

***IN-VITRO* MEDICINAL PLANT CRUDE EXTRACT AND *IN-VIVO* TOXICITY TESTING OF DICHLOROMETHANE ROOT EXTRACT OF *CITRUS LIMON* IN LABORATORY RABBITS**

ABSTRACT

Aims: To identify viable phytomedicines traditionally employed for the treatment of malaria in Kenya that could be developed into antimalarial agents.

Study design: Quantitative analysis of antiplasmodial activities and brine shrimp bioassays were carried out using standard procedures. The experiment was set in duplicate for each concentration of the drug and average IC₅₀ determined.

Place and Duration of Study: Seven indigenous plants: *Achyranthes aspera*, *Heinsia crinita*, *Bridelia cathartica*, *Citrus limon*, *Microglossa pyrifolia*, *Vernonia glabra* and *Carissa edulis* obtained from Kilifi and Homa-Bay counties in Kenya were evaluated for their anti-*Plasmodium falciparum* potential. Collection of samples and analysis took about three months.

Methodology: Both Chemical and aqueous crude extraction methods were carried out to identify the most active extracts against *P. falciparum* and then isolate pure active phytochemicals. Pure compounds were subjected to Nuclear Magnetic Resonance (NMR), Infra-Red (IR) and Mass Spectroscopy (MS) analyses for structure elucidation.

Results: Four extracts (hexane, dichloromethane (DCM), methanol and water) of seven different species of plants were analyzed for their anti-plasmodial activities. W2 and D6 strains of *Falciparum* were tested. However, the three most active extracts were from *Citrus lemon* roots (DCM) with IC₅₀ value of 7.017 µg/mL, *C. edulis* root (aqueous) with IC₅₀ value of 8.054 µg/mL and *B. Cathartica* leaves (methanol) with IC₅₀ value of 15.647 µg/mL. However, three pure compounds were obtained; suberosin IC₅₀ 26.7 (Strain W2), 53.1 (Strain D6) and xanthyletin IC₅₀ 1580 (Strain W2) from *C. Limon* (DCM) and spinasterol IC₅₀ 43.2 (Strain W2) from *M. pyrifolia* (hexane).

CONCLUSION: The three different species of plants with most active compounds have demonstrated their potentiality in treatment for *falciparum* malaria. Structures of the isolated three compounds can be modelled to synthesis of anti-plasmodial drugs as they are active *in vitro*.

Keywords: *In-vitro*, crude extract, *in-vivo*, toxicity, Dichloromethane

1. INTRODUCTION

Following an ethnomedicine survey carried out in Kilifi and Homa-Bay Counties among the Chonyi and Luo people respectively, several plants had been mentioned by herbalists as having been utilized against malaria. Rachuonyo in Homa-Bay County and Kilifi County are located in Nyanza and Coast provinces of Kenya respectively. They share almost the same climatic conditions due to proximity to Lake Victoria and Indian Ocean respectively, which favors breeding of mosquitoes resulting in high incidences of malaria in these areas. These surveys were carried out under the auspices of World Health organization (WHO) funded Multilateral initiative on Malaria (MIM) project number 990096 [32].

1.1 Ethnomedicine survey

Following an ethnomedicine survey carried out in Kilifi and Homa-Bay Counties among the Chonyi and Luo people under the auspices of WHO funded Multilateral Initiative on Malaria (MIM) project number 990096 [32], several plants were mentioned by herbalists as having been utilized against malaria (Table 3). Kilifi and Homa-Bay Counties are found in Coast and Nyanza provinces of Kenya, respectively. They share almost the same climatic conditions due to proximity to Indian Ocean and Lake Victoria, respectively, which favor breeding of mosquitoes and therefore have high incidences of malaria (Figure 1 - A, B & C).



Figure 1:County Governments". Kenya Law Reform Commission. 29th August 2012

1.2 Reasons for conducting ethno-medicine survey in Kilifi and Homa Bay

Kilifi is located in the former Coast Province, 60 kilometers (km) north of Mombasa. It borders Mombasa and Kwale to the south, Tana River to the north, and Taita to the west. The county has six sub-counties; namely, Kilifi, Ganze, Malindi, Magarini, Rabai and Kaloleni. It has 17 divisions, 54 locations, 175 sub-locations. The total area is 12,246 kilometers squared (km²). The population of the county was estimated to be 1,217,892 in 2012 as projected in the Kenya Population and Housing Census 2009, composed of 587,719 males and 630,172 females. The population was projected to rise to 1,336,590 and 1,466,856 in 2015 and 2017 respectively, at growth rate of 3.1 percent per annum.

The average annual rainfall ranges from 300millimeters (mm) in the hinterland to 1,300mm at the coastal belt. Major occupation is fishing due to its proximity to Indian Ocean. The annual temperatures range between 21°C and 30°C along the coastal belt and between 30°C and 34°C in the hinterland. These conditions are suitable for mosquito survival and therefore malaria epidemics. Health services include malaria control. The malaria positivity rate (%) is approximately 30 malaria cases (per 100,000 people) 21,945 and malaria admission 179,160. Established control measures are no longer working. Resistance against insecticides and antimalarial, with malaria morbidity and mortality are rising. There is an increase in the mean age of children admitted with *P. falciparum* infections from 20.2 months in 1990 to 45.3 months in 2014 [42]. Though residents of Kilifi rely mostly on ethno-medicine to manage human ailments, the indigenous knowledge remains largely undocumented. The objective of this study was to survey, record and report some of the medicinal plant species used to manage human ailments with particular interest on malaria in Kilifi and Homa Bay Counties [12] (Figure 1 - A & B).

Homa-Bay County is located in the former Nyanza Province along the south shore of Lake Victoria. Its capital and largest town is Homa Bay. The Luo and Basuba who primarily inhabit the area are

fishermen. The county has a population of 1,131,950 (2019 census) and an area of 3,154.7 km². Fishing in Lake Victoria is the major source of livelihood for its residents. Administrative Divisions include Asego, Ndhiwa, Nyarongi, Rangwe and Riana. The climate in Homa Bay is warm and overcast. Over the course of the year, temperatures typically vary from 65°F to 85°F and is rarely below 62°F or above 90°F. Mosquitoes thrive under these conditions. The natural resources include Lake Victoria, good arable land, game reserves, clean natural beaches, building materials such as sand, rough stones, granite, limestone and permanent rivers.

Malaria positivity rate (%) is about 46, cases (per 100,000 people) 58,820 and admission 12,479. The challenges are many in a rural county with only three doctors and 40 nurses per 100,000 persons. For example, in 2015 nearly 59% of the population had malaria. Forty percent of children were not born at health facilities, making it more difficult to track maternal and child health needs. There is therefore improved malaria data and use through Surveillance. Herbal medicines are therefore alternatives to health care in the county [13] (Figure 1 - A & C) whose documentation is lacking. Herbalists from the two counties mentioned numerous plants that were used to treat malaria seven of which were used in this study.

Herbalists have exemplified plants such as, *Achyranthes aspera* (Amaranthaceae), *Heinsiacrinita* (Rubiaceae), *Brideliacathartica* (Euphorbiaceae), *Citruslimon* (Rutaceae), *Microglossapyrifolia* (Asteraceae, Compositae), *Vernoniaglabra* (Compositae) and *Carissa edulis* (Apocynaceae) as being effective in treating malaria. They can either be used singly or combined in concoction or decoction depending on the severity of illness. Extracting the plant material (whole plant, stem, bark, leaves or roots), starts with non-polar solvents such as hexane or petroleum ether, and followed with solvents of increasing polarity such as ethyl acetate, dichloromethane (DCM), ethanol and water.

1.3 Safety screening methods

Different tissues contain a variety of enzymes in varying concentrations. Following tissue injury, plasma enzyme levels do not necessarily change in proportion to those in the damaged tissue. However, in some cases, the injuries are associated with changes of specific enzymes in the plasma. For example, myocardial infarction is associated with increased plasma lactate dehydrogenase (LDH) and aspartate transferase (AST) concentrations [4]. LDH is widely distributed as would be expected from its involvement in glucose metabolism, being found in all organ cells. Nonetheless, it is principally abundant in cardiac and skeletal muscle, kidney, liver and erythrocytes. Generally, when physicians reckon some kind of tissue damage in humans, total LDH measure may be requested together with other tests as an investigative test. Due to this determination of individual LDH, iso-enzyme concentrations can be used together with other tests, to help ascertain the disease or disorder causing tissue damage. LDH is found in virtually all body tissues, so its test is done to discover tissue changes and assist in the diagnosis of cardiac disease. A health professional/practitioner may advise avoiding drugs that may interfere with the test. Drugs that can elevate LDH levels include anesthetics, aspirin and others (www.healthatoz.com/healthatoz/Atoz/ency/lactate_dehydrogenase_test.jsp 24k, www.henryfordhealth.org/14366.cfm - 66k).

Immuno-toxicity refers to any adverse effect on an organism's normal functioning of the immune system that results from exposure to a chemical substance. Dose-response relationships could be demonstrated between immuno-globulin measurements and internal exposure markers. Immune parameters include; immuno-histopathology, total white blood cell counts, immuno-phenotyping of peripheral blood leukocytes, challenge with specific antigen, cell-mediated immunity, natural killer cell activity, phagocytosis, host resistance assays, hypersensitivity and autoimmunity, and quantification of total serum immunoglobulin levels. Total serum immuno-globulin (Ig) levels (IgG, IgM, and IgA) can be quantified in rodents [7] and in non-human primates [38] using the enzyme-linked immuno-sorbent assay (ELISA). However, the determination of total serum Ig levels in experimental animals has not proven useful, since pronounced effects on immune function are required before significant changes in total serum Ig levels can be observed. In single radial immuno-diffusion (SRID) standard antigen concentrations calibration curves are generated to determine unknown concentrations of antigens (Rabbit IgG, IgM and IgA) used to immunize goats [19].

Chemical pathology is the study of changes in the body that occur following administration of a chemical or a drug. These include changes in the normal physiology and biochemistry. When the normal values are altered, this may imply injury to some tissues of the body leading to changes in specific enzymes, blood sugar and products of immune cells [4]. The degree of alteration of the normal body parameters caused is used to assess the relative safety of the chemical or drug. It is a

standard scientific requirement that the safety assessment tests are done in animals first before recommending administration of the test substance in human beings.

Supreme Test Strips are used to determine blood glucose levels in capillary blood. The test principle is specific for β -glucose and is a glucose oxidase/oxidase-based reaction. The results obtained equate to capillary whole blood glucose values. Measuring range-visual readings with color comparison chart 2.2-27.7 mmol/l (40-500 mg/dl). The Supreme strip can be used with Supreme Plus, Extra and Petit meters. The target area of each Supreme test strip contains reactive ingredients in the following approximate proportions; Glucose oxidase-33.4 μ g, peroxidase-0.75 μ g & toluidine 8.5 μ g [20].

The following seven plants; *Achyranthes aspera* (Amaranthaceae), *Heinsiacrinita* (Rubiaceae), *Brideliacathartica* (Euphorbiaceae), *Citrus limon* (Rutaceae), *Microglossapyrifolia* (Asteraceae, Compositae), *Vernoniaglabra* (Compositae) and *Carissa edulis* (Apocynaceae) that were mentioned by the herbalist in Kilifi County and Rachuonyo in Coast and Nyanza Provinces respectively as being used to treat fever and malaria. They are either used singly or combined in concoction or decoction depending on the illness. Plant extracts were obtained using organic solvents of increasing polarity and the preparation of aqueous extract was according to the method of herbalists; n-hexane, dichloromethane, methanol and water. Antiplasmodial activity of the extracts was tested against CQ sensitive and resistant strains of *P. falciparum* *in vitro*. Brine shrimp lethality test as a way of selecting the most active candidate for toxicity testing was also done. Out of the 28 plant extracts that were tested for antiplasmodial activity, the DCM root extract of *Citrus limon* (CLR) had the highest activity (IC_{50} of 7.017 μ g/ml).

The antimalarial testing was undertaken against the background that plant kingdom offers a territory little explored for the presence of potential pharmacologically active compounds against protozoa in general including malaria, trypanosomes and leishmania parasites. Plants are the sources of the clinically used antiprotozoal drugs quinine, emetine and artemisinin. Berberine, a plant extract, has been used clinically in the treatment of cutaneous leishmaniasis. The monoterpenoid spinatol isolated from the bark of the Bolivian species *Oxandra spinata* (Annonaceae) is active against strains of promastigotes of *Leishmania* and epimastigotes of *Trypanosoma*. Diterpenoids, Triterpenoids and Naphthoquinones,

Before a new drug may be used clinically in humans or animals, it must be invariably evaluated for potential toxicity to determine whether and how the drug causes injury. Sub-chronic and chronic toxicity studies are usually done on animals and differ primarily in the duration of treatment, number of animals used, dose, and number of dose levels tested. In both sub-chronic and chronic studies, one dose should approximate the anticipated therapeutic dose, and at least one additional dose should produce some toxicity. In the sub-chronic studies, which is the basis of this study, three dose levels were employed.

By administering the drug to animals in sub-lethal doses over a long period of time it is possible to observe whether it causes any ill effects as indicated by loss of weight, a poor condition or loss of fur, inability to walk steadily and temperamental changes. By carrying out hematological, biochemical and histological tests by examining abdominal and thoracic viscera, it is possible to detect toxic changes in the liver, kidneys, heart and other organs. A sequence of testing procedures is employed when new compounds are screened for there is a possibility of overlooking a unique activity. To date, no study of a new or potential drug is complete without animal testing. By carrying out hematological, biochemical and pathological studies it is possible to detect toxic changes in the liver, kidneys, gastrointestinal tract, lungs, heart and other organs. The success of the toxicity test depends on the choice of dose. Three dose levels are normally chosen. The low dose should be asymptomatic in toxicity terms, but be a multiple of that required for pharmacological action while the top dose must be chosen to produce toxicity and the intermediate dose should have symptomatic effect but not lethal [11].

According to Van den [39], IgA is positively correlated with toxic equivalents (TEQ) values in the serum. Immuno-globulin M (IgM) showed a positive correlation with cadmium in the urine. IgE is increased with increasing O-cresol concentration in urine. Spotting of specific antibodies is a common form of medical diagnostics. Production of antibodies against self can often lead to antibodies that react with the body's own epitopes; many can be diagnosed through blood tests. Prothrombin (factual), albumin and antibodies are hepatic proteins made in this organ. Hepatic disorder is indicated by abnormal concentration of these proteins. The safety of traditional medicine can only be derived from the ethno-

medical clinical information. However, when such data are correlated with the ethno-botanical and chemical information, it is possible to determine the safety of the medicine to a high degree of accuracy. Laboratory animal experimentation should be able to confirm the information gathered [23].

The crude extract used in this study exhibited activity against both *P. falciparum* and Brine shrimps in a study conducted at the School of Pharmacy University of Nairobi (UoN) and would be interesting to test the safety of this efficacious crude drug. This study was subsequently undertaken at KALRO-BIOR where facilities were available. The specific objectives were; to determine the safety of the crude DCM extract using hematological parameters, viz; RBC, WBC and Platelet counts in rabbits, determine the safety of the crude DCM extract using biochemical parameters such as blood glucose levels, plasma LDH levels and isoenzymes and correspondingly determined the safety of the drug by examining the body weight of rabbits and histopathological studies on the major organs of the rabbits.

UNDER PEER REVIEW

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Test organisms

- a) *Plasmodium falciparum* strains:
 - (i) V1/S, a multidrug resistant strain,
 - (ii) W2-multidrug resistant strain and
 - (iii) D6, CQ sensitive strain
- b) Brine shrimp (*Artemiasalina*) eggs
- c) Male Swiss white rabbits.

3.1.2 Medicinal plants (Test articles)

- (i) *Achyranthes aspera* L.
- (ii) *Heinsiacrinite* (Afz.) G. Tayl.
- (iii) *Brideliacathartica* Bertol. f.
- (iv) *Citrus limon* L.
- (v) *Microglossapyrifolia* (Lam.) O. Ktze.
- (vi) *Vernoniaglabra* (Steetz) Vatke.
- (vii) *Carissa edulis* (Old name) changed to *Carissa spinarum* L.

3.1.3 Reagents

Chloroform, paraffin wax, agarose, coomassie blue, PBS pH 7.2, acetic acid, formalin, RPMI 1640, ethanol, DMSO, DCM, methanol, giemsa stain, immersion oil, hexane, double distilled water, CPD-adenine buffer, HEPES 25mmol/μl, sodium bicarbonate, Rhesus +ve normal human serum, liquid nitrogen, gas (CO₂ 3%, O₂ 5% and N₂ 95%), hypoxanthine solution, HYPOguaRD& ABO blood, haematoxylin and eosin stains.

3.1.4 Equipment

Rabbit cages, syringes, needles, culture flasks-250 ml, culture microtiter plates, syringe filters, microscope slides, coverslips, microscope Axioskope 40 light microscope, axio-imager z microscope (100X), clean sterile sacks, "Muharata" (hammer grinding machine), Mettler weighing balance, safety hood, freeze drier, NMR machine, IR machine, MS machine, incubator, micropipettes, heater, Mesh II harvester, liquid scintillator, GRAFIT 3.0 computer, brine shrimp hatchery box, IBM computer [28], Probit software, knives, microtome, scalpel blades, QBC II (Clay Adams), QBC blood tubes, glass plates for SRID& water bath and staining dishes.

3.2 Methods

3.2.1. Preparation of *invitro* Plasmodium test

Plasmodium falciparum standard laboratory-adapted field reference isolates V1S, W2 and D6 were used in this study. The strains were provided by the Kenya Medical Research Institute (KEMRI) where they are stored as reference stabilizers. The viabilities were confirmed before the antiparasmodial test was conducted. However, crude plant extracts were tested against V1S, while isolated compounds were tested against strains W2 and D6. Viability and parasitemia of cultured parasites were calculated by light microscopy analysis of blood smear stained with Giemsa (5000 erythrocytes counted per blood smear) as follows: thin smears were made from the donated parasitized erythrocytes. Thin air-dried blood smears were prepared on glass slides, fixed with methanol for 30 seconds, and stained with Giemsa's staining solution for a minimum of 10 minutes. Parasitemia (percentage of parasite infected-erythrocytes) was determined by counting 5000 erythrocytes using the Axioskop 40 light microscope equipped with a 100X oil-immersion objective (Zeiss, Jena, Germany). Multiply infected cells were counted as one. Microscopic pictures were taken with an Axio Imager Z1 microscope (100 X oil-immersion objective) using Axio Vision software (Zeiss).

Seven Kenyan medicinal plants were authenticated by a botanist from the University of Nairobi (UoN) as a way of confirming their botanical identity before they were collected from Kilifi and Homa Bay Counties. Specimens of these plant parts were then collected, kept individually in well labeled clean and sterile sacks in which they were transported to the laboratory for processing. A voucher specimen of each plant part was deposited at the University of Nairobi's herbarium (Table 1).

Table 1: List of plants collected and their characteristics

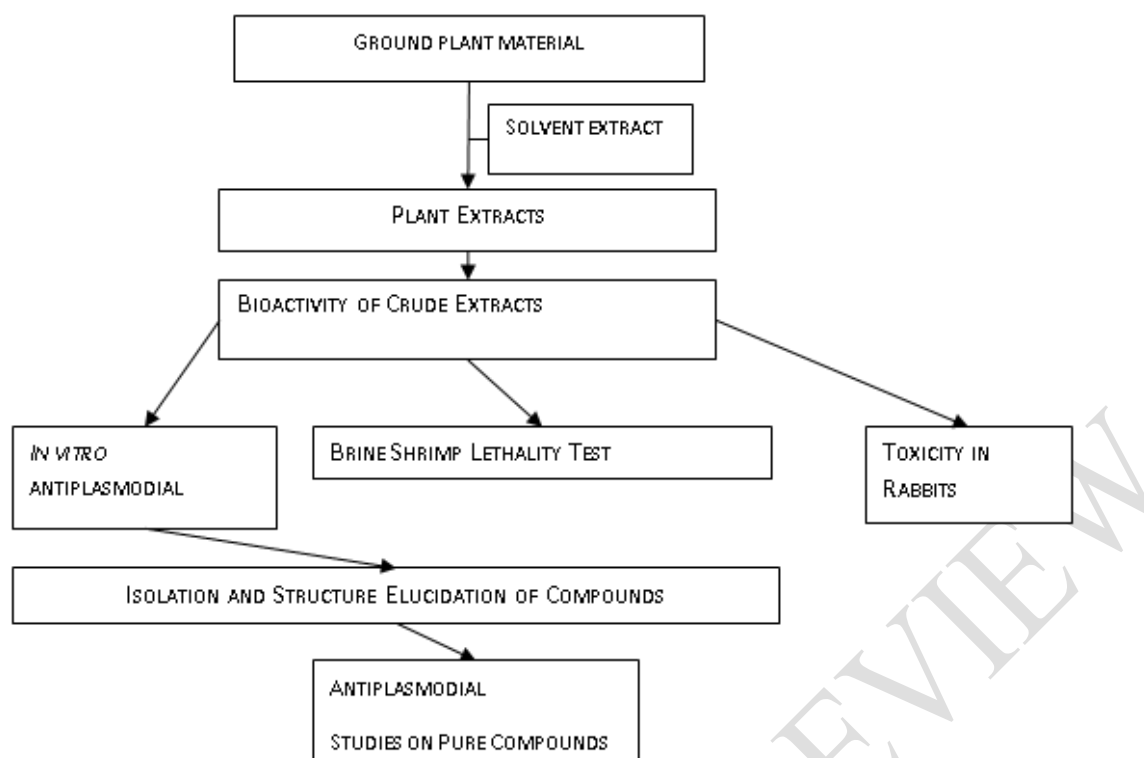
Plant Botanical Name	Voucher No.	Common/Vernacular Name
<i>Achyranthes aspera</i> L.	SG2001/01	T Tama Tama (Swahili), Prickly Chaff flower, Devil's horsewhip, Sanskrit, Apamargaama Tama (Swahili), Prickly Chaff flower, Devil's horsewhip, Sanskrit, Apamarga
<i>Heinsiacrinite</i> (Afz.) G, Tayl,	SG2001/02	Mfyofyo (Swahili), Mshosho (Giriama), Mushoka (Duruma) and Dewakiri (Nanya), Bush apple, Jasmine-gardenia, Small false gardenia
<i>Brideliacathartica</i> Bertol.f.	SG2001/03	MnembeNembe (Swahili), Blue sweetberry
<i>Citrus limon</i> L.	SG2001/04	Malimau (Swahili) Machunga Mar Ndim (Luo), Lemon
<i>Microglossa pyrifolia</i> (Lam.) O. Kutze	SG2001/05	Nyabung' Odide (Luo)
<i>Vernonia glabra</i> (Steetz) Vatke	SG2001/06	AkechMadongo (Luo), Cornflower vernonia
<i>Carissa edulis</i> (Old name) changed to <i>Carissa spinosa</i> L.	SG2001/07	Ochuoga (Luo) Mtanda-Mboo (Swahili) Fonkole, Dagams (Boran), Mutimuli (Bonjun), Molowe, Mulolwe (Duruma), Dagamsa (Gabora), Mokalakalo, Kaka- mchangani (Iwana/Malakote), Mukawa (kamba), Mukawa (Kikuyu), Olamuriaki (Maasai), Legatetwo (Marakwet/Tugen), Legetetwa, Legetetwet (Nandi, Kipsigis, Tugen), Lokotetwo (Pokot), Lmuria, Lmiriel (Samburu), Gurura, (Sanya), Kirumba (Taita) and Ekamuria (Turkana), Egyptian Carissa, Carandas plum, Karaunda (India)

For the purpose of this study, the plants were examined individually.

3.2.2 Preparation of the plant extracts

Upon collection from the field, the plant cuttings were chopped, dried at room temperature (about 30°C) in the presence of air for two weeks for adequate drying of roots and then pulverized by "Muharata" (Hammer grinding machine made locally by Kariobangi Light Industries) to powder. The dried pulverized powder, approximately 3000 grams (g), was divided into 100g and (2900g) portions. The larger portion was used to prepare organic extracts by cold percolation for 24 hours with the following purified solvents: n-hexane, dichloromethane and methanol sequentially. The filtrates from each extraction were concentrated *in vacuo*. After each extraction, the plant material was spread out in the hood and the solvent allowed to evaporate before extraction with the next solvent. The 100g portion of plant material was used to prepare an aqueous extract by boiling in water for 2 hours. The filtrate from the water extract was freeze dried to powder. All dried extracts were stored at 4°C. Aqueous and organic extracts were subjected to bioactivity guided fractionation using the *in vitro* antiplasmodial activity screening method.

The most active crude extract against *P. falciparum* was tested for its toxicity in adult Swiss white male rabbits. Pure compounds were isolated from the most active crude plant extracts against the Plasmodium using bioactivity-guided fraction. The isolated compounds were then subjected to Nuclear Magnetic Resonance (NMR), Infra-Red (IR) and Mass Spectroscopy (MS) for structure elucidation and then to *in vitro* efficacy assays against *P. falciparum* as shown in scheme 1.



Scheme 1: Screening for antiparasmodial activity

3.2.3 Preparation of parasite culture system

Antiparasmodial test was carried out according to a revised method described by [14]. *Plasmodium falciparum* laboratory reference isolate; V1/S an international multidrug resistant strain originally from a patient in Vietnam, W2-multidrug resistant strain and D6, CQ sensitive strain were used. Non-parasitized human O +ve red blood cells (RBC) (12-28 days old) were infected with the malaria parasites and cultured. Fifty (50) % RBC was prepared as outlined by [43]. The O +ve red blood cells in plasma was collected into 20 milliliters (ml) vacutainers containing citrate phosphate dextrose (CPD)-adenine buffer and was stored at 4°C for 24 hours; it was viable for 3 weeks before use. Erythrocytes were prepared for use by washing three times in WM (RPMI 1640) containing HEPES (5.94g/L), and sodium bicarbonate (7.5%, 31ml/L)). The supernatant and the buffy coat containing WBC were removed after each wash. After the final wash the RBCs were suspended in WM 50% (v/v) which would also be used in parasite cultures. Parasites stored under liquid nitrogen were rapidly thawed at 37°C and the isotonicity reconstituted as described by Watkins [43]. One ml of complete medium with serum (CMS) containing ten percent normal human serum which has been pooled and heat inactivated, Rhesus +ve, (NHS) in RPMI 1640 containing HEPES buffer 25mmol/μl and sodium bicarbonate 25 mmol/l was added to the culture, homogenized spun and the supernatant removed.

Fifty percent erythrocytes and CMs were added to the cells and homogenized to produce 6% haematocrit. A mixture of three percent carbon dioxide, five percent oxygen and ninety-five percent nitrogen gas were used to flush the parasites for 2 minutes which were then incubated at 37°C. The supernatant in each flask was renewed after every 24 hours and the cultures mixed by gently rotating the flask on a level surface before re-gassing and re-incubating. Parasitemia was assessed after every three days on Giemsa-stained thin films by counting the parasitized RBC among 10,000 RBC. When the parasitemia exceeded 2% the culture was diluted to a desired level by adding fresh 50% RBC and CMS, but maintaining the 6% haematocrit. The growth rate (GR) per 48 hours was calculated from the formula $GR = \frac{Pf - Pi}{Pi} \times \frac{1}{n}$ where Pf = final parasitemia, and Pi = initial parasitemia n = number of days in the culture [10]. The parasites were considered adapted to the *in vitro* culture and ready for drug test when they achieved a growth rate of 3-fold or greater in 48 hours.

3.2.4. Preparation of the working drug solutions for the tests

The stock solution of the drug containing 1 mg/20-50 μl DMSO was further diluted ten-fold to a concentration of 2 mg/ml with medium and was purified by filtering through a 0.22 μm filter. Twenty-five microliters (25 μl) of the working solution was dispensed in duplicates in row B of the test plate and diluted with an equal amount of CMS. A multi-tipped pipette was used to make two-fold dilutions from

one row to the next such that the highest concentration of a drug in row B was x64 that in the last row H. The final concentration after adding 200 μ l of parasites into the wells was such that row B had a drug concentration of 111.1 μ g/ml while in row H it was 1.74 μ g/ml.

3.2.5. Harvesting the malaria parasites

The malaria parasite cultures were incubated for 24 hours and labelled by adding radiolabeled [3H]-hypoxanthine solution per well and plates re-incubated further for 24 hours. The [3H]-hypoxanthine incorporation was measured by liquid scintillation on a Beta counter after drying the filter papers at 60°C for 30 minutes. The set up was that each drug concentration was tested in duplicate. The parasites were harvested using a Mesh II harvester on mini mash glass filter (Wittaker M A products) with plenty of distilled water after the second incubation period. The incorporation of [3H]-hypoxanthine was determined by liquid scintillation counting on a scintillation counter. The % inhibition was calculated using the formula: % Inhibition = [mean NTPE - mean DTPE cpm / mean NTPE - mean NPE] x 100. Where: cpm = count per minute. Mean NTPE = mean cpm for non-treated parasitized erythrocytes, mean NPE = mean cpm for non-parasitized erythrocytes and mean DTPE = mean cpm for drug treated parasitized erythrocytes.

3.2.6. Calculation of percentage parasite growth inhibition

The percentage inhibition of *P. falciparum* parasite growth by the drugs was determined using the formula:

$$\text{Inhibition} = [(cpm \text{ control} - cpm \text{ drug}) / (cpm \text{ control} - cpm \text{ background})] \times 100:$$

Where: cpm is count per minute; cpm drug = cpm for drug treated parasites; cpm control = cpm for the non-treated parasite; cpm background = cpm for wells containing medium alone (no parasite).

The percentage inhibition data were used to derive the drug concentration causing 50% inhibition of [3H]-hypoxanthine incorporation into nucleic acids (IC_{50}). A computer programme (GRAFIT 3.0 designed by Leatherbarrow [24]) was used where the data was fitted to the equation (a) below. The concentration response curve was plotted with the drug concentration displayed logarithmically on the X-axis and the percentage inhibition on the Y-axis.

Radioimmunoassay equation (a) for the concentration-response curve expressed in terms of IC_{50} value is thus given as:

$$y = [a / ((1 + 1/IC_{50}C) \times C)] + d$$

Where **a**, is the maximum **y** range; **d**, is the background **y** value; and **c**, is a slope factor. Where necessary, statistical differences between mean IC_{50} values were examined by the student's t-test.

3.3 Safety screening

3.3.1. In vivo toxicity studies

Since most bioactive plant constituents are toxic at higher doses, a possible approach to developing a useful general bioassay is to screen for plant extracts that are toxic to zoologic systems. For this purpose, the brine shrimp (*Artemia salina*) lethality test was originally proposed by Bernard [6]. It represents an easy way to detect general bioactivity in plant extracts and a handy procedure for tracking the isolation of bioactive constituents. A rectangular plastic double-chambered box with dividing wall and which had 2.3mm holes (Encia-Italy) was used to hatch brine shrimp eggs from Lake Urmia-Iran. Artificial sea salt water made by dissolving 16 grams of sea salt in five hundred milliliters of distilled water was used to fill the chamber. Dry yeast 3 milligrams (mg) were added to serve as food for the larvae. The eggs were sprinkled carefully in the dark compartment while the other compartment was illuminated by natural light through a hole in the lid of the box. After 48 hours, the larvae were collected by using a pipette from the illuminated side to which they hatched due to their phototropism behavior. They were separated from their shells by the divider wall [37].

Dimethylsulphoxide (DMSO) was used as a solvent to dissolve the plant extracts and the drug solution was then diluted with artificial sea salt water so that the DMSO content did not exceed 0.05%. Ten brine shrimps (Lake Urmia-Iran) were transferred to 1 ml of each plant sample vial containing 125, 250, 500, and 1000 μ g/ml of plant extract using a Pasteur pipette. The experiment was set in duplicate for each concentration of the drug. The control tube had only sea water and DMSO. Brine shrimp survivors were enumerated after twenty-four hours and the lethality fifties (LD_{50} values). These were determined by taking average of five assays using a Finney Probit analysis program on an IBM computer [28] or the ED_{50} values (μ g/ml) calculated using Probit, a computer program [37]. The results are shown in Table 4.

3.3.2 Experimental design

DCM extract of ground *Citrus limon* roots was subjected to toxicity studies in adult male rabbits weighing 2 kilograms (kg) which were fed daily on rabbit pellets (UNGA® feeds Ltd) and tap water *ad libitum*. The rabbits were chosen for this study for ease of handling during blood collection. The animals were divided into four groups of 5 rabbits each. The fourth group was the control. Hematological parameters such as packed cell volume (PCV), platelets (PLT), granulocytes (neutrophils (NEU), eosinophils (EOS) and basophils (BAS)) and lymphocytes (LYM), and biochemical parameters such as blood glucose (GLU) levels and serum lactate dehydrogenase (LDH) activity, immuno-globulin such as IgA, IgG and IgM levels, and general health parameters such as weight were determined twice a week for two weeks during which the drug was administered. The administration of the plant extract was as follows: group one rabbits was injected subcutaneously with 0.8 g/kg body weight, group two was injected subcutaneously with 1.6 g/kg body weight dose, group three was injected subcutaneously with 2.4 g/kg body weight while group four were not given any drug except the vehicle [48]. The administration of the drug continued on daily basis for 14 days [26]. The parameters were monitored six hours after the administration of the drug and twice a week for two weeks post extract administration. Overall, the health and general well-being were observed and recorded on daily basis (Table 2).

Table 2: Parameters used to assess safety of *Citrus limon* root DCM extract

Treatment group	Dose (g/kg body weight)	Parameters
1	0.8	Body weight Biochemistry: LDH and glucose levels Histology: liver, heart, kidneys, brain, spleen, and pancreas
2	1.6	Body weight Biochemistry: LDH and glucose levels Histology: liver, heart, kidneys, brain, spleen, and pancreas
3	2.4	Body weight Biochemistry: LDH and glucose levels Histology: liver, heart, kidneys, brain, spleen, and pancreas
4	0	Body weight Biochemistry: LDH and glucose levels Histology: liver, heart, kidneys, brain, spleen, and pancreas

3.4 Drug administration and sample collection

3.4.1 Subcutaneous injection (SI)

The crude *Citrus limon* root DCM extract was dissolved in 0.05ml 10% analytical grade ethanol in distilled water completely and then injected into the subcutaneous tissues of the rabbits daily for 14 days. The dissolved drug for parenteral injection as a requirement was warmed to rabbit body temperature before administration. This volume of ethanol cannot affect the plasma volume as a vehicle on a 2kg rabbit [15]. This route of injection is frequently used in the laboratory experiments especially in tests for toxicity [3, 39]. Absorption through this route is slow but uniform [44]. A stock of the drug of 3.2 g/kg body weight was prepared.

Body weights of the rabbits and whole blood for blood counts, plasma and serum for biochemistry (plasma glucose, LDH, IgA, IgG and IgM levels) were collected aseptically using syringe and needle as baseline data (day zero) and twice weekly for two weeks (14 days) the period for which the drug was administered. Throughout the experiment, the animals were observed for any toxic reactions twice daily.

3.4.2 Hematology

Before collecting blood from the marginal ear-vein of the rabbit, the ears were cleaned thoroughly with 70% ethanol analytical grade. Venous blood was drawn with a vacutainer containing anticoagulant ethylenediaminetetraacetic acid disodium salt (EDTA). The blood was then thoroughly mixed with the anticoagulant and analyzed for hematological parameters using the QBC II (Clay Adams®). The Clay Adams QBC II System is a seven-parameter hematology screening device with the following quantitative values from a centrifuged blood tube: packed cell volume (PCV), platelet count (PLT),

white blood cell count (WBC), granulocyte count (% and number) and lymphocyte-monocyte count (% and number). The QBC II platelet count, white blood cell counts and counts of the granulocyte and lymphocyte/monocyte white cell subpopulations are derived from electro-optical measurements of the packed cell volumes in a specially-designed QBC blood tube.

3.4.3 Biochemistry

Blood glucose levels were determined using the supreme kit [20]. The ears of the rabbits were aseptically prepared using 70% ethanol and 2 ml of blood obtained using a syringe. The blood was applied to the test strip and color development matched with the color comparison chart or the strip was inserted into the reading meter. Serum LDH levels were determined by the standard methods described by [49].

3.4.4 Immunology - Single radial immunodiffusion (SRID)

This assay was performed as outlined Mancini [27]. Agarose was melted in a microwave and transferred to 56°C water bath. 240 µl of antisera was each added to 12 ml of agarose at 56°C and mixed well. The agarose was carefully layered on to pre-coated glass plate 8x8 centimeters squared (cm²) standing on a levelled surface and allowed to set. After the gel had set, use was made of a gel punch to cut 20 wells per plate. The wells were 3mm in diameter and had vertical sides. The agarose plug was removed with a Pasteur pipette attached to a water vacuum pump. Each of the 5 wells on the left-hand side was filled with standard solutions of 50, 100, 150, 200 and 250 µg/ml. A measured volume of 10 µl for IgG and 20 µl for the rest was adequate. The remaining 15 wells were filled with the sera from the experimental and control rabbits. The plates were left in a humid chamber to equilibrate.

To measure the precipitin rings, the plates were washed for 24 hours in a few changes of phosphate buffered saline pH 7.2 (PBS) with the aim of removing free protein from the agarose. Good quality, lint free filter paper was used to cover the plates and then tissue paper was added on top with little weight to dry the gel overnight. The tissue paper and the weight were removed first then the filter paper was damped slightly with distilled water before removing it. The plates were air dried then stained submerged in the staining mixture [27] in a staining dish for 5 minutes. The staining mixture was prepared as follows: The Coomassie brilliant blue dye (1.25g) was dissolved in a solution of glacial acetic acid (50ml) and distilled water (185 ml). The plates were stained for five minutes and the same solution without the dye was used for differentiation. The dry, stained plates were placed in a photographic enlarger and the diameter of the precipitation rings was measured with a ruler. For the standards the diameters of the rings were measured and plotted on a linear scale against the log of the antigen concentration. The concentrations of both experimental and control immunoglobulins were read from the respective curves.

3.5 Determination of general health of animals

3.5.1 Determination of weight

Weights of experimental and control rabbits were determined using Shadow-Graph (Exact Weight Scale Company, Columbus, Ohio), a weighing balance with a wide maximum capacity range. The rabbits were weighed as follows: a rabbit was removed from its holding cage one at a time. The animal was carefully placed on the weighing balance. Weights were removed or added from the scale until balance was achieved. Weight determination of the animal was done by adding up the total numbers on the balance in designated metric units.

3.6 Statistical analysis

In this study, the collected biochemical, hematological, immunological and health data was initially recorded in the laboratory notebook. This was then entered into the excel spreadsheet, cleaned for errors and exported into SPSS software. The descriptive statistics including mean, SEM, median, mode, variance, standard deviation, range, minimum and maximum were generated. Statistical significance within and between means of the measured parameters including weight, packed cell volume (PCV), granulocytes (neutrophils, eosinophils and basophils) (GRAN), lymphocytes (LYM), platelets (PLT), immunoglobulins IgA, IgM, and IgG, lactate dehydrogenase (LDH), and glucose (GLU) at the doses of 0.8, 1.6, and 2.4 g/kg body weight, and control at time point 14 days were compared using ANOVA followed by Tukey's post ANOVA. An adjusted *P*-value of less than 0.00833 was considered significant.

3.7 Histology

3.7.1 Tissue harvesting

Tissues (brain, heart, liver, spleen and kidney) were harvested immediately after euthanasia to prevent postmortem autolysis and decomposition as changes occur in tissues within minutes of death. More organs and tissues were collected than needed even when the study was limited to just one organ system. Just one tissue was submitted for histology and the rest were kept in 10% formalin indefinitely, just in case the need arose to look at other tissues. One tissue for each organ was trimmed for fixation and the others stored. The fixative, 10 percent formalin, was prepared by dissolving 8.5g of sodium chloride in 900ml of distilled water and mixing with 100ml of 40 percent formaldehyde. The trimmed tissue was fixed in 10 percent formalin using 10 to 20 times its volume.

3.7.2 Histological tissue embedding, sectioning and staining

Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mold. Since the tissue blocks are very thin, they need a supporting medium in which the tissue blocks are embedded. This supporting medium is called embedding medium. In this process tissue was dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with wax. The infiltrated tissues were then embedded into wax blocks. Once the tissue was embedded, it was stable for many years. The tissues were then cut or sectioned in the microtome at thicknesses varying from 2 to 50 μm . Obtained sections from the same site of the same tissue were processed and stained by hematoxylin and eosin. These sections were then microscopically examined using X100 objective and photographs were taken.

4. RESULTS

4.1 Plant extracts

4.1.1 Yield of plant extracts

The seven plants in this study were extracted with aqueous and organic solvents. Table 3 gives weights of ground material, weights of their respective organic and aqueous extracts and the percentage yields per plant. Aqueous extracts had the highest percentage yield in each plant. The highest aqueous extracts percentage yield was obtained from the leaf of *M. pyrifolia* (39.74g) followed by *V. glabra* (25.66g). The least aqueous extracts percentage yield was obtained from *C. edulis* root (8.175g). Among the organic extracts the highest percentage yield was seen in the methanolic leaf extract of *V. glabra* (12.203 g) followed by another methanolic leaf extract of *M. pyrifolia* (10.43g). Hexane extracts had the least percentage yields followed by DCM extracts (Table 3).

Table 3: Yield of the plant extracts

Plant material	Powder (g)	Solvent	Extract weight (g)	% Yield
<i>Achyranthes aspera</i> leaves (AAL)	116	Hexane	0.3	0.259
		DCM	0.8	0.69
		Methanol	4.3	3.707
		Water	1.387	1.196
<i>Bridelia cathartica</i> leaves (BCL)	152.3	Hexane	4.6	3.020
		DCM	2.7	1.773
		Methanol	10.7	7.026
		Water	0.89	0.584
<i>Hemiacranium</i> Leaves (HCL)	92.2	Hexane	0.9	0.976
		DCM	0.7	0.759
		Methanol	2.7	2.928
		Water	1.119	1.214
<i>Citrus limon</i> roots (CLR)	31	Hexane	0.3	0.968
		DCM	0.9	2.903
		Methanol	1.0	3.225
		Water	0.764	2.465
<i>Microglossa pyrifolia</i> leaves (MPL)	16.3	Hexane	23.059	2.089
		DCM	18.225	1.651
		Methanol	1.7	10.43
		Water	3.974	39.74
<i>Vernonia glabra</i> leaves (VGL)	69	Hexane	0.6	0.870
		DCM	1.3	1.884
		Methanol	7.2	10.435
		Water	2.567	3.720

<i>Carrisa edulis</i> root (CER)	159.5	Hexane	1.1	0.690
		DCM	0.6	0.376
		Methanol	4.5	2.821
		Water	1.635	1.025

4.1.2 Results of the Brine shrimp lethality test

The results of the brine shrimp lethality test are displayed in Table 4. The DCM extract of *Achyranthes aspera* leaves was the most active against brine shrimps with an LC_{50} of 0.460 $\mu\text{g/ml}$. *Brideliacathartica* leaf DCM and methanolic extracts had LD_{50} s of 6.163 $\mu\text{g/mL}$ and 6.197 $\mu\text{g/mL}$, respectively, against the brine shrimps. Both *Citrus limon* (CL) hexane ($P = .00$) and DCM ($P = .00$) root extracts were too active at the concentrations used against the brine shrimps and the two killed all the shrimps depicted by the values. *Citrus limon* methanolic root extract had LC_{50} of 2.195 $\mu\text{g/ml}$. *Microglossapyrifolia* (MP) hexane leaf extract had LC_{50} of 3.389 $\mu\text{g/ml}$ while its DCM leaf extract had LD_{50} of 3.260 $\mu\text{g/ml}$. *Vernoniaglabra* (VG) hexane leaf extract had LC_{50} of 6.087 $\mu\text{g/ml}$, while its DCM leaf extract was more active with an LC_{50} of 2.449 $\mu\text{g/ml}$; its methanolic leaf extract was the most active against brine shrimp in this plant with an LD_{50} of 0.106 $\mu\text{g/ml}$ second to *Citrus limon* root hexane and DCM extracts. *Achyranthes aspera* (AA) hexane and methanolic leaf extracts were not active against brine shrimp larva. *Brideliacathartica* (BC) hexane leaf extract was inactive against the brine shrimps. Methanolic extracts of MPL did not show any activity against brine shrimps (Table 4).

Table 4: LC_{50} s ($\mu\text{g/ml}$) of crude plant extracts against brine shrimps calculated at 95% confidence interval using probit

Drug	Hexane (1)	DCM (2)	Methanol (3)
<i>Achyranthes aspera</i> leaves (AAL)	> 1000	0.460	> 500
<i>Brideliacathartica</i> leaves (BCL)	> 500	6.163	6.197
<i>Citrus limon</i> roots (CLR)	< 0.00	< 0.00	2.195
<i>Microglossapyrifolia</i> leaves (MPL)	3.389	3.260	> 500
<i>Vernoniaglabra</i> leaves (VGL)	6.087	2.449	0.106

Three plants including *B. cathartica*, *C. limon* and *M. pyrifolia* were further processed using their DCM and methanol extracts to obtain pure compounds.

4.1.3 In-vitro antiplasmodial activity of the plant extracts

The *in-vitro* antiplasmodial activities of the extracts against V1/S, multidrug resistant strain of *P. falciparum*. Results indicate that the most active crude extract against *P. falciparum* was a DCM root extract of *C. limon* with an IC_{50} of 7.017 $\mu\text{g/mL}$. The second crude extract in terms of antiplasmodial activity was an aqueous extract of *C. edulis* roots with an IC_{50} of 8 $\mu\text{g/mL}$. The leaves of *B. cathartica* DCM extract was the third most active crude extract against *P. falciparum* with an IC_{50} of 11.537 $\mu\text{g/mL}$. The statistical differences between mean IC_{50} values were examined by the student's t-test (Table 5).

Table 5: In-vitro antiplasmodial activity of plants extracts against V1/S Strain

Plant material	Solvent	IC_{50} s ($\mu\text{g/ml}$)
<i>Achyranthes aspera</i> leaves (AAL)	Hexane	18.087
	DCM	86.501
	Methanol	111.127
	Water	38.990
<i>Brideliacathartica</i> leaves (BCL)	Hexane	32.908
	DCM	11.537
	Methanol	15.647
	Water	25.985
<i>Hensiacrinital</i> leaves (HCL)	Hexane	34.223
	DCM	13.336
	Methanol	24.805
	Water	47.203
<i>Citrus limon</i> roots (CLR)	Hexane	30.092
	DCM	7.017
	Methanol	916.997
	Water	96.860
<i>Microglossapyrifolia</i> leaves (MPL)	Hexane	21.376
	DCM	34.88
	Methanol	313.647
	Water	203.457

Vernoniaglabra leaves(VGL)	Hexane	427.40
	DCM	53.62
	Methanol	112.495
	Water	-
	Hexane	193.599
Carrisa edulis root (CER)	DCM	30.074
	Methanol	69.969
	Water	8.054
	Hexane	193.599
Chloroquine		9.915 ng/ml

The resistance of *P. falciparum* to antimalarials and mosquitoes to insecticides, has necessitated search for new compounds against malaria making use of leads from ethnopharmacology studies. To the populace relying on medicinal plants against malaria, it is vitally important that the safety and efficacy of such medicines be determined, reproducible dosage forms be developed and availed for use, and their active components determined [35, 41].

Resistance to chloroquine is stated as an IC_{50} less than 100nM (approximately 0.052 $\mu\text{g/mL}$) [5]. However, K39 and V1 strains of *falciparum* fell far below this cut-off concentration in this study for reasons which could not be explained (IC_{50} of CQ was 0.040 $\mu\text{g/mL}$ for W2& 0.011 $\mu\text{g/mL}$ for D6, IC_{50} of Mefloquine was 0.012 $\mu\text{g/mL}$ for W2and 0.040 $\mu\text{g/mL}$ for D6 and IC_{50} of Quinine was 0.103 $\mu\text{g/mL}$ for W2&0.031 $\mu\text{g/mL}$ for D6). Most researchers consider IC_{50} values above 100 $\mu\text{g/mL}$ to be inactive [5] and that values ranging between 20-100 $\mu\text{g/mL}$ as moderate activity. Researchers have grouped plants with the following activities against malaria parasites as follows; Group A (greater than 1 $\mu\text{g/mL}$), B (1 to 5 $\mu\text{g/mL}$) and C (6 to 10 $\mu\text{g/mL}$) [5]. Most of the plant extracts in this study except DCM root extract of *C. limon* with an IC_{50} of 7.017 $\mu\text{g/mL}$, aqueous extract of *C. edulis* with an IC_{50} of 8.054 $\mu\text{g/mL}$ and DCM and methanolic extract of *B. Cathartica* with an IC_{50} of 11.537 $\mu\text{g/mL}$ and 15.647 $\mu\text{g/mL}$, respectively, DCM extract of *H. crinite* with an IC_{50} of 13.336 $\mu\text{g/mL}$ and hexane extract of *A. aspera* with an IC_{50} of 18.087 $\mu\text{g/mL}$ are considered to be within the mild or moderate activity range. Values less than 20 $\mu\text{g/mL}$ are considered to be in the high activity range for crude plants extracts.

4.4 In-vivosubacute toxicity testof DCM extract of *C.limon*root in rabbits

4.4.1 In-vivo Toxicity Test in Rabbit Results

The most active crude extract (DCM root extract of *C. limon* IC_{50} 7.017 $\mu\text{g/mL}$) was tested in rabbits for its toxicity and the results are as shown in Figures 2-10& Table 6.

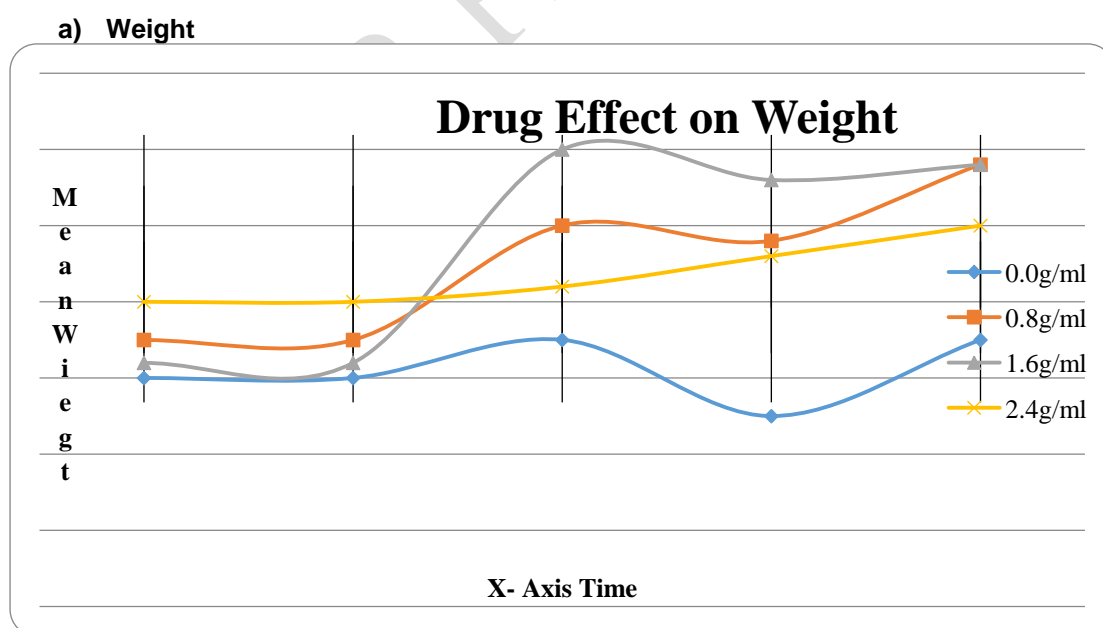


Figure 2. Mean weight in kg plotted against drug concentration in g/kg

Key: y axis represents parameter value/counts/concentration.

X axis represents number of days the parameter was determined; 1 is pretreatment baseline sampling and 2-5 is sampling during treatment within two weeks.

This was the most affected parameter as a whole. Treatment with 0.8 g/kg differed from treatment with 1.6 g/kg significantly as the P -value was 0.025, from treatment with 2.4 g/kg with a p value equal to 0.04 and from the controls with a $P = 0.00$ level. Treatment with 1.6 g/kg differed from treatment with 2.4 g/kg very significantly with a $P = 0.00$ and from the controls again very significantly with a $P = 0.00$. The increase in mean weight in treatment with 1.6 g/kg was more than the increase in treatment with 2.4 mg/kg. Treatment with 2.4 g/kg differed from the controls very significantly with $P = 0.00$. The increase in weight in treatment with 0.8, 1.6 and 2.4 g/kg increased with the drug concentration (Figure 2& Table 6).

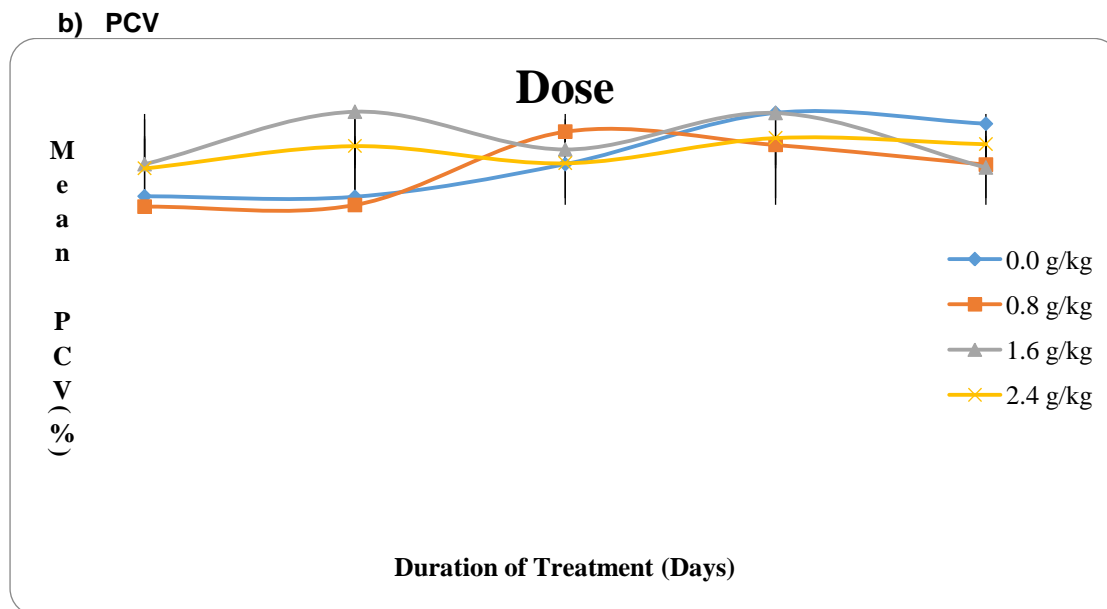


Figure 3. Mean PCV % plotted against drug concentration in g/kg

There were no significant changes in PCV in the treated groups (Figure 3& Table 6).

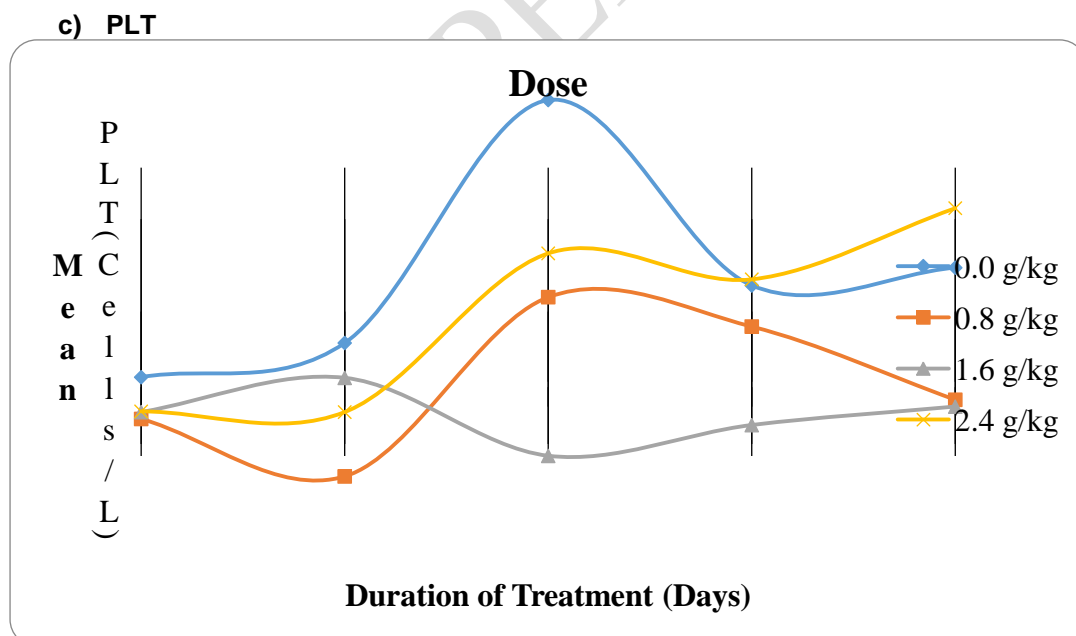


Figure 4. Mean PLT Cells/L plotted against drug concentration in g/kg

Treatment with 0.8 mg/kg crude DCM extract compared to the controls, had a significant difference of $P = 0.03$ but it did not differ significantly from treatment with 1.6 and 2.4 mg/kg. Treatment with 1.6 mg/kg compared to block treatment with 2.4 mg/kg had a significant difference of $P = 0.01$. There was an increase in PLT levels in treatment with 2.4 mg/kg. Treatment with 1.6 mg/kg compared to

controls, had a big margin in that the significance had a $P = 0.01$ level. Therefore, the treatment negatively affected the levels of platelets significantly (Figure 4 & Table 6).

d) GRANULOCYTES

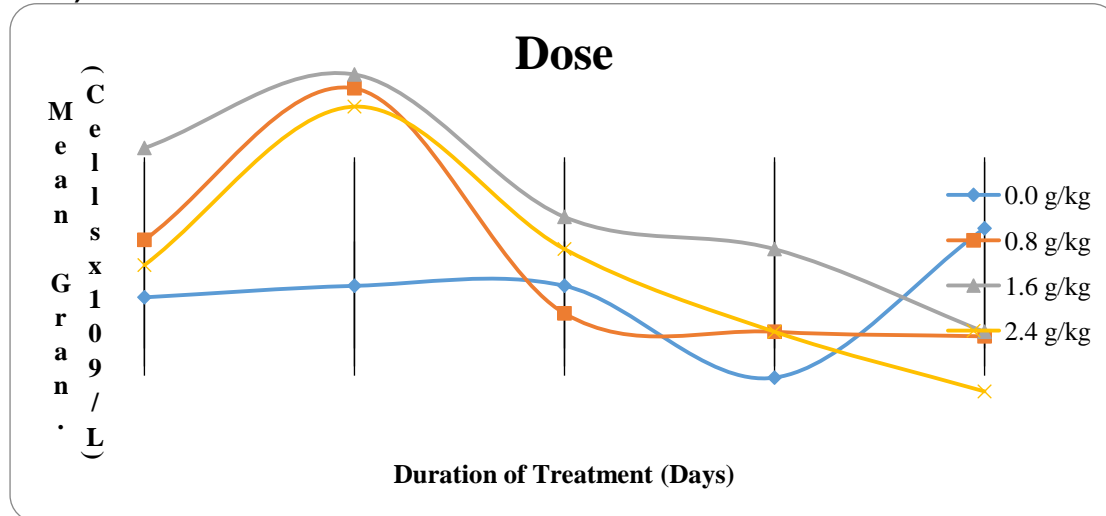


Figure 5. Mean Granulocyte count x10⁹ cells/L plotted against drug concentration in g/kg
This parameter was not affected by the treatments (Figure 5 & Table 6).

e) LYMPHOCYTES/MONOCYTE

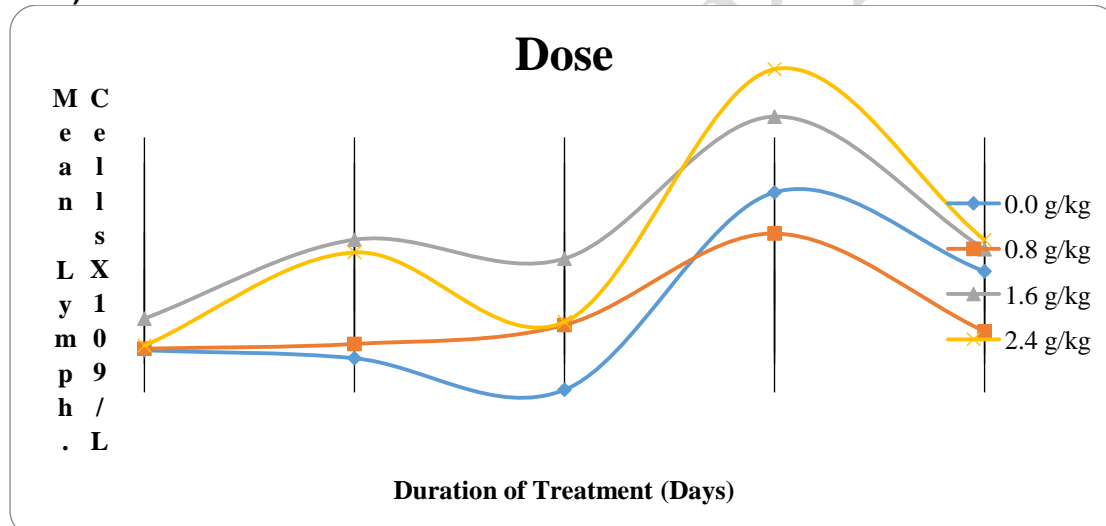


Figure 6. Mean Lymphocyte/Monocyte count X10⁹ cells/L plotted against drug concentration in g/kg

Treatment with 0.8 g/kg differed significantly from treatment with 1.6 g/kg with $P = 0.01$ at a confidence limit of 95%. The difference between treatment with 0.8 g/kg and treatment with 2.4 mg/kg was also big with $P = 0.02$ but there was no difference between treatment with 0.8 g/kg and the controls. The number of lymphocytes increased with the increase in drug administration with a significant increase seen on the 5th treatment with 1.6 and 2.4 g/kg. Controls did not significantly differ from any treatment, showing that the treatment did not affect the number of lymphocytes throughout the experiment (Figure 6 & Table 6).

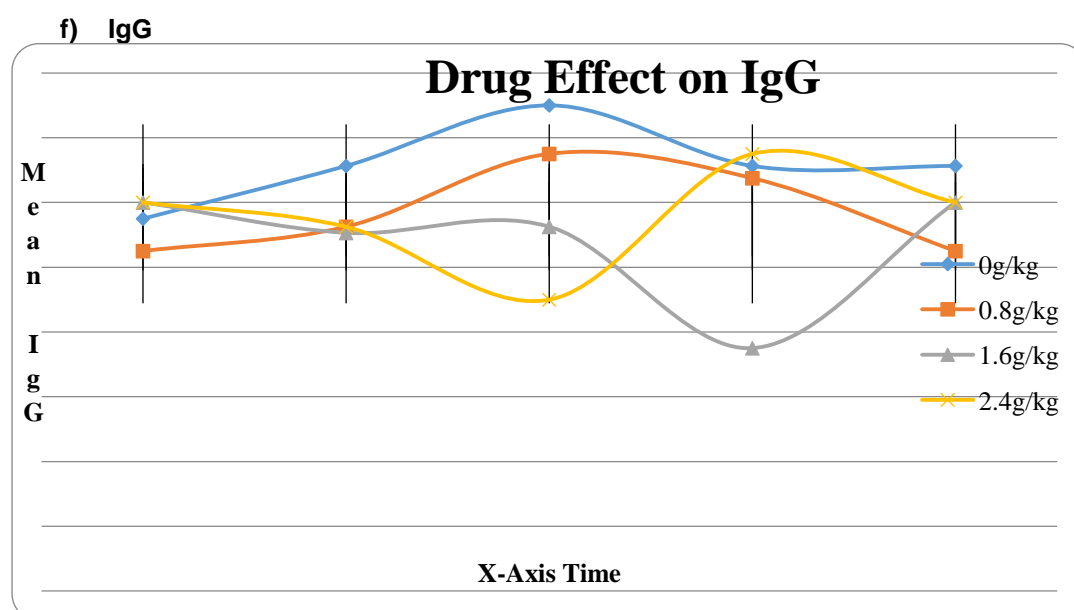


Figure 7. Mean IgG mg/L plotted against drug concentration in g/kg

Treatment with 0.8 g/kg differed from treatment with 1.6 g/kg significantly thus having $P = 0.01$. Treatment with 1.6 g/kg 2 differed from controls very significantly with $P = 0.00$. Treatment with 2.4 g/kg differed from the controls, with $P = 0.03$. The drug had a suppressive effect on the mean IgG levels as the increase in the concentration of the drug reduced the concentration of IgG (Figure 7& Table 6).

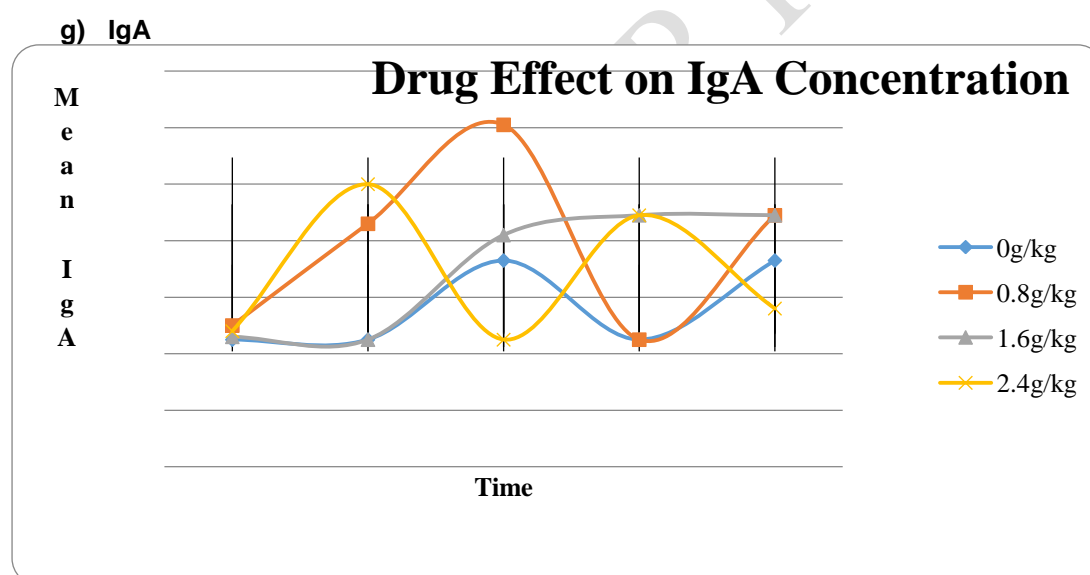


Figure 8. Mean IgA mg/L plotted against drug concentration in g/kg

This parameter was not affected by the various treatments (Figure 8& Table 6).

h) 1gM

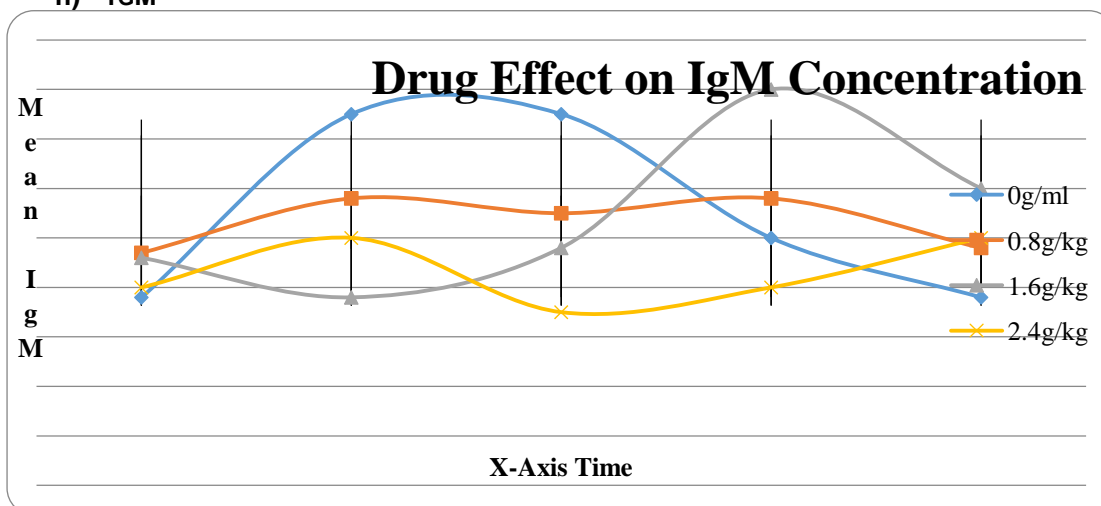


Figure 9. Mean IgM mg/L plotted against drug concentration in g/kg

Treatment with 0.8 g/kg did not differ with any treatment including the controls. Treatment with 1.6 g/kg differed from treatment with 2.4 g/kg significantly with $P = 0.05$ (Figure 9& Table 6).

i) LDH

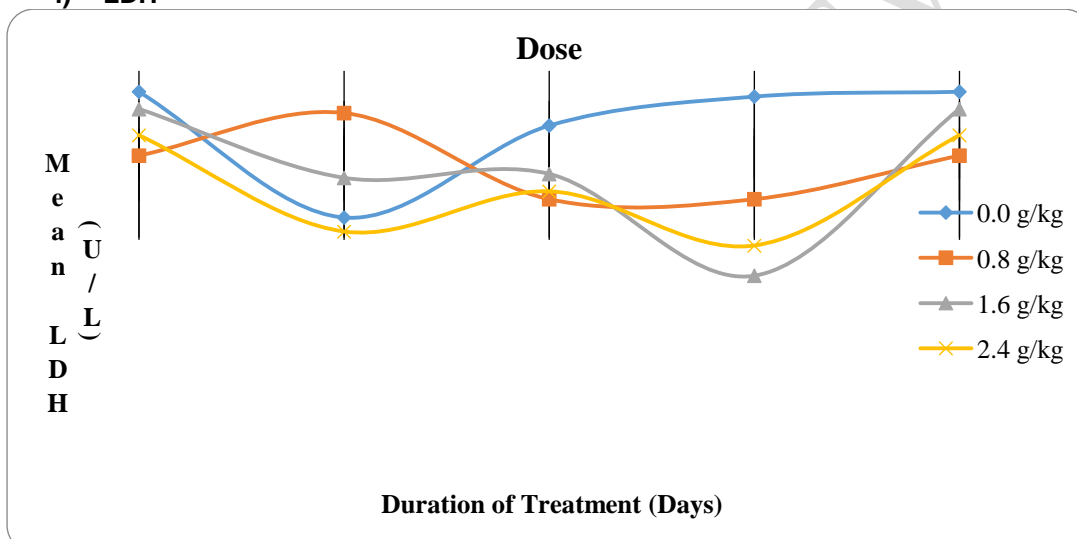


Figure 10. Mean LDH U/L plotted against drug concentration in g/kg

This parameter was not affected by treatment of animals (Figure 10& Table 6).

j) GLUCOSE

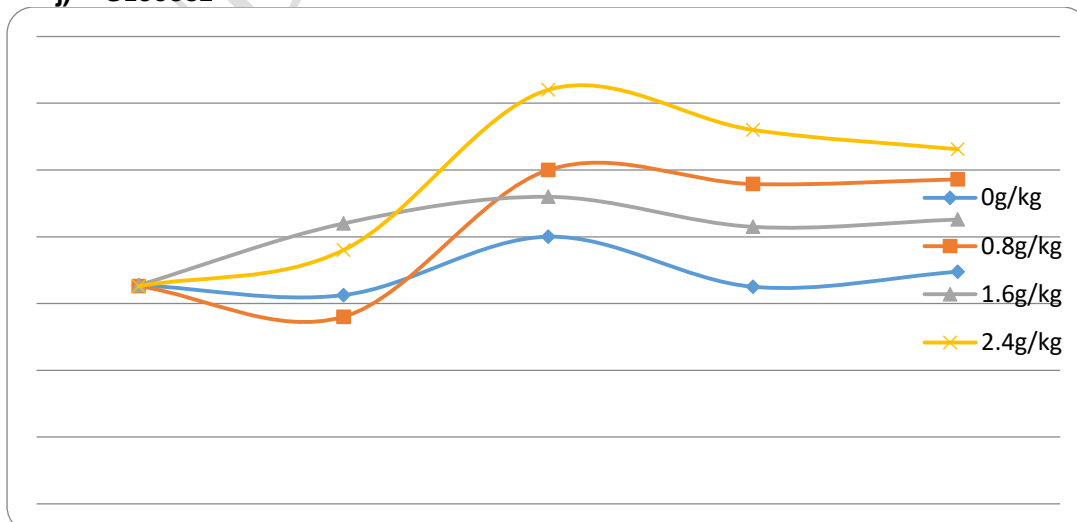


Figure 11. Mean Glucose mg/dl plotted against drug concentration in g/kg

Treatment with 1.6 g/kg differed from treatment with 2.4 g/kg significantly with $P = 0.04$. The different treatments were significant whereby treatment with 1.6 g/kg gave less glucose than treatment with 2.4 g/kg. Treatment with 2.4 g/kg differed from the controls significantly with $P = 0.01$ (Figure 11 & Table 6).

The effects of a daily subcutaneous administration of DCM extract of *C. limon* root at 0.8, 1.6, and 2.4 g/kg body weight to rabbits for 14 days on body weight, packed cell volume, platelets, granulocytes, lymphocytes, immunoglobulins IgA, IgG, and IgM, lactate dehydrogenase and glucose is presented in Table 10. Results indicate that all the measured parameters of the extract treated rabbits at all the tested doses were not significantly affected except those of platelets and IgG when compared to those of the control rabbits. The platelet levels for rabbits treated with the DCM extract of *C. limon* roots at 0.8 and 1.6 g/kg body weight were similarly decreased when compared to that of the control rabbits. Further, the IgG levels for rabbits treated with the DCM extract of *C. limon* roots at 1.6 and 2.4 g/kg body weight were similarly decreased when compared to those of the normal control rabbits.

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Table 6: Effect of subcutaneous administration of DCM root extracts on hematological, immunological and biochemical parameters in rabbits (N=5)

Treatment	Weight(kg)	PCV	PLT	GRAN	LYM	IgG	IgA	IgM	LDH	GLU
Control	2.45±0.35a	44.60±0.20a	510±109a	1.15±0.75a	1.50±0.50a	13.13±1.88a	0.73±0.30a	0.38±0.07a	89±15a	69.50±8.5a
Extract dose (g/kg body weight)										
0.8	2.68±0.16a	40.66±1.75a	317±24.6b	0.68±0.24a	1.12±0.22a	10.50±0.75a	0.89±0.11a	0.48±0.02a	75.8±4.95a	97.2±10.2a
1.6	2.58±0.24a	40.38±1.51a	307±18.1b	0.70±0.35a	1.64±0.12a	12.0±0.75b	0.89±0.11a	0.60±0.10a	85.4±6.58a	85.2±9.07a
2.4	2.60±0.21a	42.64±1.09a	597±63.1a	0.44±0.10a	1.70±0.20a	12.0±0.75b	0.56±0.11a	0.50±0.00a	80.0±6.67a	106.2±9.24a

Results are expressed as mean ± standard deviation. Statistical analysis was carried out using ANOVA followed by post-ANOVA test. $P = 0.05$ was considered significant. Values with the same small case superscript letters are statistically similar.

Key: (a) Weight (kg) (b) PCV (%) (c) PLT ($\times 10^9/L$) (d) GRAN ($\times 10^9/L$) (e) LYM ($\times 10^9/L$) (f) IgG, IgA & IgM (mg/L), (g) LDH (U/L), (h) GLU (mmol/L)

Citrus aurantiifolia is frequently used against malaria in Brazil [34] and also *M. pyrifolia*, also studied here, has been used in Ghana against malaria. The present study also established the presence of some popular herbal antimalarial plant species in Nyanza and the Coastal region that may not be common in other regions. However, though *B. carthatica* has been used in Zimbabwe against malaria, its crude extracts did not exhibit significant antiplasmodial activity in this study probably because of geographical variations. The parts utilized by the traditional healers may also not necessarily contain the most active compounds for the choice may depend on the convenience of preparation. Most of the antimalarial concoctions are obtained from roots, leaves and at times the entire plant [33].

None of the crude extracts fell in the 1st or 2nd groups. The DCM extract of *C. limon* roots with an IC₅₀ of 7.017 µg/mL, and an aqueous extract of *C. edulis* roots with an IC₅₀ of 8.054 µg/mL were the only crude extracts that fell within group C. The rest had lower activities with IC₅₀s greater than 11 µg/mL; for example, DCM extract of BCL had an IC₅₀ of 11.537 µg/mL, DCM extract of HCL had an IC₅₀ of 13.336 µg/mL, methanolic extract of BCL had an IC₅₀ of 15.647 µg/mL and hexane extract of AAL had an IC₅₀ of 18.087 µg/mL. The remaining extracts had antimalarial activities above 20 µg/mL and up to 916.997 µg/mL. Contrary to work by Jurg [21] who demonstrated that unprocessed aqueous and ethanolic extracts of the root and the ethanolic stem extract of *B. cathartica* resulted in a 50% growth inhibition of *P. falciparum* when kept at 0.05 µg/mL, the present study showed that the extracts from this plant were generally active. The difference could have been due to the different localities and therefore different soil textures and climatic conditions. Out of the 28 crude extracts tested, only five had IC₅₀s greater than 100 µg/mL and thus 82% were active. This can reflect some accuracy in the part played by the herbalist and the authenticating authority at the University of Nairobi.

The following ranges of IC₅₀s were observed per plant regardless of the chemical used for extraction: AAL with an IC₅₀ of 18.087-111.127 µg/mL, HCL with an IC₅₀ of 13.336-47.203 µg/mL, CLR with an IC₅₀ of 7.017-916.997 µg/mL, MPL with an IC₅₀ of 21.376-313.647 µg/mL, VGL with an IC₅₀ of 53.62-427.40 µg/mL, CER with an IC₅₀ of 8.054-193.599 µg/mL and BCL with an IC₅₀ of 11.537-32.908 µg/mL. The same plants that showed high activities with IC₅₀s of 7.017-11.537 µg/mL in category C against *P. falciparum*, had also significant bioactivity against brine shrimp, *Artemiasalina*. It therefore shows that these plant extracts were generally active. [1] found a low antimalarial activity of an aqueous extract of *C. sinensis*. Various workers have claimed *C. limon* to have the following attributes: antiperiodic, astringent, antibacterial, antiscorbutic, carminative, refrigerant, stimulant, miscellany, rubefacient and stomachic. Lemons being the source of the most active crude extract with an IC₅₀ of 7.017 µg/mL is an extremely good prophylactic medicine for most ailments and has many uses at home. Vitamin C in which the fruit is rich in aids the body in the fight against infections and again protects or treats scurvy infection [9] it has also been employed as a replacement for quinine against malaria and other fevers [18].

Subacute toxicity studies and other repeat-dose studies have been done to, determine the nature of toxic events, target organ for toxicity (liver, kidney, etc.), existence of dose-response relationship, differences in species response, sensitivity between the sexes, investigate accumulative effects, any tolerance and correlate findings with any other known effects. The most important is determination and identification of the target(s) for toxicity and measurement of hematology and clinical chemistry parameters during dosing and observation at autopsy and during histopathological examination of preserved tissues. In this study, the experimental and control animal did not have significant differences in the PCV values. The normal PCV range for the rabbit is between 30-45% [22]. The PCV fell within this range during and after treatment. This would probably mean that the drug did not affect the erythropoietic system in rabbits.

The functions of platelets are in hemostasis, in maintenance of vascular integrity and blood coagulation. Reference value for platelet counts in rabbits is in the range of 300-800 x10⁹/L [36]. The various drug levels (0.8 and 1.6 g/kg body weight) had a negative effect on the PLT levels except in level 3 (2.4 g/kg body weight) where PLT increased at the end of the experiment. Among cell counts, that of PLT is the most inconsistent implying that the drugs might cause bone marrow damage [25]. Granulocytes were not affected by the administration of the test crude extract. In the control rabbits, results for lymphocytes did not significantly differ from the experimental groups though there were differences among experimental animals.

The LDH levels were not significantly affected among the different groups. This enzyme is of enormous distribution in mammalian tissues, with high concentrations in the heart, liver, kidney and muscle [6]. Spectrophotometric, fluorometric, and colorimetric methods of determination have been applied to the assay of this enzyme. In the analysis the optimum pH with pyruvate as substrate is 6.8-7.5 but with lactate used in this study, it is appreciably higher at 9-10. Lactate has the advantage of being more stable than pyruvate and NAD⁺, which has been used in this study, and is cheaper than NADH. Both rate of reaction and colorimetric techniques have been used. In the former the increase/decrease in extinction of NADH is read. However, a colorimetric technique using the reaction which was introduced in 1960 and used by [2] appears to have come increasingly into favor. The method using MTT and PMS with lactate as substrate was used in this study with a few modifications. It may be concluded that the administration of this crude DCM extract of *C. limon* did not interfere with cardiac, hepatic and pancreatic cells in any way as the LDH levels were normal during and after treatment. The leakage of LDH from even a small amount of damaged tissue can effectively increase its activity in serum as its tissue concentrations are approximately five hundred times higher than its normal concentration in serum [8].

The characteristically prolonged periods of elevated LDH values in infarction with an increase in LDH isoenzymes, that is, LDH1 higher than LDH2 yields a pattern that is useful in the laboratory diagnosis of myocardial infarction [16]. High LDH levels are also found in the following drug related conditions, toxic hepatitis, cirrhosis and hepatic necrosis. The drug administered at various levels; 1 (0.8 g/kg body weight), 2 (1.6 g/kg body weight) & 3 (2.4 g/kg body weight) did not have the above listed negative effects and the levels ranged between 80 and 89 U/L which is still within the LDH normal range (94.3±28.8 U/L) [22] in rabbits.

The increase in the serum glucose might mean ineffectiveness of the pancreas, renal system and cardiac system which might have been caused by the administration of the drug though in some quarters it has been found to support all these organs [45]. However, the method used in this study to determine glucose gave a range of 69.5-106.2 mg/dL in rabbit which is quite close to the normal range as reported by [22, 34] while working with listeriosis exposed rabbits, established a normal glucose concentration of 97.5±8.4 mg/dL in control rabbits which compares very well with 97.2±10.2 mg/dL determined in rabbits injected with 0.8 g/kg body weight of the crude drug in this study. This supports statistical analysis results and histological results which showed that hematological, immunological and biochemical parameters in control and experimental rabbits were not significantly different and that tissues were not affected in the treated animals, respectively.

Work Miyake [31] showed that lemon flavonoids or eriocitrin and heparidin taken in the diet are effective antioxidant *in-vivo*. Several workers have used strips that employ the glucose oxidase test with some variations in their findings but with the majority finding they tend to overestimate glucose levels. It is not clear whether the same applied to these results but even though, the control glucose would not have been different. The strips clearly distinguish between hypo and hyperglycemia but opinions as to their value in other circumstances vary considerably [40]. Glucose oxidase is, however, highly specific for β -D-glucose, and any glucose present in the α -form must be converted to the β -form before reacting. One of the chief advantages of glucose oxidase method, that also made this resource limited study possible, is its inexpensiveness.

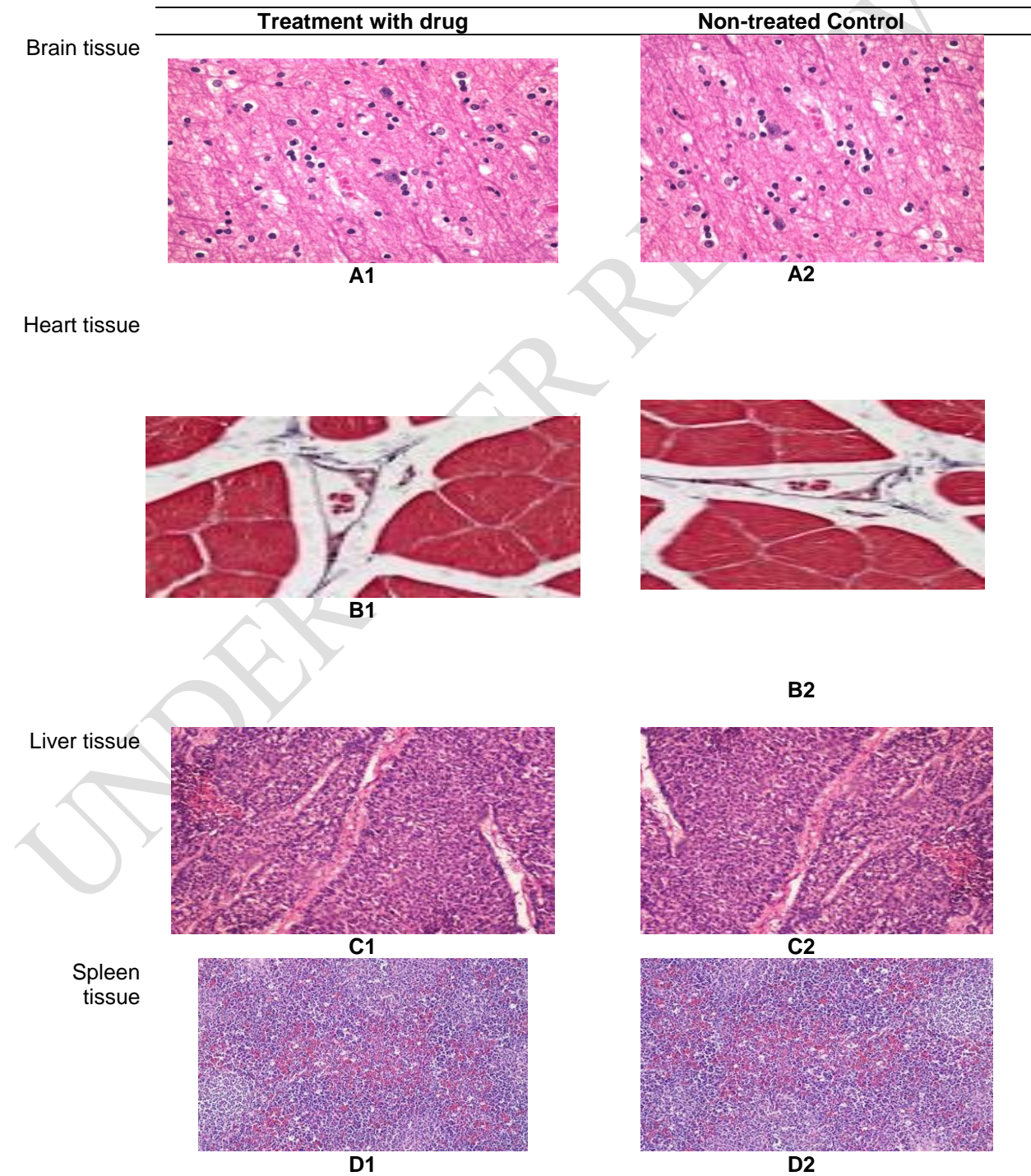
Humoral immunity involves the production of circulating immunoglobulin (IgG, IgA & IgM) by plasma cells which are derived from lymphocytes. These lymphocytes arise largely from the lymphoid tissue of the gastrointestinal tract and are known as B cells. Immuno-chemical methods used in this study and radio-immunologic methods allow for specific identification and quantitation of individual globulins. Out of the three immunoglobulins subjected to scrutiny in this study, only the IgG levels were affected by the administration of the drug but not in a dose-dependent manner. One of the two methods used for determining the levels is either radial immunodiffusion for most samples, or enzyme-linked immunosorbent assay for very low-level samples. For this study it was the radial immunodiffusion (SRID) test which was used because of its simplicity, accuracy and economic viability. The purpose of the determination was to aid in the diagnosis of immunoglobulin deficiency or increased levels during drug administration as is found in cirrhosis and hepatitis. Since only the IgG levels were affected significantly, it would not be advisable to draw strong conclusions as more information may be required to do so. In a landmark pre-clinical study published in the Journal of American Nutraceutical Association, scientists showed that Wolfberry (*Citrus limon*) juice, and

Ningxia Red TM, formerly Berry Young are immune boosters. According to a study carried out at a hospital in Beijing in 2002, Wolfberry was found to stimulate interleukin-2 and/or interferon, the two of which are anti-inflammatory substances in supporting a healthy immune system. A study by [17] concluded that concentrated juice of *Citrus aurantifolia* possessed principles with immunomodulatory effect which probably was due to the presence of the protein component in the extract.

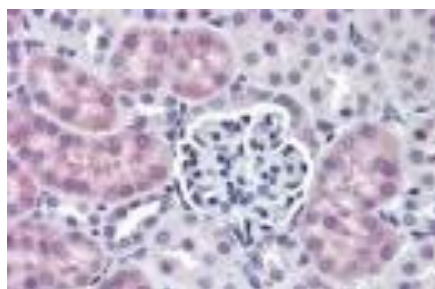
4.5 Results on Histology

Subcutaneous administration DCM rot extracts of *C. limon* to rabbits at 0.8, 1.6 and 2.4 g/kg body weight daily for two weeks demonstrated no observable histopathological effectson the liver, kidney, heart, brain, and spleen specimen of the sacrificed experimental rabbits (Figure 12 A1-E2).

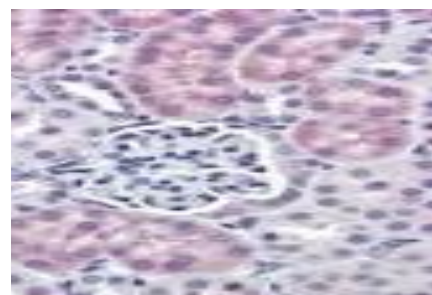
Figure 12 A1-E2: Photographs of experimental &control Rabbit tissues comparedusingx 100 objective



Kidney
Tissue



E1



E2

Weight as a parameter was very significantly increased after administration of crude drug at concentrations of 0.8, 1.6 and 2.4 g/kg body weight. The drug may have induced an increase in appetite. The study shows that there was no toxic effect observed in the sub-chronic toxicity studies conducted in rabbits. This study is in conformity with the *Costus pictus* D. Don extract study (C) 2006 Pharmainfo.net Joomla). In general, one major observation towards the end of the study was that the general health of the experimental rabbits improved as opposed to the controls.

The kidney was not affected in the drug treated rabbits. Work by [6] showed the protective effect of resveratrol in the reduction of ischemia reperfusion of rat nephritic tissue damage both by antioxidant and anti-inflammatory mechanisms. *C. limon* is also known to have the following attributes: supporting cardiovascular health, protecting and supporting the pancreas and liver and supporting eye health [44]. All organs were not affected in this study probably because *C. limon* has supportive attributes. The isolated coumarins may have medicinal value at least at the administered dosages in the crude DCM extract.

Although Miller [30] gives an exhaustive list within subacute and chronic toxicity studies; the areas of these observations can be grouped together under the categories (1) mortality, (2) clinical symptoms, (3) body weight, (4) food consumption, (5) water consumption, (6) physical examinations such as (esg), (7) urinalysis, (8) hematology, (9) clinical chemistry, (10) organ weights, (11) gross pathology and (12) histopathology. This study only concentrated on the following categories 1, 2, 3, 8, 9 and 12, thus half the number due to time constraint.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The conclusions associated with this study include:

- (i) Hexane extracts of *Citrus limon* roots (CLR), *Microglossapyrifolia* leaves (MPL), and *Vernoniaglabra* leaves (VGL), dichloromethane (DCM) extracts of *Citrus limon* roots (CLR), *Achyranthesaspera* leaves (AAL), *Brideliacathartica* leaves (BCL), *Microglossapyrifolia* leaves (PML), and *Vernoniaglabra* leaves (VGL), and methanolic extracts of *Brideliacathartica* leaves (BCL), *Citrus limon* roots (CLR), and *Vernoniaglabra* leaves (VGL) were active against the brine shrimp, *Artemiasalina*. Dichloromethane (DCM) root extract of *Citrus limon* roots (CLR) showed the highest activity *in vitro* against brine shrimp, *Artemiasalina* based on IC₅₀.
- (ii) Dichloromethane (DCM) extract of *Citrus limon* roots (CLR) (7.017 µg/mL), aqueous extract of *Carrisa edulis* roots (CER) (8.054 µg/mL), DCM extract of *Brideliacathartica* leaves (BCL) (11.537µg/mL), DCM extract of *Hensiacrinita* leaves (HCL) (13.336 µg/mL), methanolic extract of BCL (15.647 µg/mL), and hexane extract of *Achyranthesaspera* leaves (AAL) (18.087 µg/mL) demonstrated high antiplasmodial activity based on IC₅₀. Hexane extract of *Microglossapyrifolia* leaves (MPL) (21.376 µg/mL), methanolic extract of *Hensiacrinita* leaves (HCL) (24.805 µg/mL), aqueous extracts of *Brideliacathartica* leaves (BCL) (25.985 µg/mL), DCM extract of *Carrisa edulis* roots (CER) (30.074 µg/mL), hexane extract of *Citrus limon* roots (CLR) (30.092 µg/mL), hexane extract of *Brideliacathartica* leaves (BCL) (32.908 µg/mL), hexane extract of *Hensiacrinita* leaves (HCL) (34.223 µg/mL), DCM extract of *Microglossapyrifolia* leaves (MPL) (34.88 µg/mL),

aqueous extract of *Achyranthesaspera* leaves (AAL) (38.99 µg/mL), aqueous extract of *Hensiacrinita* leaves (HCL) (47.203 µg/mL), DCM extract of *Vernoniaglabra* leaves (VGL) (53.62 µg/mL), methanolic extract of *Carrisa edulis* roots (CER) (69.969 µg/mL), DCM extract of *Achyranthesaspera* leaves (AAL) (86.501 µg/mL), and aqueous extract of *Citrus limon* roots (CLR) (96.86 µg/mL) demonstrated moderate antiplasmodial activity based on IC₅₀. DCM extract of *Citrus limon* roots (CLR) (7.017 µg/mL) demonstrated the highest antiplasmodial activity based on IC₅₀.

- (iii) Dichloromethane (DCM) extract of *Citrus limon* roots (CLR) subcutaneously administered to rabbits for 14 days at a dose of 0.8, 1.6 and 2.4 g/kg body weight appears safe since rabbit weight, hematological, biochemical, and immunological parameters, and histopathology of the studied organs were normal.

5.3 Recommendations

The observation that all the seven studied plants parts extracts including *Achyranthesaspera* leaves, *Brideliacathartica* leaves, *Microglossapyrifolia* leaves, *Vernoniaglabra* leaves, *Hensiacrinita* leaves, *Carrisa edulis* roots, and *Citrus limon* roots demonstrated high to moderate antiplasmodial activity, supports their continued use as antimalarial drugs in Kilifi and Homabay.

5.3.1 Recommendations for further studies

The seven studied plants aqueous and organic extracts which demonstrated high to moderate antiplasmodial activity can further be subjected to toxicity studies to confirm their safety or otherwise in the rabbit model.

ETHICAL APPROVAL

The authors obtained all **THE** necessary ethical approval from **Institutional Animal Care And Use Committee, Kalro–Biotechnology Research Institute**

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