A SEARCH FOR ANTIMALARIAL ACTIVITY IN PLANTS TRADITIONALLY USED IN KENYA USING AN IN VITRO SYSTEM TO MEASURE ACTIVITY AGAINST P. FALCIPARUM

by MURINGI GATHERU

A PROJECT SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF PHARMACY OF THE UNIVERSITY OF NAIROBI

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A search for antimalarial activity in plants traditionally used in Kenya using an in vitro system to measure activity against Plasmodium falciparum.

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Nyina wa Gathoni (Millicent Karuiru) for typing this script.
DEDICATION

This work is dedicated to my parents
Mr. and Mrs. Habel Gatheru, my brothers and
sisters especially Nyagu and Ceke.
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ABSTRACT:

Three plants traditionally used in Kenya for the treatment of Malaria were screened for their activity against two Kenyan strains of *Plasmodium Falciparum* by an invitro method. Both strains (K39 and K33) are chloroquin sensitive. The three plants were *Catha edulis*, *Bidens pilosa* and *Caesalpinea Volkensii*. A fourth plant *Tidonia Diversifolia* which belongs to the family compositae known to contain sesquiterpenes, was also screened. From each plant an aqueous and an ethanolic extract was prepared, thus there were eight compounds (A-H) to be screened. Suppression of the uptake and incorporation of a radiolabelled precursor (G-H3) hypoxanthine into nucleic acids by the parasites was used as an indicator of activity of the compounds being screened.

It was found that there was a slight drop in the uptake of the indicator in all the compounds tested, but at the concentrations used an endpoint could not be reached and an IC50 could not be determined.
Malaria which has correctly been described as the scourge of Africa is a disease of global importance having killed more people than any other disease. It remains the major threat to life and one of the most serious Public health problems in the third world (1) Malaria is widely distributed in South east Asia the southern half of America and parts of the middle east. The disease is seen in one hundred countries, kills one million children annually in Africa alone and about 15 million people suffer from it throughout the world(2)

In Kenya many people die annually from this disease especially those living in the malariasious areas of Coast Province and around Lake Victoria.

Many modern medicines are in use both for the prevention and treatment of malaria. Resistance has been observed to some of them eg. the antifolate class of compounds, whereby resistance has been observed in the laboratory and in the field. This has led to the withdrawal of Proguanil from the United States Pharmacopoies (USP) as an antimalarial agent(3). The cause of resistance is due to spontenous genetic mutation producing resistant plasmodia(1). Chloroquine was considered to be the only available antimalarial to which resistance could not readily occur. Chloroquine resistance has been associated with a decrease in drug concentrating capacity of red blood cells infected with malarial parasites (4) (5). Nevertheless, resistance to Chloroquine is still not well understood and is very complex(5).

Malaria is best prevented and there are many ways in which this may be done:-

(a) The adult mosquito(rector) can be killed by spraying the surrounnding with insecticides like DDT.

(b) Draining all stagnant, thus old tins, bottles and pots that may hold and retain water when the rains come should be collected and destroyed.

(c) If the stagnant water is too large to be drained eg. a swamp, it can be sprayed with paraffin, this forms a thin film on the water as paraffin is lighter than water.
(d) During the night people living in mosquito infested areas should use mosquito nets to protect themselves while they sleep. These prevent the mosquito from reaching the skin of the sleeping victim. Contact between malaria patients and mosquitoes must be prevented to avoid further spread of infection.

(e) There are antimalarial drugs which kill the plasmodium when it is in the blood which are Prophylactic agents and they should be taken by a visitor to such an area. For example Chloroquine phosphate 500mg (300mg base) orally twice weekly protects travellers to malarious areas where there is no chloroquine resistance by suppressing the erythrocytic infection and thus the clinical manifestation of malaria. The drug should be started one week before arrival in the area and should be continued one month after leaving, since this continued use results in eradication of sensitive strains of P. falciparum. In other types of malaria primaquine must be given in addition to Chloroquine.

Malaria is a protozoan infection characterized by paraxysms of chills, fever and sweating, and by anaemia, splenomegally and a chronic relapsing course. Malarial parasites are four types, each with a different biologic pattern. These are Plasmodium vivax, ovale, malarial and falciparum. The infection occurs through the bite of an infected donor, or use a common syringe by drug addicts. Of the four mentioned above P. falciparum causes the most serious infections and is responsible for 99% of the deaths due to malaria.

Once plasmodium is in the blood it invades and destroys red blood cells. It causes a fever of recurring nature that can weaken the person so much and it can easily kill him. Also the number of red blood cells infected and destroyed may be so great that the patient becomes anaemic. If plasmodium gets to the liver some of them can survive there very long and thus can act as a reservoir for further attacks and the person can get fever at any time for the rest of his life.
CLINICAL MANIFESTATION OF MALARIA

Incubation period takes about 12 days for *Plasmodium falciparum*. There may be a chilly sensation and temperature rises gradually (104-106°F) i.e. (40-41°C). Headache is prominent and during intervals between paroxysms, which are exceedingly variable (36-72 hours) the patient usually has a low grade fever. One complication of *falciparum* malaria is cerebral involvement both in children and adults. This is one cause of high infant mortality in malarious areas. There is also, malaria of the gastrointestinal tract (algid malaria). The patient usually has diarrhoea and the stool is blood stained. When 40% of the total erythrocytes are haemolysed, haemolytic anaemia may result.

ANTIMALARIALS IN COMMON USE

(1) The cinchona alkaloids e.g. quinine (2) The 9 amino acridines e.g. mepacrine.
(3) The 4 aminoquinolines e.g. chloroquine and amodiaquine.
(4) The 8 aminoquinolines e.g. parnaquine, primaquine.
(5) The biguanides e.g. proginil.
(6) The dianinopyrimidines e.g. pyrimethamine

The cinchona alkaloids, aminoacrids, 4 amino, quinolines, biguonides and dianuinopryrimidines have a destructure action on the asexual erythrocytic forms of all species of human malaria parasites. The 8 aminoquinolines do not readily affect the asexual erythrocytic parasites, but they have a powerful destructive action on the secondary exoerythrocytic forms of *P. vivax*, and it is in this respect that they have their greatest value. They have inhibitory effect on the pre-erythrocytic forms of both *P. falciparum* and *P. Vivax*, but can not be used for this use without risk of toxicity. The biguonides and dianinopyrimidines are the only drugs which can be used with safety against the pre-erythrocytic forms and so far as is known they are fully effective only in *falciparum* infections. They have no apparent action on the asexual forms of *P. falciparum* incapable of completing their development in the mosquito.
EVALUATION OF ANTIMALARIAL ACTIVITY

This is done by testing the activity of the drug in the body (in vivo) or by testing outside the body (in vitro). A method that is cheap and not time consuming would be ideal. Previously evaluation of antimalarial activity was only possible in vivo, but of late methods have been developed that do so in vitro (II). Both the in vivo and the in vitro methods are useful in their own ways, but each has its own disadvantages.

Some in vivo methods of assay.

1 Albino mice inoculated intraperitoneally with one million infected red blood cells on day one, which are then treated by oral administration of the test compounds with the aid of an oesophageal sound, once daily for four days, starting three or four hours after infection. Parasite counts are made on blood films from tail blood, on day five and day seven. Control parasites counts gave from 1 - 5% infection on day five. The minimum effective dose was that which reduced parasitaemia to 2% of that of the controls.

2 Warhurst and Fohwells (1968) Technique for the evaluation of drug.
A group of ten mice is divided into two lots of five after infection with a standard inoculum of donor blood (- 10^7 infected red blood cells in 50% calf-serum - Ringer solution per recipient). A single dose of the drug to be tested is given to each mouse in one group of five between 1 - 3 hours after injection. A placebo injection is given to the other five mice. A second group of 10 mice is identically treated except that the animals receive one tenth of the inoculum received by the first ten animals. The mean latent period required to reach a 2% parasitaemia level is plotted on logarithmic graph paper for each group of five mice from counts of parasitaemia in tail blood made daily (5).

The in vivo method of drug evaluation has the advantage of giving the total picture as to the usefulness of the drug. In the animal both the exoerythrocytic and erythrocytic stages plasmodium can be acted on by the drug. The animal method is also ideal for those drugs that need to be biotransformed to the active drug before they show any activity.

The in vivo method has the disadvantage of being expensive in that you would need to keep an animal house, and somebody to look after these animals. Its also time consuming and one has to wait for a long time in order to get results. Also it cannot be used to evaluate the activity of the drug against P. falciparum.
TRADITIONAL MEDICINES.

Before modern medicines were known in Africa the traditional doctors advised the people what plants to use in the treatment of Malaria. Since the coming of the Europeans and modern medicine the traditional doctors of old have tended to be pushed into the background and is treated with some suspicion, which is not without cause sometimes.

The problem with traditional medicines is that it is not a unified practice, techniques and apprenticeship differs throughout the country, it can not be considered as on science, but rather as a collection of individually evolved practices developed in different ways.

It is quite possible that the preparations prescribed and dispensed by the traditional doctor for the treatment of malaria has only got antipyretic activity. Since malaria is associated with fever, if the herbal medicine is able to lower the raised body temperatures, then the herbalists concludes that the preparation is an antimalarial agent. The herbalists limited scientific knowledge does not allow him to give reasons any further than that malaria and fever are synonymous. They do not realise that there is more to malaria than just the fever.

The weak activity of the preparations plus the immunity factor is probably the reason that some of these preparations seem to work. People who have lived for much of their life in endemic malarious areas have humoral immunity (which is only partial) to infection. The immune response is directed primarily against the asexual parasites in the blood, the tissue forms in the liver being completely protected from the cellular-humoral immunity response(9).

In this project four plants were screened for their activity against two strains of \textit{Plasmodium falciparum} designated K39 and K31. Traditionally these plants are used both for malaria and other ailments. The four plants are:- Cathaedulis locally known as 'Miraa', Bidens Pilaosa or 'Michege' Caesalpinea volkensii or 'Mbuthi'. The fourth \textit{Tidhonia diversifolia} is not used traditionally. It was chosen because it belongs to the family compositae, which is known to contain sesquiterpenes, in antimalarial activity was observed in \textit{Qinghao} (\textit{Artemisia annual} L).
Qinghaorn, the active antimalarial principle in Qinghao, has the structure of a sesquiterpene peroxide. Qinghaosm and its derivatives, have a high level of blood schizonticidal activity against chloroquine-resistant malaria parasites both in the laboratory models and in the clinic.

Catha edulis

The shoots of Miraa are normally chewed as a stimulant. The shoots, leaves and roots have also been used in traditional medicine to treat various ailments. It is said to be good for colds and is of value in the treatment of malaria, diarrhoea and venereal diseases.

Active constituents: Fluckinger and Geroch (1887) seem to have been the first to isolate the pharmacologically active substances present in Miraa, which they called Cathine. Wolfes (1930) isolated only the alkaloid d-norpseudoephedrine and in addition 17 amino acids. According to Alles, Fairchild and Jesen (1961) there is no appreciable difference between the alkaloidal content of fresh leaves and samples that have been dried and conserved. Miraa also contains approximately 14% tannins of the condensed type (i.e., derivatives of flavanol). According to Maitai (1973) T.L.C revealed phenylallyl-amines, especially d-norpseudoephedrine, d-pseudoephedrine, L-ephedrine and amphetamine. However d-norpsendoephedrine is the only alkaloid in all parts of the plant.

Caesalpinea Volksesii

The dry mature seeds of this plant are the important part that is traditionally used as an antimalarial. The hard nut is cracked and the white inner part is ground to a fine powder. Bone soup is prepared and this powder is put into this soup which is then boiled for another, five to ten minutes. The soup is cooled and given to the patient to drink. Other uses of the plant include boiling the leaves and then inhaling the steam as a cure for the common cold. Another use is where the inner part of the seed is powdered and melted animal fat is added and made into a paste which used to treat infections of the ear.
Bidens Pilosa

Activity of extracts from the flowering heads of Bidens pilosa against plasmodium was investigated and no activity was noted (16). Traditionally the flowering heads are dried and ground. The dry powder is boiled and the resulting dark brown fluid drunk either on its own or mixed with bone soup.

In this project ethenolic and acqueos extracts of each of the four plants will be tested by an invitro method to ascertain whether these plants have any antimalarial activity.
1. Plant materials - This includes seeds of C.vulkensii, petals of T.diversifolia, flowering heads of B.pilosa and shoots of C.edulis.

2. Soxhlet extractor and thimbles.

3. Heating mantle.

4. Extracting solvents - Ethanol and water.

5. Laboratory glassware.

6. Pipettes - graduates 10 mls, 5 mls and 1 ml.

7. Vacuum dessicator.


9. Rotavapor Buchi

10. Aseptic screen.

11. Alcoholic cetrimide.

12. Whatmann No1 filter paper and cotton wool.

13. Incubator.


15. 25 ml tissue culture flasks (corning Glass works corning N.Y.)

16. Microtiter plates (cooke laboratory products, Alexandria, Va)

17. Eppendof pipette (Cooke Laboratory Products).

18. Automatic diluter (Cooke Laboratory Products) - This makes a 2-fold dilution across the plates in each column.

19. Humidified air-tight box (instrumentation department, Walter Reed Army Institute of Research, Washington, D.C.)
Materials and Equipment

1. Plant materials - This includes seeds of C. vulkensii, petals of T. diversifolia, flowering heads of B. pilosa and shoots of C. edulis.

2. Soxhlet extractor and thimbles.

3. Heating mantle.

4. Extracting solvents - Ethanol and water.

5. Laboratory glassware.

6. Pipettes - graduates 10 ml, 5 ml and 1 ml.

7. Vacuum desiccator.


9. Rotavapor Buchi

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17. Eppendorf pipette (Cooke Laboratory Products).

18. Automatic diluter (Cooke Laboratory Products)

   - This makes a 2-fold dilution across the plates in each column.

20. G-\(^3\)H Hypoxanthine (Amersham/Searle Corp; Arlington Heights, III).
21. MASH II automated cell harvester (Microbiological Associates Bethesda, MD)
22. Small disks of filter paper (no. 934-AH, Whatman, Inc; Clifton, N.J.)
23. Glass scintillation vials.
24. Toluene based scintifluor for counting.
25. Searle Model Delta 300 liquid scintillation Spectrometer.
26. Gas mixture containing 5\%O\(_2\), 5\% CO\(_2\) and 90\% N\(_2\).
27. Fresh erythrocyte Suspension.
28. Parasitized erythrocytes.
29. Small gas burner.
30. 70\% ethanol.
31. Plenty of distilled water.
32. Laminar flow aseptic screen.
**Bidens Pilosa**

- A common garden weed that grows wild around K.N.H. The flowering heads were collected and dried in the sun. When completely dry they were crushed to a fine sample. This was weighed and stored awaiting extraction. Half of the material was used for the aqueous extraction, the other half was used for the alcoholic extraction.

**Tidhonia diversifolia**

- This plant grows near Kenyatta Hospital and its quite common in most parts of the country. The yellow flower petals were collected and dried in the sun. They dry sample was crushed to a fine sample which was weighed and stored. Half of the sample was used for aqueous extraction, the other half for alcoholic extraction.

**Caesalpinea Volkensii**

- This grows near Thika and in Murang'a, also in Mweiga in Central Province. The seeds are the ones used for preparation of a soup which is drunk by the sick person. The seeds were bought in Nairobi, along River Road. The hard outer nut was cracked and the soft inside part was removed and dried, then crushed to a fine white powder. The sample was weighed, half of this was used for the aqueous extraction while the other half was used for the alcoholic extraction.
Catha edulus:

- This plant grows very well in Meru district. The shoots are the important part of the plant. These were bought in Nairobi along River Road; where they are openly sold but for another purpose. The shoots were dried in the sun and crushed. It was weighed. Half the sample was used for aqueous extraction, other half for alcoholic extraction. An alcoholic and aqueous extract was important so that the water soluble constituents could go into the aqueous extract and those constituents that are soluble in organic solvents could go into the alcoholic extract.
Aqueous Extraction

(1) The dried sample was boiled in distilled water for three hours.
(2) After cooling it was passed through cotton wool. Particulate matter was left on the cotton wool.
(3) This crude filtrate was filtered through Whatmann No1 filter paper twice.
(4) The filtrate was placed in a preweighed round bottomed flask and fixed to a rotavapour until all water was removed.
(5) The flask was placed in a vacuum dessicator and dried to constant weight.
(6) The weight of the sample was determined.
(7) The contents of the flask were then dissolved in an appropriate volume of 70% Ethanol.

Alcoholic Extraction

The dried sample was placed in a thimble and this was put into the soxhlet extractor. The soxhlet apparatus was assembled and clamped onto the stand connected to the heating mantle. Ethanol was placed in a round bottomed flask and this was connected to the soxhlet. The continuous extraction was carried on for three hours. After this the same procedure starting from (2) above was followed.

N3 All four plant samples were subjected to the same extractions as described above.
From the four plants there were eight samples i.e. four aqueous extracts and four alcoholic extracts.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SAMPLE SIZE (g)</th>
<th>EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.1170</td>
<td>C. volkennii Alcoholic</td>
</tr>
<tr>
<td>B</td>
<td>0.1258</td>
<td>B. pilosa alcoholic</td>
</tr>
<tr>
<td>C</td>
<td>0.3726</td>
<td>C. volkensii aqueous</td>
</tr>
<tr>
<td>D</td>
<td>0.9070</td>
<td>B. pilosa aqueous</td>
</tr>
<tr>
<td>E</td>
<td>1.8006</td>
<td>T. diversifolia aqueous</td>
</tr>
<tr>
<td>F</td>
<td>1.8308</td>
<td>T. diversifolia alcoholic</td>
</tr>
<tr>
<td>G</td>
<td>0.1096</td>
<td>C. edulis alcoholic</td>
</tr>
<tr>
<td>H</td>
<td>0.3145</td>
<td>C. edulis aqueous</td>
</tr>
</tbody>
</table>

The samples are dissolved in 70% ethanol. The volume of ethanol to be used is calculated so that we get a concentration of 100ng/ml. This is the highest concentration, which gets diluted to 500ng/ml by the microdiluter across the microtiter plate, as shown below:—

<table>
<thead>
<tr>
<th></th>
<th>PARASITE CONTROL</th>
<th>NON-PARASITE CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 2 3 4 5 6 7</td>
<td>9 10 11 12</td>
</tr>
<tr>
<td>B</td>
<td>100ng</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>500ng</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>31.25</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>15.525</td>
<td></td>
</tr>
</tbody>
</table>
Representation of the microtiter plate, with 96 well arranged in 8 rows (A-H) and 12 columns (1-12) Each compound is present in duplicate columns over a 64-fold range, with the highest concentration in row B, and in two-fold dilutions to the lowest concentrations in row H.

In each well of the microtiter plate is placed 200ml of parasitized erythrocyte suspension, except the last four wells of row A to which is placed 200 ml of an equivalent suspension of non-parasitized human erythrocytes. 25 ml of test sample solution was then added to each well giving a total volume of 225 ml in each well. The first eight wells of row A contained no drug'solution and served as a control (parasite control). The last four wells of row A contained neither 'drug' nor parasite - this serves as the non-parasitized erythrocyte control.

The parasitized erythrocytes were exposed to each compound in two columns with the highest concentration in row B and the lowest concentration in row H.

**CALCULATIONS REGARDING DILUTIONS:**

To get a concentration of 1000 ng/ml in row B we have to prepare a 2000ng/ml concentration of test sample due to an initial two-fold dilution.

This 2000 ng/ml is to be contained in 225ml of which 25ml is 'drug' solution. This gives the following concentration.

\[
\frac{2000 \times 225 \text{ ng}}{25} = 18000 \text{ ng/ml} = 18 \text{ ug/ml}
\]
Since 0.1 ml of 'drug' solution is placed in the microtubes to be diluted with 0.9 ml CMS, we must prepare a solution that is ten times strong i.e. 180 µg/ml

... stock concentration = 180 µg/ml.

**SAMPLE A**

Sample weight = 1.1170 g

= 1117 mg = 1117000 µg

To get 180 µg/ml, 1117000 µg should be dissolved in

\[
\frac{(1117000)}{180} mls = 6200 Mls.
\]

(1) Dissolve sample in 62 mls of 70% Ethanol
This gives a concentration of 18,000 µg/ml.

(2) Take 0.25 mls of the above solution and place in a 25 ml volumetric flask, add 70% Ethanol to the mark. This dilution gives the desired concentration of 180 µg/ml.

**SAMPLE B**

Sample weight = 0.1258 g

= 125.8 mg = 125800 µg

To get 180 µg/ml, 125800 µg should be dissolved in

\[
\frac{(125800)}{180} ml = 698 mls.
\]

(1) Dissolve sample in 69.3 ml of 70% Ethanol
This gives 1800 µg/ml

(2) Take 2.5 mls of the above solution and place in a 25 ml volumetric flask. Add 70% Ethanol to the mark.
This final dilution gives the desired concentration of 180 µg/ml.
SAMPLE C

Weight of sample = 0.3726 g.

= 372.6 mg = 372600 μg.

To get 180 μg/ml, 372500 g should be dissolve in

\[
\frac{372600}{180} \text{ mls}
\]

= 2070 mls.

Therefore: (1) Dissolve sample in 20.70 mls of 70% ethanol.

This gives a concentration of 18000 μg/ml.

(2) Take 0.25 mls of the above solution and place in a 25 ml volumetric flask.

Make up volume with 70% Ethanol. This final dilution gives the desired concentration of 180 μg/ml.

SAMPLE D

Weight of sample = 0.9070 g

= 907 mg = 907000 μg.

To get 180 μg/ml, 907000 g of sample is dissolved in

\[
\frac{907000}{180} \text{ mls}
\]

= 5038 mls.

(1) Dissolve sample in 50.38 mls of 70% Ethanol

This gives a concentration of 18000 μg/ml.

(2) Take 0.25 mls of the above solution and put in a 25 mls volumetric flask. Make up to volume with 70% ethanol.

This final dilution gives the desired concentration of 180 μg/ml.
SAMPLE E

Weight of sample = 1.8006g
= 1800.6mg = 180060ug.

To get 180ug/ml 1800600 g should be dissolved in
\[ \frac{1800600}{180} \text{ ml} = 10003.333 \text{ ml}. \]

(1) Dissolve sample in 100 ml of 70% Ethanol to give a concentration of 18000ug/ml.
(2) Take 0.25 ml of the above solution and put in 25 ml volumetric flask make up to volume with 70% Ethanol. This final dilution gives 180ug/ml.

SAMPLE F

Weight of sample = 1.8308g
= 1830.8 mg = 1830800ug.

To get 180ug/ml, 1830800ug should be dissolved in
\[ \frac{1830800}{180} \text{ ml} = 10170 \text{ ml}. \]

(1) Dissolve sample in 101.70 ml of 70% ethanol.
(2) Take 0.25 ml of the above solution and place in 25 ml volumetric flask. Add 70% Ethanol to make up the volume to the mark. This gives the desired concentration of 180ug/ml.

SAMPLE G

Weight of Sample = 0.1096 g
= 109.6mg = 109600 g.

To get 180ug/ml, 109600 g should be dissolved in
\[ \frac{109600}{180} = 608.8888 \text{ ml of 70% Ethanol}. \]
(1) Dissolve sample in 60.9 ml of 70% ethanol. This gives a concentration of 180 \mu g/ml.

(2) Take 2.5 ml of the above solution and place in a 25 ml volumetric flask. Make up to volume with 70% Ethanol. This final dilution gives 180 \mu g/ml.

**SAMPLE H**

Weight of sample = 0.3145 g.

\[
= 314.5 \text{mg} = 314500 \text{\mu g}.
\]

To get 180 \mu g/ml, 314500 \mu g should be dissolved in

\[
\frac{314500}{180} \text{ ml of 70\% Ethanol} = 1747.2222 \text{ ml}.
\]

(1) Dissolve flask contents in 69.8 ml of 70\% ethanol. This gives a concentration of 4500 \mu g/ml.

(2) Take 1.00 ml of the above solution and place in a 25 ml volumetric flask and make up to volume with 70\% ethanol. This final dilution gives the desired concentration of 180 \mu g/ml.

**NB** During the experimental work I noted that not all the sample dissolved, even after trying to warm it up. The resulting suspension was passed through a filter paper that was already weighed. The sample that remained in the flasks was weighed. The sample on the filter paper was also weighed. Total weight of undissolved sample was subtracted from the original weight of the sample to give the size of sample that actually dissolved.

Hence the 180 \mu g/ml concentration is only a theoretical value.
It follows therefore that our desired highest concentration of 1000 ng that goes into row B of the microtitration plate is only theoretical. The actual values are given below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight</th>
<th>Theoretical g/ml</th>
<th>Amount Dissolved</th>
<th>Con. (actual) g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.1170</td>
<td>180</td>
<td>0.3347</td>
<td>53.935524</td>
</tr>
<tr>
<td>B</td>
<td>0.1258</td>
<td>180</td>
<td>0.0474</td>
<td>67.82193</td>
</tr>
<tr>
<td>C</td>
<td>0.3726</td>
<td>180</td>
<td>0.2815</td>
<td>135.99032</td>
</tr>
<tr>
<td>D</td>
<td>0.9070</td>
<td>180</td>
<td>0.6735</td>
<td>133.66041</td>
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<tr>
<td>E</td>
<td>1.8006</td>
<td>180</td>
<td>0.800</td>
<td>79.973342</td>
</tr>
<tr>
<td>F</td>
<td>1.8308</td>
<td>180</td>
<td>0.1024</td>
<td>10.067729</td>
</tr>
<tr>
<td>G</td>
<td>0.1096</td>
<td>180</td>
<td>0.0171</td>
<td>28.083941</td>
</tr>
<tr>
<td>H</td>
<td>0.3145</td>
<td>180</td>
<td>0.0502</td>
<td>28.731319</td>
</tr>
</tbody>
</table>

0.1 ml aliquots of all eight samples were aseptically transferred to microtubes. For each sample there were 5 x 0.1 ml aliquots, hence a total of 40.

These were left to stand for 2 hours, after which they were ready for testing.
The parasite inoculant used in these experiments consisted of two isolates of Plasmodium falciparum. These are designated K39B and K33. The test solutions prepared and diluted in 70% ethanol was allowed to stand at room temperature for 30 min. Further dilutions were then made by addition of the compound to a measured volume of culture medium with constant mixing. The final solution contained less than 0.1% ethanol which has no measurable effect on the parasites.

Preparation of the microtitration plates:

Microtitration techniques were used to measure the activity of the test samples efficiently. The microtiter plate consisted of 96 flat-bottomed wells (already described. The plates were prepared and the parasites were harvested by using strict aseptic techniques inside a Laminar flow hood in the following sequence. An Eppendorf pipette or a microdrop multiple-well filling pipette was used to place 25 µl of the culture medium in each well of the microtiter plate. 25 µl of the test solution was then added to each of two adjacent wells in the second row (B), thus six compounds were accommodated by each plate. After the test compounds were placed to the wells of row B, an automatic diluter was used to make serial two fold dilutions across the plate in each column. When this was complete, row A remained free of any test compound, and each of the test compound was present in duplicate columns at seven concentrations over a 64-fold range in rows B through H. The parasitized erythrocytes were exposed to each compound in two columns with the highest concentration in row B and the lowest concentration in row H.
After preparation the plates were placed in a humidified airtight box, which was then flushed with a gas mixture of 5% O₂, 5% CO₂ and 90% N₂ and sealed. The box was then placed in an incubator at 37°C for 24 hours.

Preparation of Isotope and Labelling of Parasites:
Uptake of (G-³H) hypoxanthine was used as an index of growth of the parasites. The final isotope solution consisted of 20 G of (G-³H) hypoxanthine per ml of culture medium.
After the 24 hr incubation period the plates were removed from the box and 25 l of the isotope in culture medium (0.5 Ci) was added to each well. The plates were then returned to the box, flushed with the gas mixture, sealed and incubated at 37°C for an additional 18 hours.

Harvesting Parasites and Scintillation Counting
At the end of the second incubation period, each plate was harvested on a MASH II automated cell harvester. This instrument aspirates and deposits the disks of filter paper, which were then washed with copious volumes of distilled water.
Each disk was dried and placed in a glass scintillation vial containing 10 ml of toluene-based scintiflour for counting. All 96 vials corresponding to the 96 wells of the microtiter plate, were counted in a searle model delta 300 liquid scintillation spectrometer for a sufficient period of time to ensure a counting error of less than 5% for each sample.
Significance of Uptake of (G³H) hypoxanthine

Growing parasites take up the labelled hypoxanthine and thus a high reading is expected of the PRBC control (column 1-8)(row A). The RBC control column (9-12) (row A) contained no parasites, hence there was no uptake of the labelled hypoxanthine, hence the particulate contents deposited on the small disks of filter paper should contain little or no labelled hypoxanthine.

Strains of Plasmodium used

K39 and K33 were used in these experiments. When tested with antimalarial drugs chloroquine, cycloguenil and pyrimethamine the following was found.

<table>
<thead>
<tr>
<th>Drug</th>
<th>K39</th>
<th>K33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>Sensitive</td>
<td>sensitive</td>
</tr>
<tr>
<td>Cycloguenil</td>
<td>Resistant</td>
<td>sensitive</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>Resistant</td>
<td>sensitive</td>
</tr>
</tbody>
</table>

Both strains were obtained from school children in Kisumu.
RESULTS

DATA OBTAINED

K39 + Plant extracts

RBC Control: 42, 40, 31, 35, 25, 33, 50 - 36.125
PRBC Control: 3427, 2627, 3726, 3676, 3655, 3307, 3480, 3330 - 3403.625
Midpoint - 1719.875

TABLE ONE

<table>
<thead>
<tr>
<th>THEORETICAL nglm</th>
<th>ACTUAL nglm</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
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<td>0.5852</td>
<td>3630</td>
<td>3858</td>
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<tr>
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<td>3552</td>
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<tr>
<td>7.8125</td>
<td>2.3409</td>
<td>3238</td>
<td>3408</td>
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<tr>
<td>15.625</td>
<td>4.3638</td>
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<td>3689</td>
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<td>9.3638</td>
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<td>3780</td>
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<td>62.5</td>
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<td>4028</td>
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<td>2252</td>
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Sample A - Caesalpenia volkensii (ethanolic extract)

TABLE TWO

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<th>B</th>
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</thead>
<tbody>
<tr>
<td>1.9531</td>
<td>0.7359</td>
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<td>4569</td>
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Sample B = Bidens Pilosa (ethanolic extract)
### TABLE THREE

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<th>THEORETICAL nglml</th>
<th>ACTUAL nglml</th>
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<th>C</th>
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<tr>
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Sample C = Caesalpinea Volkensii (aqueous extract)

### TABLE FOUR

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<th>C</th>
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</thead>
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Sample D = Bidens Pilosa (aqueous extract)
K33 + Plant Extracts.

RBC Control: 158, 116, 196, 223 \( \bar{X} = 173.25 \)
Midpoint = 3203.375

PRBC Control: 5764, 5815, 5478, 6576, 7079, 5971, 6225, 6962 \( \bar{X} = 6233.75 \)

**TABLE 5**

<table>
<thead>
<tr>
<th>THEORETICAL (ng/ml)</th>
<th>ACTUAL (ng/ml)</th>
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</thead>
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SAMPLE A = *Caesalpine volkensii* (ethanolic extract)

**TABLE 6**

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Sample B = *Bidens Pilosa* (ethanolic extract)
# TABLE 7

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<th>ACTUAL ngl/ml</th>
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</thead>
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<td>15.62</td>
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Sample C - Caesalpinea Volkensii (water extract)

# TABLE 8

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<th>ACTUAL ngl/ml</th>
<th>D</th>
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<tbody>
<tr>
<td>15.62</td>
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<tr>
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<td>4925</td>
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Sample D - Bidens Pilosa (water extract)
### TABLE 9

<table>
<thead>
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<th>ACTUAL nglml</th>
<th>E</th>
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Sample E = Tidhonia diversofolia (water extract)

### TABLE 11

<table>
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Sample G = Catha edulis (ethanolic extract)
### TABLE 12

<table>
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<th>ACTUAL ngl/ml</th>
<th>H</th>
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Sample H = Catha edulis (water extract)

### TABLE 10

<table>
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<th>ACTUAL ngl/ml</th>
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</thead>
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Sample F = Tidhonia diversifolia (ethanolic extract)
DATA ANALYSIS

The counts and external standardization ratio for each vial were recorded on a paper punch tape for input to a desktop computer, the Tektronix 4051 Graphic system. The counts were converted by standard equations to disintegrations per minute (DPM) for each well, which were tabulated in an 8 - by - 12 matrix corresponding to the 8 rows and 12 columns of the plate. The mean values for parasite control uptake and non-parasitized erythrocyte control uptake of \( (G-^3H) \) hypoxanthine were calculated from the disintegration per minute in row A, well 1 through 8, and wells 9 through 12 respectively.

The automated system used in these experiments generated twofold serial dilutions with a 64 fold range of concentrations for each compound (using the K35 strain of \( P. falciparum \)) and a 512-fold range of concentrations for each compound (using the K39B strain of \( P. falciparum \)).

Where possible concentration - response curves of the plant extracts will be given. These will be graphs of DPM versus concentration in ngl/ml.

To show how the graphs should look like with active compounds a graph of concentration response curve of chloroquine versus Uganda I strain of \( P. falciparum \), will be given. The \( ID_{50} \) will also be given. This gives the concentration of the active compound corresponding to 50% inhibition of the uptake of \( (G-^3H) \) Hypoxanthine by the \( P. falciparum \) in the system.
FIGURE (1) Activity of the alcoholic extract of Cassalpanicus vulgaris against Pathogen: (1775) in
from Table 11.
ACTIVITY of the ameboid strain of \textit{BodiniFallan} against \textit{Plasmodium faliparum} (10) in vivo.

\textbf{Chartwell Graph Data Ref. 5531}

Log 3 Cycles x mm, 1/2 and 1 cm

\textbf{FIGURE (2)}
Activity of the aqueous extract of *Caesalpinia volkensii* against *Plasmodium falciparum* (1957) in vitro.
Activity of the aqueous extract of *Bidens pilosa* against *Plasmodium falciparum* (K39) *in vitro* from results in Table (4)
Activity of the aqueous extract of *Tithonia diversifolia* against *Plasmodium* (see Table 3)
Activity of the ethanolic extract of *Trychonía diversifolia* against *Plasmodium falciparum* in vitro from results given in Table (10)
Activity of the aqueous extract of *Anka edulis* against *Plasmodium falciparum* (K33) in vitro from results given in Table (12)
DISCUSSION

The adaptation of automated microtitration equipment as described in the text provides a rapid and quantitative measurement of antimalarial activity for compounds against Plasmodium falciparum cultivated in vitro.

Incorporation of (G-^H) hypoxanthine into nucleic acids by the parasites in vitro has been used as an indicator of activity of the compounds under testing. Hypoxanthine is capable of crossing the malaria parasite membrane. It is ultimately incorporated into both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) and therefore provides a reasonably broad index of parasite metabolism.

From the results given in Tables (1-12) and figures (1-12) it can be seen that there is hardly any activity in any of the eight compounds. The strains of falciparum used are Chloroquine sensitive. One drawback was that the concentrations used were much lower than 1000 ng/ml. This was due to the fact that not all the sample was able to dissolve in the calculated volume of the solvent. In one case the highest concentration was lower than 100 ng/ml, this was the ethanolic extract of Tithonia diversifolia (55.9318 ng/ml). The highest concentration was that of the aqueous extracts of Caesalpinea Volkensii (755.5 ng/ml) if the concentrations were as high as expected (theoretically 1000 ng/ml) probably some activity would have been seen. A concentration-response curve of the figure 13 type is expected with active compounds against sensitive strains. A sigmoidal curve is expected whereby:-

\[
\frac{C_{pm}}{C_{pm}} = \frac{1}{1 + \left(\frac{C}{K}\right)^n}
\]

\[C\text{ng/ml}\]

\[C_{pm}\text{cpm}\]
A = Here the concentration of the compound being screened is too low to affect the uptake of the radiolabelled precursor by the parasites.

B = The concentration is high enough to cause inhibition of the uptake of the indicator by the parasites. It's in this region that we get the $\mathrm{ID}_{50}$.

C = Total inhibition. Here the concentrations of the compounds are at their higher limits.

$\mathrm{ID}_{50}$ = The concentration of the active compound corresponding to 50% inhibition of the uptake of the indicator by the parasites.

CPM = This indicates the uptake of $(G-3H)$ hypoxanthrine by the parasites at each concentration of the drug.

Clearly none of the graphs drawn (figures 1-12) from results given in Tables (1-12) show the type of sigmoidal curve that one would expect with an active compound against a sensitive strain.

One has to bear in mind that this method of essay is in vitro and may be these compounds would show activity is assay by an in vivo method using the owl monkey or man. Such a method though ideal would be very expensive and the legal requirements are almost prohibitive.

Though this in vitro method is useful in measuring the activity of potential antimalarial drugs against Plasmodium falcinorum it cannot replace the very efficient primary monse screen or the secondary screen in owl monkeys. Rather it provides supplementary information with respect to activity, against $P. falciparum$ of the compounds being screened.

From the results of this project one can conclude that none of the compounds show antimalarial activity of measurable magnitude by this in vitro method of assay.
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