaquine. Combinations of chloroquine and sulfadoxine pyrimethamine are recommended in conditions where microscopy is not available (WHO, 2001). Quinine administration is practical as alternative drug in hospitals, especially among inpatients (Mengesha *et al.*, 1998).

Now day's artemisinin and artemisinin containing combined treatment had in use in African countries such as South Africa, Kenya and Sudan during epidemics (MSF, 2003). In Ethiopia still artemisinin is not registered in malaria drug regimens, but Ministry of Health is aware of the problem of resistance to current first line drugs and in collaboration with the WHO Ministry of Health was conducting a nation wide resistance study and clinical trials with artemisinin combination treatment (Coartum) (MSF, 2003).

The resistance of *Plasmodium falciparum* has expanded over the years and by now almost all countries where *Plasmodium falciparum* malaria is endemic have recorded chloroquine resistance. In view of this serious malaria situation, WHO adopted a Global Malaria Control Strategy. The strategy called for disease management programs rather than parasite-oriented control programs. The objectives of the Global Strategy are to prevent mortality, reduce morbidity, and economic loss resulting from disease through the progressive improvement and strengthening of local and national capabilities (WHO, 1993).

This strategy has four basic technical elements:

1. Providing early diagnosis and immediate treatment
2. Planning and implementing selective and sustainable preventive measures, including vector control.
3. Detecting epidemic conditions as early as possible and preventing it soon.
4. Strengthening local capacities in basic and applied research to permit and promote the regular assessment of a country's malaria situation, in particular the ecological, and economic determinants of the disease.

specificity, low toxicity and effectiveness for treatment of multi-drug resistant *Plasmodium falciparum*, and active against cerebral malaria (Bilia *et al.*, 2002), stimulated interest for the search of anti-malarial substances from other plants.

1.2 ANTI-MALARIA TRADITIONAL MEDICINES

In vivo screening of extracts from forty-eight Brazilian medicinal plants in white albino mice revealed that *Vernonia brasiliana, Eupatorium squalidum, Acanthospermum australe, Esenbeckia febrifuga, Lisianthus speciosus and Tachia guianensis*, showed 40-50% inhibition of *Plasmodium berghei* multiplication. *In vitro* test using *Plasmodium vivax* cultures indicated that *Verononia brasiliana* and *Acanthospermum australe*, caused about 50% inhibition (Carvalho *et al.*, 1991).

In vitro evaluation of anti-malarial activities of Guatemalan medicinal plants namely *Simaroubae glauca* (Simaroubacea), *Sanseveria guineensis* (Agavaceae), *Croton guatmalensis* (Euphorbiaceae), and *Neurolaena lobata* (Asteraceae) indicated that dichloromethane exacts were active against both chloroquine resistant and susceptible *Plasmodium falciparum*. *In vivo* water extract of *Croton guatemalensis* cortex significantly inhibited *Plasmodium berghei* (Franssen *et al.*, 1997).

In Malaysia, *Piper sarmentosum, Tinospora crispa, Goniothalmus scortechinii* (Annonaceae) and *Andrographis paniculata* (Acanthaceae) are commonly used by local people for treatment of fever. *In vitro*, the chloroform extract of *Andrographis paniculata* showed complete parasite growth inhibition at 0.05 mg/ml dose within 24hr incubation period. It also showed high anti-malarial activity *in vivo* (Najib *et al.*, 1999). Methanol extract of leaf of

sensitive strain indicated that; water extracts of *Alchornea cordifolia*, and *Terminalia glaucescens* actively inhibited development of the above strains (Mustafa *et al.*, 2000). *In vitro* tests of *Lippia multiflora*, *Venoria colorata*, *Aladirachata indica*, *Combretum micrathum*, *Cinnamonum camphora*, and *Xinenia americana* indicated that extracts of *Xinenia americana*, *Lippia multiflora*, and *Combretum micrathum*, showed strong anti-malarial activity to both chloroquine resistant and sensitive strains of *Plasmodium falciparum* (Benoit *et al.*, 1996).

In vivo test of *Quassia amara* and *Quassia undulata* two Nigerian medicinal plants indicated that the hexane and methanol extracts of the leaf of the two plants have showed highest suppressive activity (Ajaiyeoba *et al.*, 1999). The *in vivo* test result of *Cassia occidentalies* root bark, *Morinada morindodiesa* leaf, and *Phyllanthus niruri* the whole plant, indicated that ethanol and dichloromethane, extracts produced significant chemosuppression, with dichloromethane extracts of *Morinada morindodiesa*, and *Phyllanthus niruri* showing highest suppressive effect (Tone *et al.*, 2001).

In Zimbabwe *Cussonia spicata* (Araliaceae), *Artemisia afra*, *Vernonia colorata*, *Veronia natalensis* (Asteraceae), *Parinari curatellifolia* (Chrysobalanaceae), *Clutia hirsuta*, *Flueggea virosa*, (Euphorbiaceae), *Adenia gummifera* (Passifloraceae) and *Hymenodictyon floribundum* (Rubiaceae) are used to treat malaria locally. *In vitro*, extracts from the aerial parts of *Artemisia afra* and *Vernonia colorata* are the most active against chloroquine sensitive strain PoW and chloroquine resistant clone Dd2 of *Plasmodium falciparum* (Kraft *et al.*, 2003). A tea prepared from *Artimesia annua* hybrid was administered orally to five malaria patients. The parasite disappeared in all individuals within 2-4 days. An additional trial with 48 malaria

patients showed a disappearance of parasitemia in 44 patients within 4 days (Mueller *et al.*, 2000).

In vitro test on parts collected from eleven medicinal plants in the Sudan indicated that 59% of plant extracts exerted activity on 3D7 chloroquine sensitive *Plasmodium falciparum* strain where as 43% of extracts exerted activity on chloroquine resistant and pyrimethamine sensitive Dd2 strains. Extracts of *Gardenia lutea*, *Haplophyllum tuberculatum*, *Cassia tora*, *Acacia nilotica* and *Aristolochia bracteolate* showed activity to both strains (El-Thair *et al.*, 1999).

In vitro test on 43 species of medicinal plants used in Tanzanian indicated that about 37% of the plants showed strong anti-malarial activity. Among these *Cissampels mucronta*, *Maytenus seneglensis*, *Salacia madagacriesis*, and *Zanthooxylum chalybeun* have the most active effect (Gessler *et al.*, 1994).

In Kenya local people use *Phyllanthus reticulatus* Poir., *Suregada zanzibariensis* Baill. (Euphorbiaceae) *Terminalia spinosa* Engl. (Combretaceae) and *Dissotis brazzae* Cogn. (Melastomataceae) for treatment of fever. *In vitro* preliminary anti-malarial screening indicated that, out of 16 extracts, 12 were active against chloroquine resistant ENT36 strain; seven were active against chloroquine sensitive K67 strain. The most active extracts on both strains were leaves of *Phyllanthus reticulatus* Poir, and *Suregada zanzibariensis* Baill. (Omulokoli *et al.*, 1997).

Since the whole extract might not be responsible for chemosuppression, after testing the crude extract further fractionations may provide specific and effective compounds. For example, extracts of *Brucea javanica* fruit had shown that the anti-malarial activity of the fruit was attributable to its quassinoid constituents (O'Neili *et al.*, 1987). Similarly among extracts obtained from the root bark of *Cryptolepis sanguinolenta in vitro tests* indicated that cryptolepine and neocryptolepine are responsible for the strong activity against *Plasmodium falciparum* chloroquine resistant K-1, and W-2 strains. Also *in vivo* tests on infected mice had shown that cryptolepine, has significant effect against *Plasmodium berghei yoelii* and *Plasmodium berghei berghei* (Cimanga *et al.*, 1997).

It was also necessary to consider plants that can serve both as medicinal and as food, because as some studies indicate human population adaptation to malaria is both biological (genetic) and cultural (dietary) (Greene, 1999). A case control study in Thailand indicated that consumption of *Vicia faba* seed, a leguminous plant, which is a dietary staple in the Mediterranean region, significantly enhanced anti-malarial protection in individuals who were heterozygous or homozygous for hemoglobin E (Kitayapuran *et al.*, 1992).

Among the *Housa* population in Northern Nigeria, out of 309 plants tested in cell culture for their efficacy against *Plasmodium falciparum*, 126 had anti-malarial effect among which 82 plants are used as food. Aqueous or ethanol extracts of 22 of the plants showed an *in vitro* activity against *Plasmodium falciparum.* In general among 54 most commonly used *Hausa* anti-malarial remedies, 44(82%) are used in diet, and 39(89%) of these were used both as anti-malarial and food, 26(67%) were available during the period of highest risk of malaria in the area (Etkin, 1997).

Ethiopia is a country rich in its flora and fauna having different climatic conditions and altitudes. Such a condition makes the country a potential home of different indigenous plants most of which are used as traditional remedies. Most of the rural population in Ethiopia depends on enormous resources of plant species that are used in traditional medicine. It has been reported that, among the 700 higher plant species that are known to exist in the country, there are an estimated 800 that are employed in the traditional health care delivery system to prevent and treat nearly 300 physical and mental disorders (Kelbessa *et al.*, 1992). As in other African countries due to shortage of health facilities and economic constraints, the local people are forced to use traditional medicinal plants for treatment of different diseases at the primary health care level (Kaba, 2003).

Ethiopian traditional medical system is characterized by variation and is shaped by the ecological diversities of the country, socio-cultural background of the different ethnic groups as well as historical development, which are related to migration, introduction of foreign culture and religions (Tilahun, 2003). Plants such as *Withania somnifera*, *Phytolaca dodecandra*, *Jasmincum abssynicum*, *Combretum molle*, *Cucumis prophetrum*, are widely used in treatment of fever most likely caused by malaria, in different parts of the country (Abate, 1989). Around Butajira, *Adhatoda schimperiana*, *Veronia amygdalia* Del, *Artemisia rehan*, *Croton macrostchys*, *Hallium sativum*, *Foenicum vulgare, and Withania Somnifera* parts are used in traditional treatment of malaria (Gedif and Hahn, 2002).

Withania Somnifera leaf and root powders are used in West Africa for treatment of fever and chills. In South Africa the root powder was used as anti-helmentic agent. The root and leaf contain withanolide glycosides and stiroindosides, which have been shown to be immuno stimulant that mobilize macrophages (James, 1985).

In Arbaminch and villages nearby roots and leaves of *Moringa stenopetala* chopped up and mixed with water are used for treatment of malaria; the seeds were crushed and used for water purification as a flocculating agent and disinfectant (Mekonnen and Gessese, 1998). The seeds are also effective against skin infecting bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Mark, 1998).

Mekonnen and Drager (2003) have isolated glucosinolates from the seeds, leaves and roots, which are claimed to have therapeutic importance. The fresh root wood ethanol and the dried leaves acetone extracts were active against *Trypanosoma brucei* (Mekonnen *et al.*, 1999). All parts of the tree except the wood is edible and leaves, flowers, fruit pods are rich sources of calcium and iron, and good source of vitamins A, B and C (when consumed raw) and protein (Rams, 1994). It is an important food plant in Southern Ethiopia, where it is cultivated as a crop plant, and the leaves are boiled and eaten as cabbage and sold in local market (Mekonnen and Gessese 1998).

Since the vast knowledge surrounding traditional medicine is conveyed from one generation to the next by word of mouth with no documentation (Addis, *et al.*, 2001), and persisting negative attitudes of traditional healers and modern health professionals for collaboration, almost no scientific researches have been done on the clinical effectiveness of herbal remedies as they are used in real life. Recently researchers in the country have focused on finding more scientific information and trying to isolate and purify medically active ingredients from medicinal plants based on traditional values and claims.

In vitro test carried by Sorsa (1992) indicated that methanol extract of the fruit of *Croton macrostachys* completely inhibit schizont maturation at concentration of 15.4 gm/ml. Chloroform and methanol extract of the leaves of *Withania somnifera* and chloroform extract of the leaves of *Veronia amygdalina* also showed substantial antimalarial activity *in vitro* (Bogale and Petros, 1996). Also *in vitro* investigation of *Bersama abyssinca* (Meliathaceae) extract exhibited a significant antimalarial activity against *Plasmodium falciprum* (Kassa *et al.*, 1996).

In vitro experiment carried by Asress and Balcha (1998) on the stem bark of *Combretum molle* (Combertaceae) indicated that the acetone and methanol extract possessed remarkable schizont inhibition to *Plasmodium falciparum*. *In vitro* study on extract of five Ethiopian medicinal plants against the Ethiopian isolate *Plasmodium falciparum* line FCA-2/ Ethiopia indicated that ethanol extracts of *Artemisia afra* and *Artemisia rehan*, and *Adjuga remota* were found to have high anti-malarial activity (Kassa *et al.*, 1998). An *in vivo* test carried by Animut (2002) also showed that water extracts of *Vernonia amygdalyna* leaf and methanol extract of *Croton macrostachyus* fruits have significant suppressive effect against *Plasmodium berghei*.

In Ethiopia, as in the rest of Sub-Saharan Africa, malaria remains the major health problem with *Plasmodium falciparum* accounting for most deaths. Provision of adequate treatment is presently one of the main tools of malaria control strategies in the country. However, the growing problem of drug resistance of the parasite to currently used safe and affordable drugs (chloroquine and sulfadoxine/ pyrimethamine) make the problem serious. Such a situation will have a dramatic impact in the future, since the cost of treating malaria with anti-malarial

drugs other than chloroquine and sulfonamide pyrimethamine, is prohibitive for a large majority of the populations in the country.

Therefore, there is a need to search for new cost effective and safe drugs from traditional medicinal plants that are used by local people for treatment of malaria. Thus the present study was designed to examine the safety and anti-malarial property of some medicinal plants *in vivo* using *Plasmodium berghei* (rodent malaria, ANKA strain) whose natural host is the tree rat *Grammomys surdaster*. The parasite is a valuable experimental model for research in parasitology, immunology, and chemotherapy of malaria (Jans and Waters, 1995).

Objectives of the study:

1. General objective

To evaluate *Withania somnifera* and *Moringa stenopetala*, two plants popularly reported with anti-malarial property by traditional medicinal practitioners in an *in vivo Plasmodium berghei* mice model system.

2. Specific objectives

2.1. To evaluate the safety level of water and ethanol crude extracts of the leaves and roots of *Withania somnifera* and *Moringa stenopetala*, in mice.

2.2. To validate the ethnopharmacological information about their anti-malarial effect and the reported *in vitro* suppressive effect through an *in vivo* efficacy test of the crude extracts of *Withania somnifera* and *Moringa stenopetala* in mice infected with *Plasmodium berghei*.

Withania somnifera the local name in Amharic *Gizawa* is a plant widely distributed in drier tropical Africa and Asia. It is an erect much brunched shrub growing up to 1 meter high with distinctively hairy stem, the leaves are simple about 10 cm long elliptic to broadly ovate-lanceolate, with entire or wavy margins. The flowers are small about 1cm long, greenish or yellow occurring in short axillary clusters. It produces red globose fruits 6mm in diameter enclosed in the inflated and membranous calyx (Maurice, 1993) (Fig. 2).

2.2 EXPERIMENTAL PROCEDURES

2.2.1 PLANT MATERIAL EXTRACTION

Plant parts collected were air dried at room temperature and ground into powder by electrical grinding mill in the Biomedical Laboratory in the Department of Biology, Addis Ababa University. Then the powder was carefully sieved, and weighed and (600 gm and 355 gm of leaf and root powder of *Moringa stenopetala*; 480 gm, and 328 gm of the leaf and root powder of *Withania somnifera* respectively) were collected in plastic bags and put in deep freezer at (-70^0c) until extraction.

Distilled water and 100% ethanol, were extraction solvents; 30 gm of the dry powder of the root and the leaf of *Moringa stenopetala* and *Withania somnifera* were measured using weight balance and mixed with 600ml. of the selected solvents in Erlenmeyer flasks. After shaking well flasks containing the solution were put on orbital shaker and left for 24 hrs at a speed of 120 revolutions per minute.

After 24 hr each solution was filtered separately using cotton and 15 cm size Whatman filter paper (Made in England by Whatman limited; it is 15 cm size, ultra-fine filter with retention down to 0.7 μm in liquids) respectively. The filtrates were dried using lyophilizer for water extracts and rotary evaporator for ethanol extracts at a temperature of about 40-45^{0}c. The dried extracts (from 210 and 250 gm of water and ethanol extract of the leaf of *Moringa stenopetala* 21.7 and 12. 6 gm, from 150 and 155gm of water and ethanol extract of the root of *Moringa stenopetala* 11 and 8gm; In the same way from 210 and 260 gm of water and ethanol extract of leaf of *Withania somnifera* 17.20 gm and 14. 8 gm, from 150 and 175 gm of root of *Withania somnifera* 13 and 9 gm) were collected in labeled sterile small bottles and put in deep freezer until needed for the experiment.

2.2.2 *IN VIVO* ACUTE AND SUBACUTE TOXICITY TEST OF EXTRACTS

Toxicity test was carried out using Swiss albino male mice, which are 6 to 7 weeks old and weighing 23-27 gm. The mice were bred in the Animal House of the Department of Biology, Addis Ababa University. The mice were fed with standard mouse cubs and also given clean drinking water. The parameters measured were red blood cell (RBC), white blood cell (WBC) counts using counting chamber; percentage of packed cell volume (PCV) using hematocrit reader; weight in grams using a weighing balance; physical condition and feeding behavior of the mice by careful cage side observation. The parameters considered have the following normal values in mice, RBC = 6.7-12.5 million, WBC= 5.4-16 thousand, PCV= 32-54 percent and adult weight 25-40 gm (Tuffery, 1987).

The acute and sub acute toxicity test for each of the above crude extract was conducted by using four groups of Swiss albino male mice weighing 23-27 gm each containing three. One of the groups was a control group that was given extraction solvent the other three are the test groups. The acute toxicity effect was measured in 24 hrs period after giving single oral doses of 900, 750 and 600 mg/kg body weight of the crude extracts. Careful cage side visual observations on changes in the fur, feeding and movement activities of mice were made at every 4hr interval after the administration of extracts.

The subacute toxicity test was evaluated based on the method of Witthawaskul *et al.* (2003). On day 0 after physical observation RBC, WBC count, PCV values and weight are measured. Then 0.5 ml/mouse of each extract 900, 750 and 600 mg/kg/day was given for group 1, 2 and 3 respectively and 0.5 ml/mouse of the extraction solvent was given for group 4 mice for three consecutive days at 24 hr interval. The measured RBC, WBC, PCV, body weight and cage side physical observation values were recorded on day 0 before administration the extracts and on day 4 after giving the extracts. Doses which induce any change in the physical condition of mice and significant difference on the above measured parameters would not be included in the efficacy test.

2.2.3 *IN VIVO* ANTI-MALARIAL SUPPRESSIVE TEST OF EXTRACTS

Male Swiss albino mice weighing 23-27 gm were used for the test. The mice were bred at the Animal House of the Department of Biology Addis Ababa University. The animals were supplied with mouse cubs and clean drinking water. During the test the animals were put randomly into five groups each group containing four mice. The parasite strain used for the

test was rodent malaria *Plasmodium berghei* that was maintained by serial passage of blood from mouse to mouse (Ajaiyeoba *et al.*, 1999).

The test based on the four-day suppressive test described by Peters *et al.* (1975). Further investigation on extracts with significant effect was made by giving extracts until infected mice died (Satayavivad *et al.* 1998). The parasitized erythrocytes for each test were collected from tip of tail of an infected mouse by taking snip of blood in sterilized syringe and diluted with proportional amount of sterile saline. Each mouse was inoculated with 0.2 ml of blood containing about $9.5x10^6$ *Plasmodium berghei* infected erythrocytes on day 0 intravenously at the tail region. Intravenous administration was advantageous for even distribution of the parasite and to avoid variation in the course of infection (Peters, 1987).

Infected mice were treated with different extracts, chloroquine and extraction solvents orally based on their weight. Group 1 mice were the negative control groups given 0.5 ml/ mouse of the extraction solvent either water or ethanol. For those taking ethanol, 10% ethanol was given since such amount has no any toxic effect on the mice (Mekonnen and Gessese, 1998). Group 2, 3, and 4 were the test groups administered with 600, 750 and 900 mg/kg of the extract, and each mouse was given 0.5 ml of the extract. Group 5 mice were the positive controls and were given chloroquine (10 mg/kg/day) manufactured in Adigrat, Ethiopia, which was effective to drug sensitive strain of *Plasmodium berghei.*

Oral administration of the extracts, chloroquine and extraction solvent to infected mice started after three hrs of parasite inoculation. Further doses were administered on day 1, 2 and 3 and mice received a total of 4 doses following O'Neill *et al.* (1987) procedure. Plant extracts

which showed significant suppressive effect on parasitemia level during the 4th day efficacy test were further evaluated by continuous treatment every 12 hr until the infected mice died using the protocol of Satayavivad *et al.* (1998). The progress of the parasite was checked by parasitemia count per field on slide by taking tail snip blood smears that were fixed by methanol and Giemsa stained, on day 4 and 6 for the 4 day efficacy test and for further investigation of extracts with significant effect smears were made daily until mice died.

2.3 DATA ANALYSIS

The data for toxicity (Table 1-4) and efficacy tests (Table 5-10) are presented in M±SEM. The data were analyzed using SPSS version 10. The toxicity test was analyzed using one-way ANOVA followed by Posthoc Scheffe's procedure to compare between groups on the same day and Student's paired t-test to compare with in groups taking the same dose at different day. The efficacy test data were also analyzed by one-way ANOVA followed by Posthoc Scheffe's procedure to compare parasitemia level between groups on the same day. Student's paired t-test was used to compare the parasitemia level, with in group taking the same dose at different day (Kirkwood, 1988). All data were analyzed at 95% confidence interval (α = 0.05). Percentage of suppression will be calculated by using the formula proposed by Munoz *et al.* (2000). That is;

$$\textbf{\% of suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia with extracts}}{\text{Parasitemia in negative control}} \times 100.$$

3. RESULTS

3.1 ACUTE AND SUBACUTE TOXICITY TEST

The acute test on mice administered with the water and ethanol extracts of the root and the leaf of *Moringa stenopetala* and *Withania somnifera* indicated that single dose of all the extracts administered orally were non lethal to mice up to dose of 900 mg/kg in 24 hr. The subacute toxicity test also indicated all extracts at dose 900, 750 and 600 mg/kg did not produce death of the mice administered with the extract every 24 hr for 4 days. The mice did not show change in general behavior or other physiological activities such as movement and feeding and weight loss as compared to the control group.

The overall ANOVA between group 1, 2, 3, and 4 mice on day 0 before the administration of water extracts of the leaf and the root of *Moringa stenopetala* did not show a statistically significant difference in all parameters measured. The analysis of variance of group 1, 2, and 3 mice administered with 900, 750 and 600 mg/kg, of the leaf and the root extracts on day 4 was not statistically significant in all parameters measured, in comparison to control group 4 The Student's paired t-test result also indicated that all parameters measured did not show statistically significant difference between day 0 and day 4 in-group 1, 2 and 3 mice administered with 900,750 and 600mg/kg (Table1).

Table 1. Subacute toxicity test of water extract of leaf and root of *Moringa stenopetala* in mice.

Part	Group	Dose	Day	RBC (10^6)	WBC (10^3)	PCV (%)	Weight(gm)
Leaf	1	900 mg/kg	0	9.21± 0.24	9.85± 0.32	50.16 ± 0.66	24.50± 0.28
			4	9.22± 0.24	9.86± 0.33	50.20 ±0.69	24.53± 0.18
	2	750 mg/kg	0	9.65±0.19	9.84± 0.10	51.13 ± 0.56	24.87± 0.21
			4	9.67±0.19	9.85± 0.09	51.19 ±0.89	25.03± 0.13
	3	600 mg/kg	0	9.20± 0.03	9.81± 0.30	51.03 ± 0.52	24.83± 0.22
			4	9.21± 0.04	9.81± 0.29	51.08 ±0.52	25.10± 0.12
	4 (control)	0.5 ml. Dist. H_2O	0	9.41±0.16	9.78± 0.75	50.63 ± 0.44	24.66± 0.33
			4	9.42±0.16	9.78± 0.77	50.65 ±0.77	25.00± 0.34
Root	1	900 mg/kg	0	9.55 ±0.34	9.92± 0.04	51.00±0.32	24.70± 0.36
			4	9.55± 0.34	9.93± 0.06	51.10± 0.58	24.90±0.49
	2	750 mg/kg	0	9.67±0.21	9.91 ±0.08	50.83± 0.49	24.67± 0.33
			4	9.68±0.21	9.93± 0.07	50.87±0.73	24.93± 0.52
	3	600 mg/kg	0	9.91±0.03	9.84± 0.10	50.80± 0.46	25.16±0.16
			4	9.92± 0.03	9.86± 0.10	50.83± 0.46	25.18± 0.21
	4 (control)	0.5 ml. Dist.H_2O	0	9.59±0.20	9.80±0.57	51.40±0.25	24.44±0.20
			4	9.60±0.21	9.80±0.04	51.42± 0.14	25.46± 0.31

N.B All tests were compared against the control group that were given the extraction solvent.

Table 2. Subacute toxicity test of ethanol extract of leaf and root of *Moringa stenopetala* in mice.

Part	Group	Dose	Day	RBC (10^6)	WBC (10^3)	PCV (%)	Weight (gm)
Leaf	1	900 mg/kg	0	9.62 ± 0.32	10.04± 0.08	50.31±0.37	24.33±0.18
			4	9.62 ± 0.32	10.39±0.08*[1,4], *a	50.32±0.37	24.43±0.23
	2	750 mg/kg	0	9.51± 0.02	9.89± 0.05	49.97 ±0.51	25.00±0.23
			4	9.52± 0.18	9.91± 0.04	50.00±0.61	25.35 ±0.24
	3	600 mg/kg	0	9.38±0.24	9.93± 0.02	49.70±0.36	25.00±0.16
			4	9.39±0.25	9.94± 0.02	49.80±0.26	25.33±0.33
	4 (control)	0.5ml.10% EOH	0	9.38 ± 0.20	9.81± 0.03	50.31± 0.49	24.40±0.17
			4	9.38 ± 0.15	9.82± 0.02	50.33± 0.54	25.00 ±0.33
Root	1	900 mg/kg	0	9.55±0.34	9.96 ± 0.04	51.00 ±0.51	24.26 ± 0.17
			4	9.55 ± 0.34	10.08±.0.06*[1,4,] *a	51.00 ±0.57	24.60±0.49
	2	750 mg/kg	0	9.67 ± 0.21	9.90 ± 0.08	50.83 ±0.49	24.70± 0.15
			4	9.67 ± 0.21	9.91±0.07	50.85 ±0.57	24.93± 0.52
	3	600 mg/kg	0	9.91± 0.02	9.80± 0.10	50.90± 0.46	24.36± 0.12
			4	9.92± 0.03	9.81± 0.10	50.93± 0.46	25.56± 0.28
	4 (control)	0.5 ml.10% EOH	0	9.59± 0.2	9.70± 0.03	51.40 ±0.25	24.43± 0.21
			4	9.60±0.21	9.70± 0.03	51.41 ±0.14	25.00± 0.31

Keys: * = P < 0.05,

a = comparison between Day 0 and Day 4.

1, 4 = Comparison between Group 1 and the respective control group.

N.B All tests were compared against the control group that were given the extraction solvent

Among mice given 900, 750, and 600 mg/kg of the leaf extracts, the multiple comparison analysis showed statistically significant difference on the WBC count of group 1 mice given 900 mg/kg on day 4 ($P = 0.021$) and the root extract also showed significant difference ($P = 0.035$) in comparison to control group mice. The Student's paired t-test analysis showed statistically significant difference on the WBC count of group 1 mice given 900 mg/kg of the leaf extract ($P = 0.036$) and the root extract also showed significant difference ($P = 0.034$) between day 0 and day 4. Multiple comparison and Student's paired t-test analysis of extracts at doses 750 and 600 mg/kg did not show statistically significant difference in all parameters measured (Table 3).

Table 3. Subacute toxicity test of water extract of leaf and root of *Withania somnifera* in mice.

Part	Group	Dose	Day	RBC (10^6)	WBC (10^3)	PCV (%)	Weight (gm)
Leaf	1	900 mg/kg	0	9.24± 0.32	9.71± 0.03	50.63± 0.06	24.70± 0.15
			4	9.25± 0.32	9.99± 0.04*[1,4,*]a	50.66± 0.06	24.74± 0.16
	2	750 mg/kg	0	9.25± 0.18	9.87± 0.27	50.33± 0.37	24.70± 0.20
			4	9.26± 0.18	9.88± 0.27	50.36± 0.29	25.00± 0.33
	3	600 mg/kg	0	9.64± 0.10	9.92± 0.09	50.70± 0.50	24.53± 0.29
			4	9.65± 0.09	9.92± 0.03	50.90± 0.31	25.00± 0.33
	4 (control)	0.5ml. Dist. H_2O	0	9.40± 0.25	9.68± 0.27	50.00± 0.4	24.40± 0.03
			4	9.40± 0.25	9.68± 0.05	50.16± 0.17	24.83± 0.44
Root	1	900 mg/kg	0	9.42 ±0.04	9.73 ± 0.06	51.83±0.93	24.18± 0.15
			4	9.43 ±0.04	9.96 ±0.06*[1,4,*]a	51.90±0.91	25.76± 0.23
	2	750 mg/kg	0	9.38 ± 0.13	9.74 ± 0.05	50.53±0.74	25.13± 0.06
			4	9.38 ± 0.13	9.76 ± 0.05	50.53±0.76	25.13 ± 0.00
	3	600 mg/kg	0	9.56 ± 0.14	9.78 ± 0.03	50.33±0.88	25.13± 0.06
			4	9.57 ± 0.14	9.78 ± 0.03	50.56±0.56	25.21± 0.33
	4 (control)	0.5ml. Dist.H_2O	0	9.68 ± 0.04	9.58± 0.03	50.69±0.81	25.33± 0.33
			4	9.69 ± 0.03	9.58 ± 0.03	50.79 ±0.57	25.33± 0.33

Key: * = $P < 0.05$,

a = variation between % parasite of Day 0 and. Day 4.

1, 4 = Comparison between Group 1 and the respective control group.

N.B All tests were compared against the control group that were given the extraction solvent.

The overall analysis of variance of all parameters measured on day 0 before administration of the ethanol extract of the leaf and the root of *Withania somnifera* did not show statistically significant difference between groups. As the multiple comparison analysis of parameters measured after administration of 900, 750 and 600, mg/kg, of ethanol extracts of the leaf of *Withania somnifera* indicated, statistically significant difference was seen on WBC count of mice administered with 900 mg/kg on day 4 ($P = 0.042$) and the root also showed significant difference ($P = 0.044$) in comparison with the control group. The Student's paired t-test analysis of mice administered with 900 mg/kg of the leaf extract indicated that the WBC count showed statistically significant difference between day 0 and day 4 ($P = 0.040$) and the root extract also showed significant difference ($P = 0.035$). The multiple comparison analysis of all parameters measured at dose 750 and 600 mg/kg of ethanol extracts of the leaf and the root of *Withania somnifera* did not show statistically significant difference on day 4 in comparison to the control group and the Student's paired t-test result between day 0 and day 4 also did not show statistically significant difference (Table 4).

Table 4. Subacute toxicity test of ethanol extract of leaf and root of *Withania somnifera* in mice.

Part	Group	Dose	Day	RBC (10^6)	WBC (10^3)	PCV (%)	Weight (gm)
Leaf	1	900 mg/kg	0	9.62±0.32	9.94±0.05	51.00±0.33	24.33±0.33
			4	9.62±0.31	10.19±0.03*[1,4,*]a	51.20 ±0.33	24.53±0.33
	2	750 mg/kg	0	9.51±0.02	9.86±0.01	50.77±0.33	25.00±0.33
			4	9.52±0.02	9.88±0.01	50.77±0.33	25.00±0.33
	3	600 mg/kg	0	9.49±0.13	9.90±0.04	50.33±0.33	25.00±0.33
			4	9.50±0.13	9.91±0.04	50.63±0.33	25.03±0.33
	4 (control)	0.5ml 10% EOH	0	9.71±0.13	9.83±0.05	50.67 ±0.67	24.33±0.33
			4	9.71±0.13	9.84±0.05	50.67 ±0.67	24.65±0.33
Root	1	900 mg/kg	0	9.67 ± 0.33	10.00± 0.02	50.00± 0.57	24.33 ±0.33
			4	9.68 ± 0.33	10.23±0.04*[1,4,*]a	50.88± 0.57	25.00 ±0.33
	2	750 mg/kg	0	9.43 ± 0.28	9.92 ± 0.02	50.00± 0.57	25.00± 0.16
			4	9.44 ± 0.48	9.93± 0.11	50.00± 0.57	25.20 ±0.17
	3	600 mg/kg	0	9.45 ± 0.11	9.94 ± 0.05	50.00± 0.57	25.00± 0.33
			4	9.46 ± 0.11	9.96 ± 0.06	50.00± 0.57	25.21 ±0.34
	4 (Control)	0.5ml. 10% EOH	0	9.69 ± 0.09	9.84± 0.07	51.00± 0.57	25.00 ±0.33
			4	9.70 ± 0.09	9.84± 0.07	51.00± 0.57	25.00 ±0.33

Keys: * = $P < 0.05$.

a = Comparison between Day 0 and Day 4.

1, 4 = Comparison between group 1 and respective control value.

N.B All tests were compared against the control group that were given the extraction solvent.

3.2 *IN VIVO* ANTI-MALARIAL SUPPRESSIVE TEST

As determination of parasitemia indicated, the percentage of parasitemia was lower in all the mice administered with the extracts of *Moringa stenopetala* and *Withania somnifera* than the negative control groups given the extractions solvent only. Though the mice treated with all the extracts showed reduced parasite load as compared to the control group they were not cleared completely while positive control groups administered with chloroquine cleared the parasite on day 4. The range of the mean survival time of mice given the extract was between (9.08 $\pm$ 0.40) at higher dose and (6.05 $\pm$ 0.17) at lower dose, while the mean survival time of the negative control group mice was (5.25$\pm$0.21) (Table 5-8). In addition, as seen on the slide preparations of the blood smears taken from mice administered with the extracts, most of the parasitized erythrocytes were at the ring stage on day 4 (specially at higher dose), while schizont stages were seen in the smears taken from the untreated negative control mice.

The multiple comparison analysis using Scheffe's procedure indicated that group 3 mice administered with 750 mg/kg of *Moringa stenopetala* root water extract showed statistically significant difference on day 4 parasitemia level (P = 0.014) and group 4 mice administered with 900 mg/kg of the root extract also showed significant difference (P = 0.004) compared to the negative control group. But at dose 600 mg/kg, suppressive effect of the above extract on day 4 parasitemia level was not statistically significant. The suppressive effect of the extract at the above doses on day 6 parasitemia level was also not statistically significant in comparison to the negative control group (Table 5).

Table 5. *In vivo* suppressive test of *Moringa stenopetala* root water and ethanol extracts against *Plasmodium berghei* in mice.

Solvent	Group	Dose	Day 4	Day 6	Mean survival time (day)
			% Parasitemia	% Parasitemia	
Water	1	0.5ml.Dist.H_2O (- control)	11.75± 0.20	20.17±0.35	5.38±0.14
	2	600 mg/kg	10.90± 0.26	19.88±0.34***a	6.41±0.15
	3	750 mg/kg	10.60± 0.18* [1,3]	19.22±0.32***a	7.25±0.22
	4	900 mg/kg	9.5±0.18** [1,4]	18.98±0.32***a	8.60±0.31** [1,4]
	5	10 mg/kg CQ. (+ control)	0.00	0.00	
Ethanol	1	0.5 ml.10% Eth. (- control)	11.56± 0.45	18.72±0.49	5.08±0.19
	2	600 mg/kg	11.45± 0.44	18.64±0.49***a	6.33±0.22
	3	750 mg/kg	11.37± 0.45	18.55±0.48***a	6.91±0.20
	4	10 mg/kg CQ(+ control)	0.00	0.00	

Key: * = P < 0.05, ** = P< 0.01*** = P< 0.001.

a = Comparison between % parasitemia of Day 4 and Day 6.

1, 3/ 1, 4 = Comparison between negative controls and 750 and 950 mg/kg doses.

N.B: All tests were compared against the negative control group 1.

The percentage of suppression of parasitemia on day 4 and 6 in mice treated with 900 mg/kg of the water extract of the root of *Moringa stenopetala* was found to be about 19% and 4.5% respectively (Fig.3).

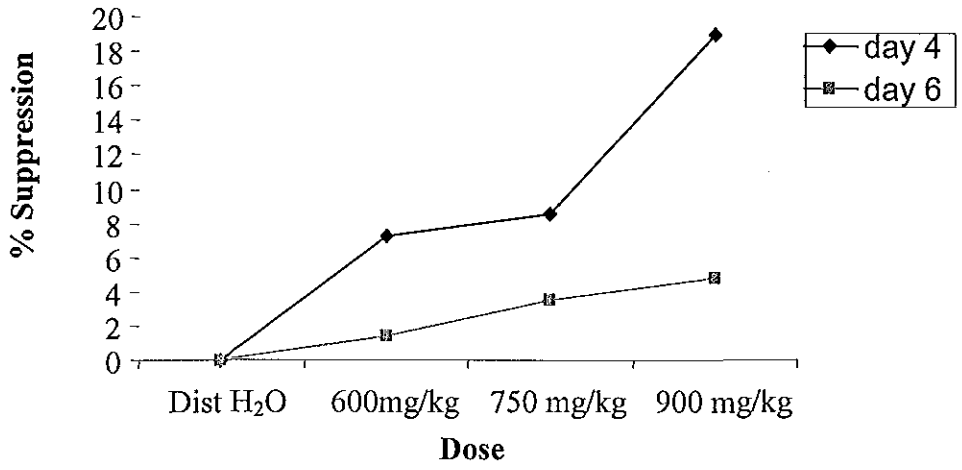

Fig.3. Suppressive effect of water extract of the root of *Moringa stenopetala* on *Plasmodium berghei* in mice.

The multiple comparison result indicated that the suppressive effect induced by the water extract of the leaf of *Moringa stenopetala*, ethanol extract of the root and the leaf of *Moringa stenopetala*, at dose 600, 750 and 900, mg/kg was not statistically significant on day 4 and 6 parasitemia level compared to the negative control group (Table 6).

Table 6. *In vivo* suppressive test of *Moringa stenopetala* leaf water and ethanol extracts against *Plasmodium berghei* in mice.

Solvent	Group	Dose	Day 4	Day 6	Mean
			% Parasitemia	% Parasitemia	survival time
Water	1	0.5 ml.Dist.H_2O(- control)	10.62± 0.15	20.70±0.20	5.41±0.19
	2	600 mg/kg	10.45± 0.17	20.56±0.20***a	6.66±0.30
	3	750 mg/kg	10.40±0.16	19.55±0.18***a	7.10±0.31
	4	900 mg/kg	9.90± 0.22	18.52±0.18***a	7.67±0.35* [1,4]
	5	10 mg/kg CQ. (+ control)	0.00	0.00	
Ethanol	1	0.5 ml.10% Eth. (-control)	11.77± 0.24	18.71±0.46	5.25±0.21
	2	600 mg/kg	11.67± 0.24	18.63±0.46***a	6.08±0.25
	3	750 mg/kg	11.59± 0.25	18.18±0.27***a	6.68±0.25
	4	10 mg/kg CQ (+ control)	0.00	0.00	

Key: * = $P < 0.05$, *** = $P<0.001$.

a = Comparison between % parasitemia of Day 4 and Day 6.

1, 3/ 1, 4 = Comparison between negative controls and 750 and 950 mg/kg doses.

N.B: All tests were compared against the negative control group 1.

The suppressive effect induced by the water extract of the leaf of *Withania somnifera* was statistically significant at doses 750 (P = 0.015) and at dose 900 mg/kg (P = 0.004) on level of day 4 parasitemia in comparison to the negative control mice. The extract also induced statistically significant suppressive effect on day 6 parasitemia at dose 750 mg/kg (P = 0.045) and at dose 900mg/kg (P = 0.038) as compared to the negative control group (Table 7).

Table 7. *In vivo* suppressive test of *Withania somnifera* leaf water and ethanol extracts against *Plasmodium berghei* in mice.

Solvent	Group	Dose	Day 4 % Parasitemia	Day 6 % Parasitemia	Mean survival time
Water	1	0.5 ml.Dist.H_2O(-control)	12.46± 0.40	19.31±0.21	5.58±0.15
	2	600 mg/kg	11.95± 0.38	19.01±0.18*[1,2]***a	6.91±0.34
	3	750 mg/kg	10.53± 0.43* [1, 3]	18.16±0.24*[1,3]***a	7.91±0.33* [1, 3]
	4	900 mg/kg	9.40± 0.43** [1, 4]	16.89±0.24*,[1, 4] ***a	9.08±0.40** [1, 4]
	5	30 mg/kg CQ. (+ control)	0.00	0.00	
Ethanol	1	0.5 ml.10% Eth.(- control)	10.76± 0.28	19.23±0.22	5.58± 0.15
	2	600 mg/kg	10.70± 0.28	19.17±0.22***a	6.33± 0.18
	3	750 mg/kg	10.59± 0.29	19.06±0.23***a	6.91±0.23
	4	900 mg/kg	10.57± 0.29	19.03 ±0.24***a	7.25±0.22
	5	10 mg/kg CQ. (+ control)	0.00	0.00	

Key: * = P < 0.05, ** = P<0.01*** = P<0.001.

a = variation between % parasitemia of Day 4 and Day 6.

1, 3/ 1,4 = Comparison between negative controls and 750 and 950 mg/kg doses.

N.B: All tests were compared against the negative control group 1.

In mice administered with 900 mg/kg of water extract of the leaf of *Withania somnifera* about 33% and 13% suppression of parasitemia was obtained on day 4 and 6 respectively (Fig. 4).

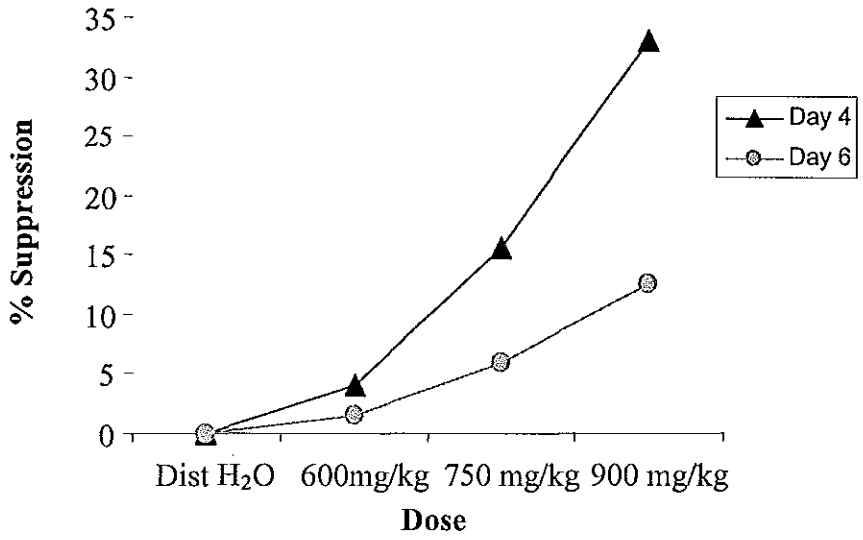

Fig.4. Suppressive effect of water extract of the leaf of *Withania somnifera* on *Plasmodium berghei* in mice.

The multiple compassion test indicated that the suppressive effect induced by ethanol extract of the leaf and the root of *Withania somnifera*, and water extract of the root of *Withania somnifera* on day 4 and 6 parasitemia level was not statistically significant, in comparison with the negative control group (Table 7 and 8).

The Student's paired t-test analysis after terminating treatment on day 4 showed that there was statistically significant difference between day 4 and day 6 parasitemia level of all extracts at dose 600 mg/kg, 750 mg/kg and 900 mg/kg ($P < 0.001$ in all cases) (Table 5 -8). Such condition initiated for further test by giving extracts continuously up to death of mice.

Table 8. *In vivo* suppressive test of *Withania somnifera* root water and ethanol extracts against *Plasmodium berghei* in mice.

Solvent	Group	Dose	Day 4	Day 6	Mean survival time
			% Parasitemia	% Parasitemia	
Water	1	0.5 ml. Dist.H_2O (- control)	11.99± 0.29	18.29±0.63	5.46± 0.14
	2	600 mg/kg	11.94± 0.29	18.25±0.42***a	6.16 ±0.21
	3	750 mg/kg	11.90± 0.28	17.32±0.41***a	6.71 ±0.31
	4	900 mg/kg	10.85± 0.28	17.03±0.42***a	7.41±0.34
	5	30 mg/kg C(+ control)	0.00	0.00	
Ethanol	1	0.5 ml.10% Eth.(-control)	10.50± 0.18	19.77±0.26	5.33 ±0.18
	2	600 mg/kg	10.42± 0.18	19.70±0.26***a	6.05 ±0.17
	3	750 mg/kg	10.33± 0.18	19.62±0.26***a	6.51± 0.19
	4	900 mg/kg	9.73± 0.28	18.60 ±0.25***a	7.08± 0.20
	5	30 mg/kg CQ. (+ control)	0.00	0.00	

Key: *** = P<0.001.

a = variation between % parasitemia of Day 4 and Day 6.

1, 3/ 1, 4 = Comparison between negative controls and 750 and 950 mg/kg doses.

N.B All tests were compared against the negative control group 1.

Further investigation on water extract of the leaf of *Withania sominfera* at 750 and 900 mg/kg dose, by giving the extract every 12 hr until the infected mice died indicated that giving the extracts every 12 hr prolonged the survival time of mice by more than twice to that of the negative control group. The mice treated with 750 and 900 mg/kg of the leaf of *Withania sominfera* have the mean survival time (12.43±0.21) and (13.85 ± 0.17) respectively, while the mean survival time of the control group mice was (5.86 ± 0.14) (Fig. 5).

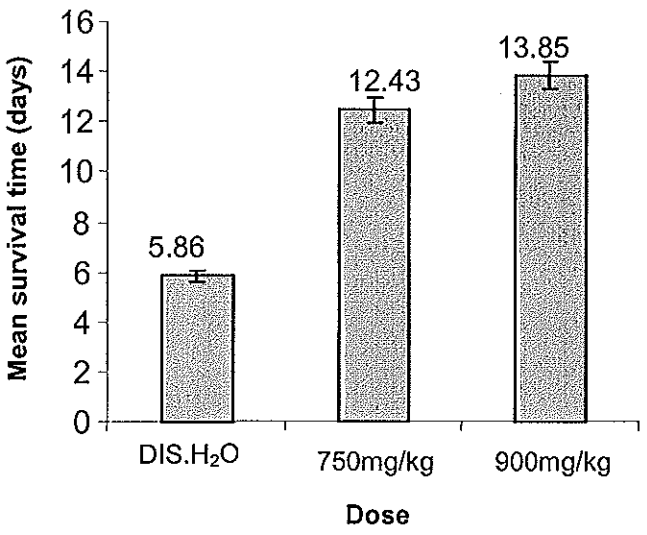

Fig.5. The effect of water extract of the leaf of *W. somnifera* on survival time of *Plasmodium berghei* infected mice on every 12 hr treatment schedule.

The water extract of the root of *Moringa stenopetala* also extend the survival time mice by twice as compared to the non-treated control group during every 12 hr treatment. The mean survival time of mice treated with 750 and 900 mg/kg of water extract of the root of *Moringa stenopetala* was (10.00± 0.31) and (11.85± 0.26) respectively where as that of the negative control group was (5.42 ±0.21) (Fig. 6).

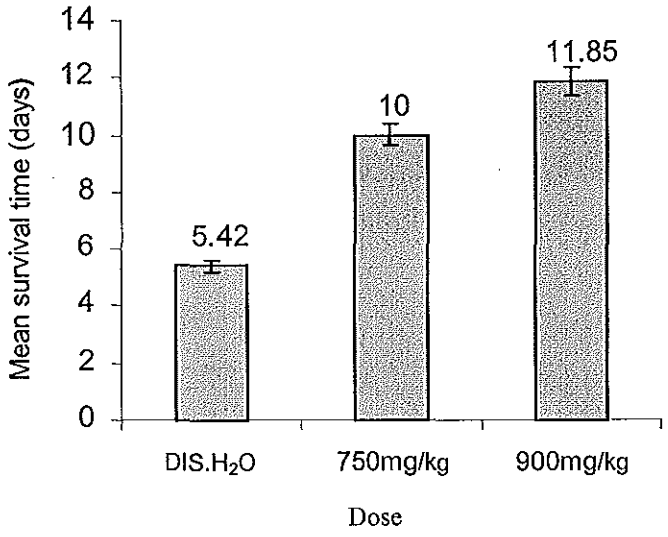

Fig.6. The effect of water extract of the leaf of *M. stenopetala* on survival time of *Plasmodium berghei* infected mice on every 12 hr treatment schedule.

The over all ANOVA of further test on the suppressive effect of water extract of the leaf of *Withania somnifera* and the root of *Moringa stenopetala* showed that at time 0 before administration of the extracts statistically no significant difference was seen between all parameters measured in comparison to the control groups, indicating that all parameters measured are with in the same range of the normal value. Multiple comparison analysis using the Scheffe's procedure indicated that water extract of the leaf of *Withania somnifera* induced statistically significant suppressive effect on the parasite load at dose 750 mg/kg ($P = 0.03$) and at dose 900 mg/kg ($P < 0.001$) on day 4. The water extracts of the leaf of *Withania somnifera* also suppress the parasite to statistically significant level ($P < 0.001$) up to day 9 in comparison to the lethal parasite load of the negative control mice, then after the rise in parasite load and the test group mice were died between days 10 and 14, while mice in the control group died between day 5 and day 6 (Table 9).

Water extract of the root of *Moringa stenopetala* also suppresses the parasite to significant level at dose 750 mg/kg ($P = 0.035$) and at dose 900mg/kg ($P < 0.001$) on day 4. The water extract of the root of *Moringa stenopetala* also suppresses the parasite to statistically significant level ($P< 0.001$) up to day 9 in comparison to the lethal parasite load of the negative control mice, then after the parasite load rise up and the test group mice were died between day 10 and 14, while mice in the control group died between day 5 and day 6 (Table 10).

Table 10. The effect of *Moringa stenopetala* root water extract on every 12 hr treatment schedule against *Plasmodium berghei* in mice.

Day	Parameters	Group			
		1. 0.5ml.H_2O	2. 900 mg/kg	3. 750 mg/kg	4. 10 mg/kg CQ
0	PCV	50.1±0.21	50.80 ±0.15	50.27± 0.27	50.33±0.33
	Wt	25.70±0.19	26.5±0.24	26.1±0.34	25.03±0.33
4	PCV	48.79±0.20	50.82±0.14^{*1,2}	50.39±0.27	50.33±0.33^{***1,4}
	Wt	23.70±0.24	28.27±0.24^{***1,2}	27.67±0.29^{***1,3}	25.03±0.33^{***1,4}
	% Para.	12.75 ± 0.20	8.70±0.32^{**1,2}	9.45±0.31^{**1,3}	0.00
	% Supp.	0	28.10	21.50	100
6	PCV	43.20±0.42	51.01±0.14^{***1,2}	50.68±0.21	50.63±0.33^{***1,4}
	Wt	23.8± 0.51	29.14 ± 0.23^{***1,2}	28.47±0.32***1,3	25.70±0.19^{***1,4}
	% Para.	23.68 ± 0.79	7.26±0.25^{***1,2}	8.23±0.22***1,3	0.00
	% Supp.	0	68.80	64.96	100
8	PCV	DEAD	51.23± 0.12^{*1,2}	50.64±0.19***	50.67 ±0.67^{***1,4}
	Wt		29.54± 0.20^{***1,2}	28.64±0.26^{***1,3}	26.5±0.24^{***1,4}
	% Para.		6.13±0.15^{***1,2}	7.48±0.33^{***1,3}	0.00
	% Supp.		73.90	67.90	100
9	PCV	DEAD	51.33± 0.12^{*1,2}	50.64±0.19***	50.67 ±0.67^{***1,4}
	Wt		28.84±0.18^{***1,2}	28.02±0.28^{***1,3}	26.5±0.24^{***1,4}
	% para.		5.59 ± 0.25***1,2	7.26 ± 0.31^{***1,3}	0.00
	% Supp.		76.00	68.80	100
10	PCV	DEAD	49.35±0.38^{***1,2}	48.41±0.30^{**1,3}	50.77±0.33^{***1,4}
	Wt		26.65±0.26^{***1,2}	25.25±0.37^{***1,3}	28.37±0.24^{***1,4}
	% Para.		9.67 ±0.36***1,2	13.82±0.71^{***1,3}	0.00
	% Supp.		58.5	44	100
12	PCV	DEAD	44.31±0.38	DEAD	51.00±0.33^{***1,4}
	Wt		24.37±0.53		29.54±0.20^{***1,4}
	% para		19.78 ±0.89^{*1,2}		0.00
	% Supp.		42		100
14	PCV	DEAD	DEAD	DEAD	51.70 ±0.21***1,4
	Wt				31.54±0.27***1,4
	% para				0.00
	% Supp.				100

Key: * = P < 0.05** = P <0.01*** = P<0.001.

1, 2/ 1, 3 = Comparison between negative controls and 750 and 900mg/kg.

N.B: All tests were compared against the negative control group1.

Observation of behavioral patterns revealed that the mice were active in movement and feeding up to day 10 in comparison to the negative control mice and then after their feeding and movement activity decreased day to day and finally the mice were unable to feed and set lonely at cage corner where they collapsed and died (Table 9 and 10).

Following treatment with water extracts of the leaf of *Withania somnifera* and the root of *Moringa stenopeteala* parameters such as PCV and weight increased up to day 9 post infection and showed statistically significant difference ($P< 0.001$) in comparison to the negative control group of mice. After day 9 the weight and PCV values of mice decreased while the % Parasitemia increased. Administration of the leaf extract of *Withania somnifera* every 12 hours reduced the parasitemia to 4 % and the extract of root of *Moringa stenopeteala* reduced parasitemia to 5.5 % on day 9, and the % of suppression was 79.84% in case of 900 mg/kg of water extract of the leaf of *Withania somnifera* and 76% in case of 900 mg/kg water extract of the root of *Moringa setnopetala* on day 9 (Table 9 and 10). After the 9^{th} day post infection parasitemia began increasing and continued treatment had no effect (Fig.7 and 8).

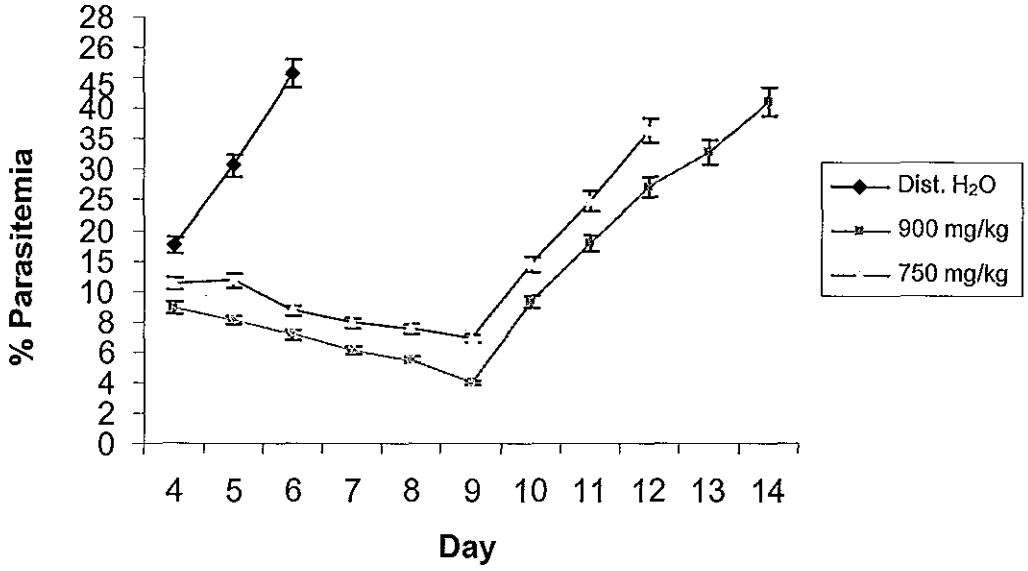

Fig. 7. *In vivo* effect of water extract of leaf of *Withania somnifera* on *Plasmodium berghei* on every 12 hr treatment Schedule.

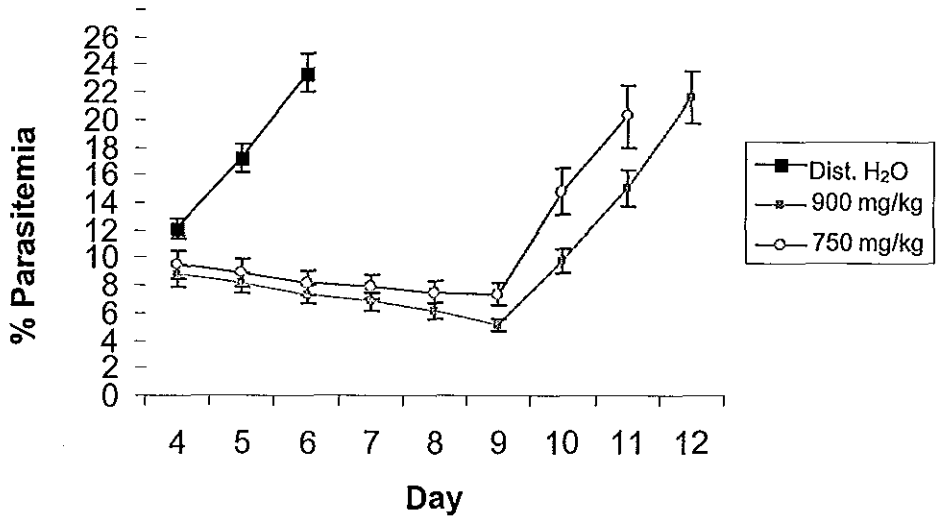

Fig. 8. *In vivo* effects of water extract of root of *Moringa stenopetala* on *Plasmodium berghei* on every 12 hr treatment Schedule.

The *Njemps*, in Kenya chew the bark as a treatment against coughs, and use the bark extracts to make fortified soups (Jahn, 1991). Nomadic peoples in the Omo valley in Ethiopia use the roots of wild *Moringa stenopetala* to purify clayey water, where it is known to clarify water more than the chemical alum which is used in purification of water and non toxic (Mayer & Steltz, 1993). The *dama*, which is eaten in the Konso district in southern Ethiopia on average two or three times a day, is made of flour of maize and leaf powder of *Moringa stenopetala*, is undoubtedly the main dish; these people also use the leaf of *Moringa stenopetala* in the preparation of local drink *Cheka* by mixing equal amount of the leaf with flour of sorghum and no symptoms of acute or subacute toxicity is observed in children or adults (Jiru, 1995).

Toxicity test has indicated *Withania somnifera* extracts to be non-toxic at high doses. The report of Gandhi, *et al.* (1994) indicated that in mice administered orally with extracts of the leaf, and the root of *Withania somnifera* the extracts were non lethal up to a dose of 1750 mg/kg. The result indicated that *Withania somnifera* appeared to have a wide safety margin. *Withania somnifera*, extracts are also found to reduce toxicity and inflammation induced by chemicals. In mice administered orally with water extracts of the leaf and the root of *Withania sominfera* 3-4 hours prior to induction of inflammation reduction of inflammation by 75% have been demonstrated (Gandhi, *et al.* 1994).

The lack of *in vivo* toxicity of root and leaf of *Moringa stenopetala* was also demonstrated in an *in vitro* toxicity test on hepatocytes by using water extracts of the leaf and the root of *Moringa stenopetala* (Negussu Mekonnen, personal communication). This is good proof that the water extract does not contain toxic substances and is consistent with the use of *Moringa stenopetala* leaves as a vegetable by population in Gamo Goffa, southern Ethiopia. It is also consistent with the fact that the local population around Arbaminch uses large quantities of

toxic substances with the physiology of the mice used in drug efficacy test, the toxic dose of 900 mg/kg of ethanol extracts of the root and the leaf of *Moringa stenopetala* were not included in the efficacy test.

With *Withania somnifera* the significant increase in WBC count, at 900 mg/kg dose may not only relate to acute toxic response because according to the report of Davis and Kuttan (2000), *Withania somnifera* extract was found to increase the number of WBC count and such increment was shown to be a sign of stimulation of the immunological activity of the Balb/c mice. It has also been suggested that the significant increase in WBC count might be due to constituents extracted by water and ethanol that enhance cytotoxic T cell activity (Davis and Kuttan, 2002). Therefore, the increase in WBC count may not only indicate the toxic effect of the extracts but also may be an indication of boosting of the immune system.

The significant suppression of parasitemia by the water extract of the root of *Moringa stenopetala* on day 4 (Table 5), was in agreement with the anti-malarial activity of the water extract of the leaf and the root of *Moringa oliefra*, which showed significant anti-malarial activity against *Plasmodium falciparum in vitro* (Kohler *et al.*, 2002). The suppression induced by water extract of the root of *Moringa stenopetala* is a confirmation of the information obtained by Mekonnen and Gessesse (1998) through their enquiry of the community in Arbaminch area that the water decoction of leaf and root of this plant is used for treatment of malaria, and people get relief from fever.

The 19% suppression of parasitemia induced by *Moringa stenopetala* extract was comparable to the 21.7% suppression induced by the anti-malarial medicinal plant *Azadirachta indica*

(Obih and Makinde, 1985). Evaluation of the anti-malarial properties of the tablets made from extract of *Azadirachta indica* had indicated that the tablet suspensions at a concentration of 800 mg/kg produced parasite suppression of 79.6% (Isah *et al.*, 2003), indicating that similar evaluation of ingredients of *Moringa stenopetala* could also have promising results to that of *Azadirachta indica* .

The significant relapse in parasitemia on day 6 following significant suppression on the 4th day post infection indicated that treatment with *Moringa stenopetala* extract may have to be continued for much longer period of time, to be more effective.

Since *Moringa stenopetala* is widely used as a vegetable by the local population its medicinal value can be surmised. Protection from malaria in the *Hausa* people (North Nigeria) has been shown to be due to the consumption of plants that have medicinal value and also serve as food (Etkin, 1997) is an important indicator. Similarly, a daily food ration of *Moringa stenopetala* can be expected to have a malaria protective effect in Ethiopia.

The possible explanations given for the lack of anti-parasitic effect of extracts of medicinal plants are that the extracts may have large particle size and may not be absorbed during their pass through the gastrointestinal tract, or it also be that of the ingredients required for protection against malaria may not have been extracted by the solvents used (Franssen, 1997).

Another possible reason for reduced parasite suppression by the extracts could be that the active ingredients may have disintegrated in dried powder used. Drying may reduce much of the potential medicinal value, because when plant parts are dried the plant cells lose water and

collapse. And once a plant material is dried, it cannot be re-hydrated, and many medicinal molecules are trapped in the dried cellulose mass, impairing extraction of the active principles (Martin, 2002). In this study drying of the medicinal plants could not be avoided because the specimens had to be collected from long distance from the laboratory.

The 33% malaria parasite suppression of *Withania somnifera* leaf water extract was comparable to suppression induced by water extract of the leaf of *Solanum indicum* (31.9%) (Abatana and Makinde, 1986), which is an effective anti-malarial. Studies had shown that *Withania somnifera* extracts boost the immune system of mice by increasing WBC count, enhancing the circulating antibody titer and increasing cytotoxic T cell production (Davis and Kuttan, 2002). Hence, the significant level of parasite reduction might be due to the immune potentiating effect of the extract in the infected mice.

According to Carvalho *et al* (1991) drugs are considered to be active against malaria if they can suppress 30% of the parasitemia. Thus, the water extract of the leaves of *Withania somnifera* would have a good potential as a source of anti-malarial active ingredient. This conclusion is supported by the report of Bogale and Petros (1996) whereby a very high anti-malarial *in vitro* effect was shown for methanol extract of the leaves of *Withania somnifera.*

The significant suppression of parasitemia by the water extract of the leaf of *Withania somniferaon* (Table 6) was in agreement with the report of Gedif and Hahn (2002), which indicated that the water decoction of dried and powdered leaf of *Withania somnifera* was prescribed by local healers around Butajira area for treatment of malaria. High level of

reduction of fever in patients presumptively treated for malaria with administration of the decoction may be due to reduction of parasite load in the patients.

The study by James (1985), on the effect of *Withania somnifera* crude extracts on mice infected by microbial pathogens, had shown that in neutropenic mice infected with *Staphylococcus aureus* treatment with aqueous extract of the leaf of *Withania somnifera*, reduces mortality due to sepsis from 75% to 50%. It was suggested that the reduction in mortality in the treated mice was due to the immuno-stimulatory effect of the extract, which led to a significant increase in the level of neutropihlia and leukocytes.

The significant parasite suppressive effect induced by water extract of the root of *Moringa stenopetala* by a longer dosing schedule was a further confirmation of the 4-day suppressive test. It also was similar to the *in vitro* test with water extract of the root of *Moringa olifera* on *Plasmodium falciparum* which significantly reduced parasite load (Kohler *et al.*, 2002). The suppressive effect might be due to ingredients such as alkaloids in the leaf (Keville, 2000).

Weight and PCV values significantly increased up to day 9 in mice treated with water extracts of the root of *Moringa stenopetala* and the leaf of *Withania somnifera* (Table 9 and 10). The increase in the vital values was in correlation with suppression in parasitemia. These findings were in agreement with the report of Mishra *et al.* (2000), which indicated that hemoglobin concentration, PCV and weight of mice treated with water extract of *Withania somnifera* increased to significantly as a reflection of improved health.

In vivo suppressive test results by other workers (Satayavivad *et al.* 1998) had shown that the plant extract that prolonged the survival time of treated mice twice as compared to the infected non-treated mice would be considered as good candidates for further study. The findings of the present study, showed that water extract of the leaf of *Withania somnifera* and the root of *Moringa stenopetala*, prolonged survival time of mice by more than twice as compared to the negative control groups (Fig. 5 and 6), indicates that the water extracts of the leaf of *Withania somnifera* and the root of *Moringa stenopetala* are good candidate for further study.

Furthermore, Munoz and his collaborators (2000) had used percentage suppression of extracts as a predictor of their potential source of anti-malaria active ingredients. That is, extracts that induce greater than or equal to 50% inhibition can be considered to be containing active ingredients. Therefore the water extracts of the leaf of *Withania somnifera* and root of *Moringa stenopetala* that suppressed parasitemia by 79.84% and 76 %, respectively could contain active anti-malarial ingredients.

Parasitemia increases after day 9 post infection indicating that the suppressive effect of the extracts has been overcome by the parasite. Such condition may be related to the survival strategies of malaria parasites, one of which is having branched respiratory chain mainly alternative oxidase and the cytochrome pathway. The alternative oxidase pathway has been shown to contribute to the survival of the parasite under conditions in which the cytochrome chain is blocked (Ramaya *et al.*, 2002). The functioning of the alternative oxidase pathway has been demonstrated for Atovaquone an anti-malarial drug designed to act on the cytochrome pathway. Clinical trials in which atovaquone was used alone to treat *Plasmodium falciparum*

malaria resulted in an initial clearance of parasites from the blood followed by recrudescence in most of the patients (Looareesuwan *et al.*, 1996). It was suggested that the high recrudescence rate seen when atovaquone is used alone to treat *Plasmodium falciparum* could be due to the presence of branched respiratory pathway whereby the alternative oxidase pathway is activated up on blockage of the cytochrome pathway by atovaquone.

Murphy and Lang-unnasch (1999) had suggested that the survival of some parasites in the presence of atovaquone was due to the presence of alternative oxidase pathway which enables the survival of some parasites although atovaquone blocked the cytochrome path way. Based on biochemical assay Murphy and Lang-unnasch (1999) predicted that atovaquone could be potentiated by using a combination anti-malarial drug therapy to inhibit the alternative oxidase pathway.

Likewise *Plasmodium berghei* could have the alternative oxidase pathway which may be used by the parasite to overcome the suppression induced by the leaf of *Withania somnifera* and the root of *Moringa stenopetala* extracts by possible act on the cytochrome. Hence, combination of extracts could block both pathways and suppress or eradicate the parasite more effectively.

The increment of parasitemia could also be due to reduction of WBC count, as seen in mice treated with *Withania somnifera* the WBC count increase up to day 10 then after decreases down to the normal level (Davis and Kuttan, 2000), other studies also indicated that extracts from *Withania somnifera, Emblica officinale and Tinospora cordifolia* increase the WBC count up to day 10 then the count decreased (Pallabi, 1998), indicating that day 10 could be the period where WBC count reached the maximum level. The decrease in the WBC count may give opportunity for parasitemia to reproduce at fast rate and attack more red blood cells and

result in cell lyses and will cause significant reduction on PCV, feeding activity of mice and weight and finally death of mice. Similar result was also obtained by Satayaviviad *et al.* (1998) by giving the extract till death of mice where mice died on 12^{th} day at highest dose.

5. CONCLUSION AND RECOMMENDATIONS

The *in vivo* toxicity test showed that *Moringa stenopetala* and *Withania somnifera* ethanol and water extracts showed no major toxic effects on mice up to 900 mg/kg dose. *In vivo* anti-malarial test using male Swiss albino mice against rodent malaria *Plasmodium berghei* (drug sensitive ANKA strain) indicated that among eight extracts tested, water extracts of the root of *Moringa stenopetala* and the leaf of *Withania somnifera* showed significant suppressive effect in comparison with the negative control. The percentage of suppression induced by the above two extracts on day 4 was 19% and 33% respectively. Further continued treatment with water extract of leaf of *Withania somnifera* and root *Moringa stenopetala* result in 79.84% and 76 % suppression respectively and extended the survival time of the infected mice more than twice that of the negative control groups.

Therefore, bioassay-guided phytochemical screening is an important step in the search for safe, low cost and more effective chemotherapeutic anti-malarial agents from the rich flora of the country.

On the basis of the results of the study on the anti-malaria effect of the plant extracts, the following recommendations may be made

1. The validity of the anti-malaria suppressive effect of *Moringa stenopetala* and *Withania somnifera* need further investigation by isolating the active ingredients.

2. Further investigation by using other solvents, and fresh plant materials soon after collection might be a useful area of investigation.

3. Research on anti-malarial medicinal plants should also focus on combining of extracts, as combining different solvent extracts would better approximate traditional medicinal practice (Martin, 2002).

4. It is possible that plants such as *Moringa stenopetala* that have dietary effect may have been contributing to protection against malaria in Ethiopia. Therefore, research in Ethiopia dealing with anti-malarial medicinal plants may have to focus on plants of dual purpose as in the case of *Moringa stenopetala*.

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