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

UNIVERSITY OF NAIROBI
LIBRARY

NAIROBI

SEPTEMBER 1982

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

by Muringi Gatheru

Supervisor: Dr. W. M. Watkins,
Head Pharmaceutics Section,
Department of Pharmacy,
Faculty of Medicine,
University of Nairobi,
NAIROBI, KENYA.

ACKNOWLEDGEMENT

I wish to thank the following people

- Dr. W. M. Watkins, my Project Supervisor, who patiently and tirelessly helped and guided me through this project.

- Laboratory technicians in the Department of pharmacy, especially Mr. Kinai, Mr. Tolo, Miss Kibaya and Mr. Mureithi.

- 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.

CONTENTS PAGE

Abstract...................... 1

Introduction.................. 2

Methods and Materials.........

Results.......................

Discussion....................

Reference.....................

ABSTRACT:

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

It was found that there was a slight drop in the uptake of the indicator in all the compunds tested, but at the concentrations used an endpoint could not be reached and an ID_{50} could not be determined.

INTRODUCTION :

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 malacious 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 resitance 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). Nevetheless, 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 sorrounding 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 mosquitos 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(6). For example Chloroquine phosphate 500mg(300mg base) orally twice weekly protects travellers to malarious areas where there is no chloroquine resistance by suppressing the enythrocytic 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(7).

Malaria is a protozoan infection characterized by paraxysms of chills, fever and sweating, and by anaemia, splenomegally and a chronic relapsing course. Malrial 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 of a common syringe by drug addicts(7). Of the four mentioned above P falciparum causes the most serious infections and is responsible for 99% of the deaths due to malria.

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 plamodium gets to the liver some of them can survive there very long and thus can act as a resevoir for further attacks and the person can get fever at any time for the rest of his life(8).

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) ie. (40-41°C). Headache is prominent and during intervals between parxysms, which are exceedingly variable (36-72 hours) the patient usually has a low grade fever(7). 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 diahoea and the stool is blood strained. When 40% of the total erthrocytes are haemolysed, haemolytic anaemia may result.

ANTIMARALIALS IN COMMON USE

(1) The cinchona alkaloides 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. prognil.
(6) The dianinopyrimides e.g. pyrimethamine (9) (10) (4)

The cinchona alkaloids, aminoacrids, 4 amino, quinolines, biguonides and dianuinopryrimidines have a destructure action on the asexual erthrocytic forms of all species of human malaria parasites. The 8 aminoquinolines do not readily affect the asexual erthrocytic 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 bignonides and diaminopyrimidines 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(9)

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 does 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 inoculom of donor blood (- 10^7 infected red blood cells in ice-cold 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 exoerythocytic and erythrocytic stages plasmodium can be acted on by the drug. The animal method is also ideal for those drugs that need to be biotranstormed to the active drug before they show any activity.

The invivo 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.

TRADIONAL MEDICINES.

Before modern medicines were known in Africa the traditonal 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 through out 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 malria 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 ~~go as give~~ reasons any further than that malaria and fever are synoymous. They do not realise that there is more to malaria than just the fever.

The weak activity of the prepararions plus the immunity factor is probably the reason that some of these preparations seem to work. People who have ~~hired~~ 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 cellulo-humoral immunity response(9).

In this project four plants were screened for their activity against two strains of Plasmodium falciparum designated K39 and K33. Tradionally these plants are used both for malaria and other ailments. The four plants are:- Cathaedulis locally known as 'Miraa' Bidens Pilosa or 'Michege' Caesalpinea volkensii or 'Mbuthi'. The fourth Tidhonia diversifolia is not used tradionally. It was chosen because it belongs to the family compositae, which is known to contain sesquiterpenes in antimalarial activity was observed in Qinghao (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(20)

Catha edulis

The shoots of Miraa are normally chewed as a stimulant(13). The shoots, leaves and roots have also been used in tradional medicine to treat various ailments. It is said to be good for colds and is of value in the treatment of malaria, diarrhoea and veneral diseases (14) (17)

Active constituents: Fluckinger and Geroch (1887) seem to have been the first to isolate the pharmocologically 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 (ie. derivatives of flavanol). According to Maitai (1973) T.L.C revealed phenylalleyl-amines, especially d-norpsendoephedrine, d-psendoephedrine, L-ephedrine and amphetamine. However d-norpsendoephedrine is the only alkaloid in all parts of the plant.

Caesalpinea Volkesii

The dry mature seeds of this palnt 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.

METHODS AND MATERIALS

Materials and Equipment

1. Plant materials - This includes seeds of C.volkensii, 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. Vaccum dessicator.
8. Electric balance.
9. Rotavapor Buchi
10. Aseptic screen.
11. Alcoholic cetrimide.
12. Whattmann №1 filter paper and cotton wool.
13. Incubator.
14. Microtubes.
15. 25 ml tissue culture flasks (corning Glass works corning N.Y.)
16. Microtiter plates (cooke laboratory products, Alexndria, 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 Insitute of Research, Washington, D.C.)

METHODS AND MATERIALS

Materials and Equipment

1. Plant materials - This includes seeds of C.volkensii, 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. Vaccum dessicator.
8. Electric balance.
9. Rotavapor Buchi
10. Aseptic screen.
11. Alcoholic cetrimide.
12. Whattmann Nº1 filter paper and cotton wool.
13. Incubator.
14. Microtubes.
15. 25 ml tissue culture flasks (corning Glass works corning N.Y.)
16. Microtiter plates (cooke laboratory products, Alexndria, 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 Insitute of Research, Washington, D.C.)

20. G-^{3}H Hypoxanthine (Amersham/Searle Corp; Arlington Heights, III).

21. MASH II automated cell harvester (Microbiological Associates Bethsda, 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 sreen.

COLLECTION AND PREPARATION OF PLANT MATERIAL

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 Whattmann 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 vaccum 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 samplw 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 continous extraction was carried on for three hours. After this the same procedure starting from (2) above was followed.

NB All four plant samples were subjected to the same extrations as described above.

From the four plants there were eight samples i.e. four aqueous extracts and four alcoholic extracts.

SAMPLE	SAMPLE SIZE (g)	EXTRACT
A	1.1170	C.volkenii Alcoholic
B	0.1258	B.pilosa alcoholic
C	0.3726	C.volkensii aqueous
D	0.9070	B.pilosa aqueous
E	1.8006	T.diversifolia aqueous
F	1.8308	T.diversifolia alcoholic
G	0.1096	C.edulis alcoholic
H	0.3145	C.edulis aqueous

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

	PARASITE CONTROL								NON-PARASITE CONTROL			
A	1	2	3	4	5	6	7	8	9	10	11	12
B	100ng											
C	500ng											
D	250											
E	125											
F	62.5											
G	31.25											
H	15.525											

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 nglml in row B we have to prepare a 2000nglml concentration of test sample due to an initial two-fold dilution.

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

$$2000 \times \frac{225}{25} \text{ ng/ml}$$

$$= 18000 \text{ ng/ml}$$

$$= 18 \text{ng/ml}.$$

UNIVERSITY OF NAIROBI LIBRARY

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. 180μgl/μl

∴ stock concentration = 180 μg/μl.

SAMPLE A

Sample weight = 1.1170 g

= 1117 mg = 1117000μg

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

$$\frac{(1117000)}{180} \text{ mls} = 6200 \text{ Mls.}$$

(1) Dissolve sample in 62 mls of 70% Ethanol
This gives a concentration of 18,000μg/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 180μg/μl.

SAMPLE B

Sample weight = 0.1258g

= 125.8mg =125800μg

To get 180μg/ml, 125800 g should be dessolved in

$$\frac{(125800)}{180} \text{ ml}$$

= 698mls.

(1) dissolve sample in 69.8 ml of 70% Ethanol
This gives 1800μglml

(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 180μglml

SAMPLE C

Weight of sample = 0.3726 g.

= 372.6mg = 372600μg.

To get 180μglml, 372500 g should be dissolve in

$\frac{(372600)}{180}$ mls

= 2070 mls.

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

This gives a concentration of 18000μglml.

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

Make up volume with 70% Ethanol. This final dilution gives the desired concentrati of 180μglml.

SAMPLE D

Weight of sample = 0.9070g

= 907 mg = 907000μg.

To get 180μ glml, 907000 g of sample is dissolved in

$\frac{(907000)}{180}$ mls

= 5038 mls.

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

This gives a concentration of 18000μglml.

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

This final dilution gives the desired concentration of 180μglml.

SAMPLE E

Weight of sample = 1.8006g

= 1800.6mg = 1800600μg.

To get 180μglml 1800600 g should be dissolved in

$$\frac{(1800600)}{180} \text{ mls.}$$

= 100003.333 mls.

(1) Dissolve sample in 100 mls of 70% Ethanol to give a concentration of 18000μglml.

(2) Take 0.25 mls of the above solution and put in 25 mls volumetric flask make up to volume with 70% Ethanol. This final dilution gives 180μglml.

SAMPLE F

Weight of sample = 1.8308g

= 1830.8 mg =1830800μg.

To get 180 glml, 1830800μg should be dissolved in

$$\frac{(1830800)}{180} \text{ml}$$

= 10170 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 180μglml.

SAMPLE G

Weight of Sample = 0.1096 g

= 109.6mg = 109600 g.

To get 180μglml, 109600 g should be dissolved

in $\frac{(109600)}{180}$ml = 608.8888 ml of 70% Ethanol.

(1) Dissolve sample in 60.9 mls of 70% ethanol. This gives a concentration of 1800μglml.

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

SAMPLE H

Weight of sample = 0.3145g.

= 314.5mg = 314500μg.

To get 180 glml, 314500 g should be dissolved in

$$\left(\frac{314500}{180}\right) \text{ mls of 70\% Ethanol.}$$

$$= 1747.2222 \text{ ml.}$$

(1) Dissolve flask contents in 69.8 mls of 70% ethanol. This gives a concentration of 4500μglml.

(2) Take 1.00 ml of the above solution and place in a 25 ml volumetric flask and make upto volume with 70% ethanol. This final dilution gives the desired concentration of 180μglml.

NB During the experimental work It was 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 180μglml concentration is only a theoritical value.

Sample	Weight	Theoretical glml	Ammount Dissolved	Con. (actual) glml
A	1.1170	180	0.3347	53.935524
B	0.1258	180	0.0474	67.82193
C	0.3726	180	0.2815	135.99032
D	0.9070	180	0.6735	133.66041
E	1.8006	180	0.800	79.973342
F	1.8308	180	0.1024	10.067729
G	0.1096	180	0.0171	28.083941
H	0.3145	180	0.0502	28.731319

It follows therefore that our desired highest concentration of 1000 ng that goes into row B of the microtitration plate is only theoritical. The actual values are given below:-

Sample		
Sample A	180 glnl → 1000ng.	53.935524 → 299.642 ng.
B	"	67.82193 → 376.788 ng.
C	"	135.99032 → 755.5 ng.
D	"	133.66041 → 742.557 ng.
E		79.973342 → 444.2963 ng.
F	"	10.067729 → 55.9318 ng.
G	"	28.083941 → 156.0218 ng.
H	"	28.731319 → 159.6184 ng.

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

These were left to stand for 2 hours, after which they were ready for testing.

The parasite inocular 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 I multiple-well filling pipette was used to place 25μl of the culture medium in each well of the microtiter plate. 25μl of the test solution was then added to each of two adjacent wells in the second row(B), thus six compunds were accommodated by each plate. After the test compunds 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 compund 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% O_2,5% Co_2 and 90% N_2 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-^{3}H) nypoxanthine was used as an index of growth of the parasites. The final isotpe solution consisted of 20 G of (G-^{3}H) hypoxathine 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 dired 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 ($G^{3}H$) hypoxanthine

Growing prasites take up the labelled hypoxanthine and thus a high reading is expected of the PRBC control (column 1-8)(row A). The RBC control colum (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 amd pyrimethamine the following was found.

Drug	K39	K33
CHoroquine	Sensitive	sensitive
Cyclognenil	Resistant	sensitive
Pyrimethamine	Resistant	sensitive

Both strains were obtained from school children in Kisumu.

RESULTS

DATA OBTAINED

K39 + Plant extracts

	Average
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

THEORETICAL ng/ml	ACTUAL ng/ml	A	A
1.9531	0.5852	3630	3858
3.90625	1.17045	3225	3552
7.8125	2.3409	3238	3408
15.625	4.3638	4141	3689
31.25	9.3638	3698	3780
62.5	18.727	3159	4028
125	37.455	3164	3234
250	74.910	3559	3846
500	142.821	3274	3245
1000	299.642	2192	2262

Sample A - Caesalpenia volkensii (ethanolic extract)

TABLE TWO

THEORETICAL ng/ml	ACTUAL ng/ml	B	B
1.9531	0.7359	3908	4569
3.90625	1.472	3714	3901
7.8125	2.934	4257	3436
16.625	5.887	4257	4052
31.25	11.774	3666	4122
62.5	23.549	3560	3778
125	47.098	3760	3624
250	94.197	3782	3772
500	188.394	3148	3956
1000	376.788	2257	3541

Sample B = Bidens Pilosa (ethanolic extract)

TABLE THREE

THEORETICAL nglml	ACTUAL nglml	C	C
1.9531	1.475	3219	3806
3.90625	2.951	3488	3469
7.8125	5.902	3668	3234
15.625	11.8047	3982	3699
31.25	23.609	3247	4187
62.5	47.218	3220	4160
125	94.437	3300	3859
250	188.87	3437	4036
500	377.75	2805	3748
1000	755.5	2426	3032

Sample C = Caesalpinea Volkensii (aqueous extract)

TABLE FOUR

THEORETICAL nglml	ACTUAL nglml	C	C
1.9531	1.4503	3728	3860
3.90625	2.9006	3778	3891
7.8125	5.8012	3307	4117
15.625	11.6024	4256	3937
31.25	23.2045	3967	3682
62.5	46.409	3438	3956
125	92.8197	3705	3571
250	185.6394	3959	3659
500	371.2708	3764	3142
1000	742.557	3264	2158

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

THEORETICAL nglml	ACTUAL nglml	A	A
15.62	4.6819	6171	5911
31.25	9.3638	5455	5604
62.5	18.727	5340	5515
125	37.455	5252	5299
250	74.910	5921	5471
500	149.821	4790	5599
1000	299.642	4424	4489

SAMPLE A= Caesalpinee volkensii (ethanolic extract)

TABLE 6

THEORETICAL nglml	ACTUAL nglml	B	B
15.62	5.887	7304	6877
31.25	11.774	5199	5949
62.5	23.549	6699	6153
125	47.098	6404	5722
250	94.197	6047	5938
500	188.394	5866	5527
1000	376.788	5365	5653

Sample B= Bidens Pilosa (ethanolic extract)

TABLE 7

THEORETICAL nglml	ACTUAL nglml	C	C
15.62	11.8047	8303	5713
31.25	23.609	6164	6283
62.5	47.218	7959	6058
125	94.437	5994	5935
250	188.87	7096	6052
500	377.75	6140	5888
1000	755.5	6545	5843

Sample C- Caesalpinea Volkensii (water extract)

TABLE 8

THEORETICAL nglml	ACTUAL nglml	D	D
15.62	11.6024	8049	6966
31.25	23.2049	8871	7225
62.5	46.409	6821	6996
125	92.8197	5331	6043
250	185.6394	6175	5733
500	371.2708	5570	5556
1000	742.557	4925	5505

Sample D - Bidens Pilosa (water extract)

TABLE 9

THEORETICAL nglml	ACTUAL nglml	E	E
15.62	6.9421	5766	7065
31.25	13.8842	5603	7629
62.5	27.7685	6538	6614
125	55.537	5408	6256
250	111.074	5901	6332
500	222.1481	6011	7005
1000	444.2963	5781	5761

Sample E = Tidhonia diversofolia (water extract

TABLE II

THEORETICAL nglml	ACTUAL nglml	F	F
15.62	2.4378	7130	6169
31.25	4.8756	6336	6049
62.5	9.7513	5650	6900
125	19.5027	5293	6025
250	39.0054	5354	5901
500	78.0109	5088	5249
1000	156.0218	4802	4982

Sample G= Catha edulis (ethanolic extract)

TABLE 12

THEORETICAL nglml	ACTUAL nglml	H	H
15.62	2.494	7594	6126
31.25	4.988	6154	6621
62.5	9.9761	6452	6368
125	19.9523	55270	6170
250	39.9046	5270	5474
500	79.8092	4954	4958
1000	159.6184	4750	5699

Sample H = Catha edulis (water extract)

TABLE 10

THEORETICAL nglml	ACTUAL nglml	.F	F
15.62	0.8739	8205	7272
31.25	1.7478	6013	7087
62.5	3.4957	6091	5894
125	6.9914	6009	5603
250	13.9829	5856	5288
500	27.9659	5073	4959
1000	55.9318	4090	4560

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 desk top computer, the Tekronix 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-^{3}H) hypoxanthine were calculated from the disintegration per minute in row A, well 1 through 8, and wells 9 through 12 respectively.

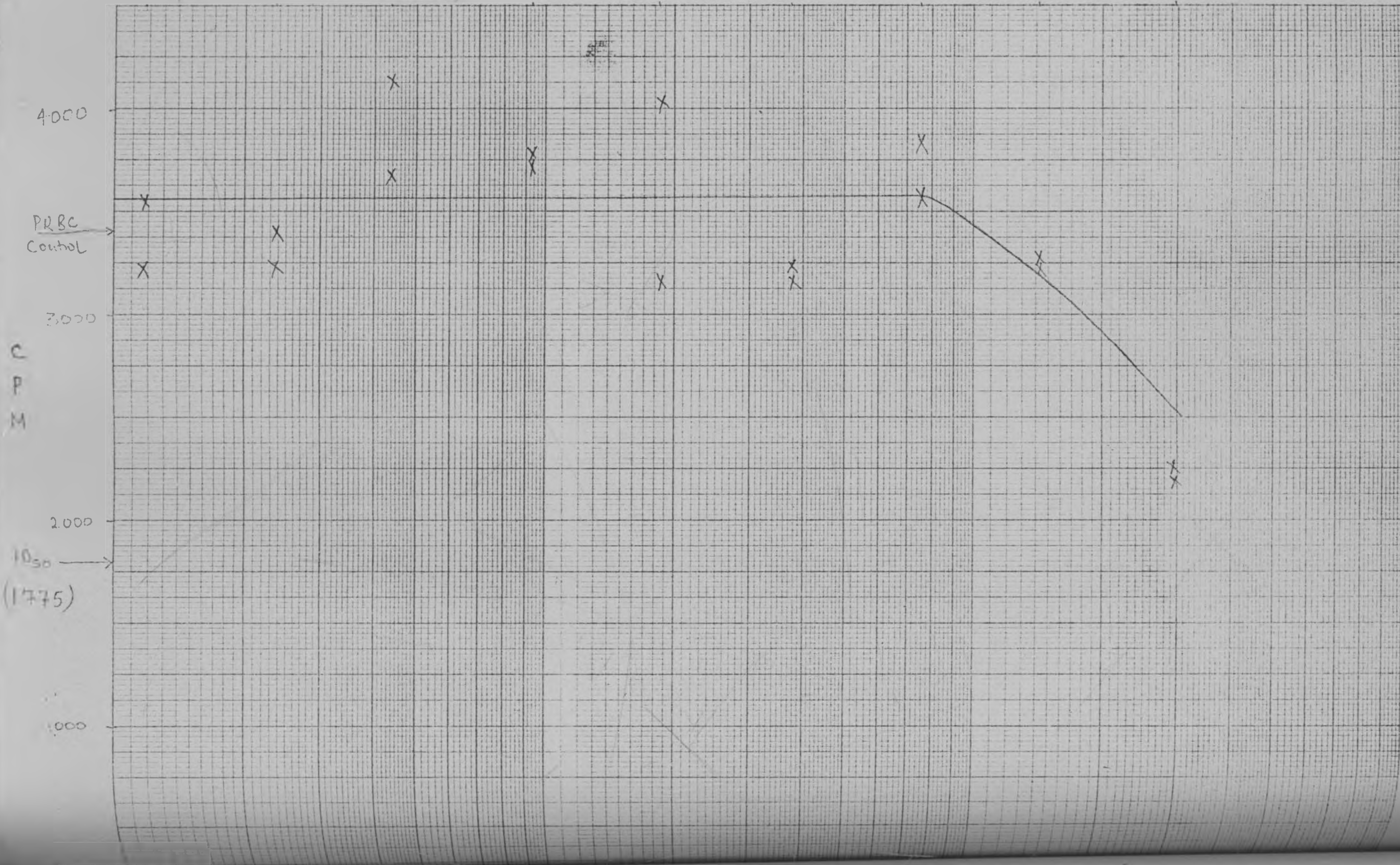
The automated system used in these experiments generated twofold serial dilutions with a 64 fold range of concentrations for each compound (using the K33 strain of P. falciparum) and a 512-fold range of concentrations for each compound (using the K39B strain of P. falciparum.

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

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% inhibitation of the uptake of (G-^{3}H) Hypoxanthine by the Plasmodium falciparum in the system.

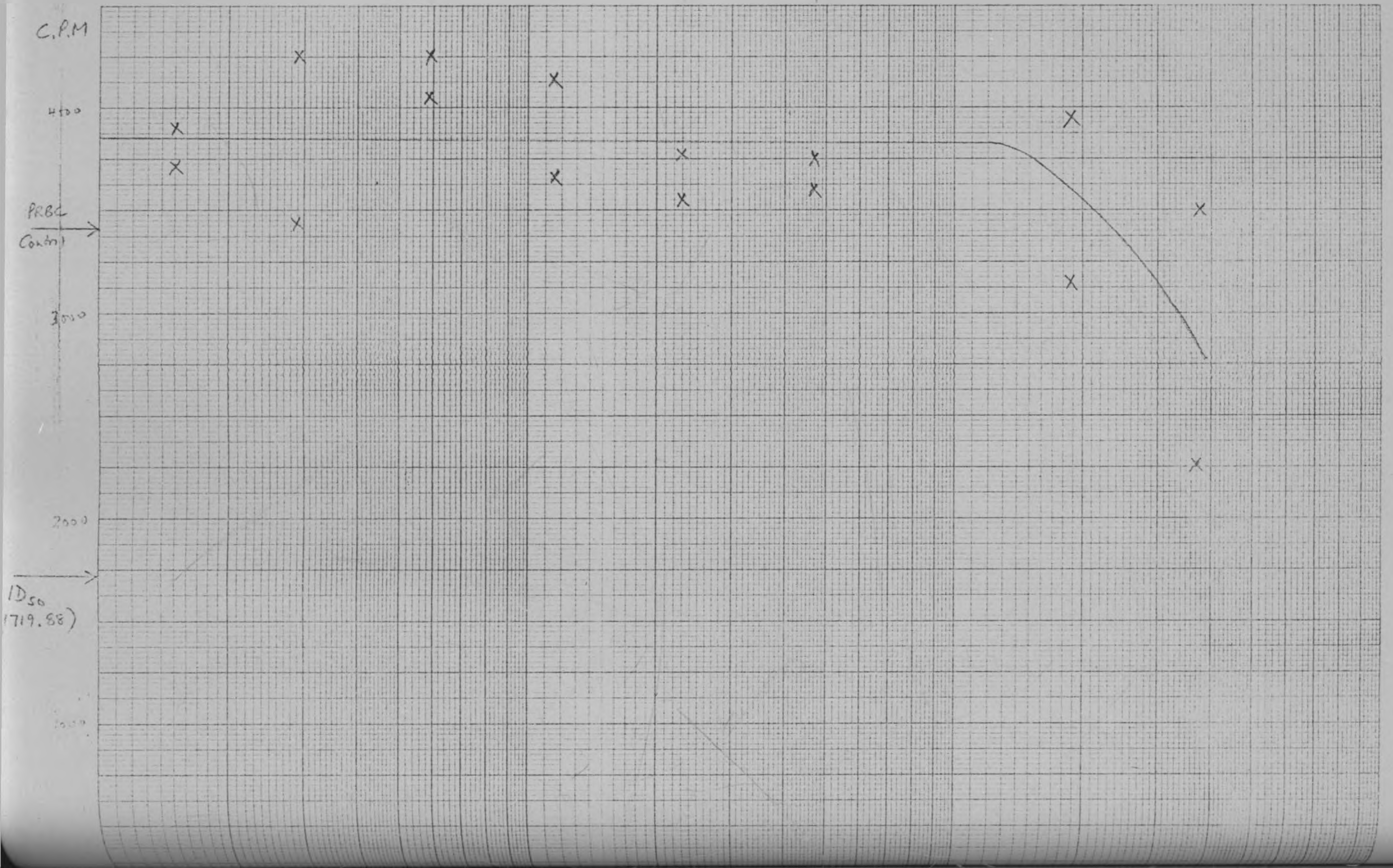
Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

FIGURE (1) Activity of the ethanolic extract of Caesalpinia volkensii against Plasmodium falciparum (K39) in vitro from results in table (1)



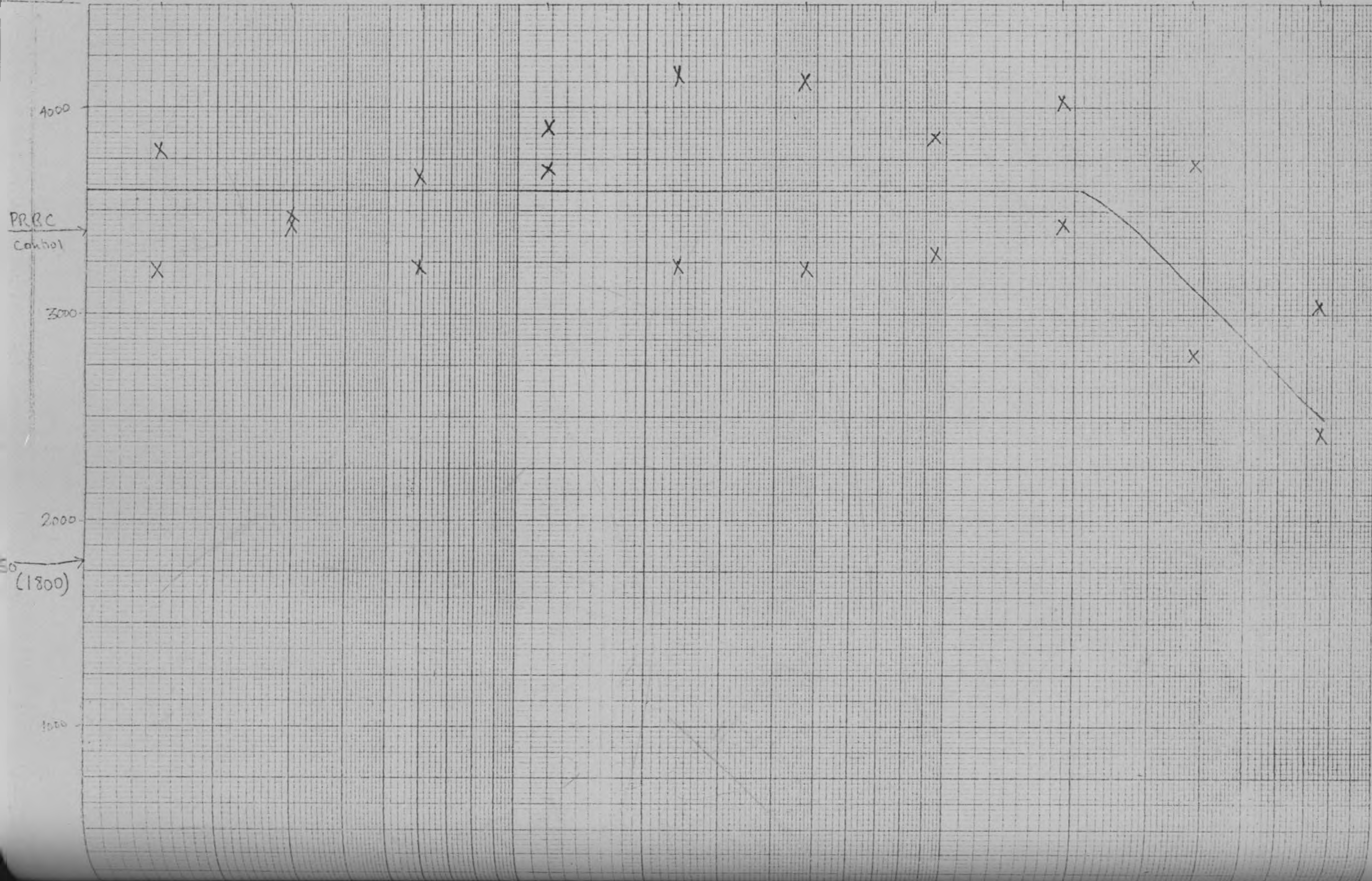
Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

FIGURE (2) Activity of the ethanolic extract of Bidens pilosa against Plasmodium falciparum (K39) in vitro from results in table (2).



Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

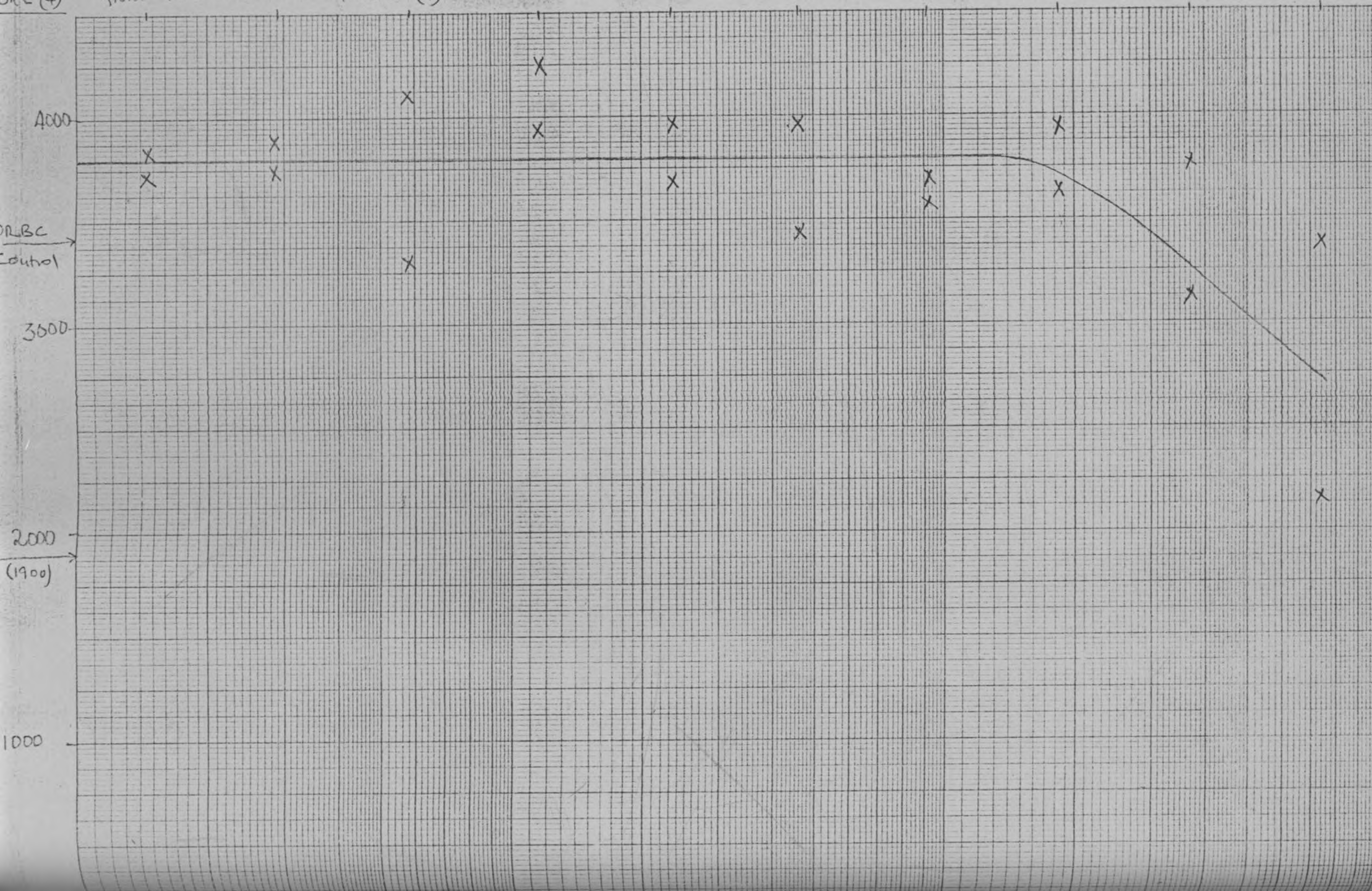
URE (3) Activity of the aqueous extract of Caesalpinea volkensii against Plasmodium falciparum (K39) in vitro from results in Table (3)

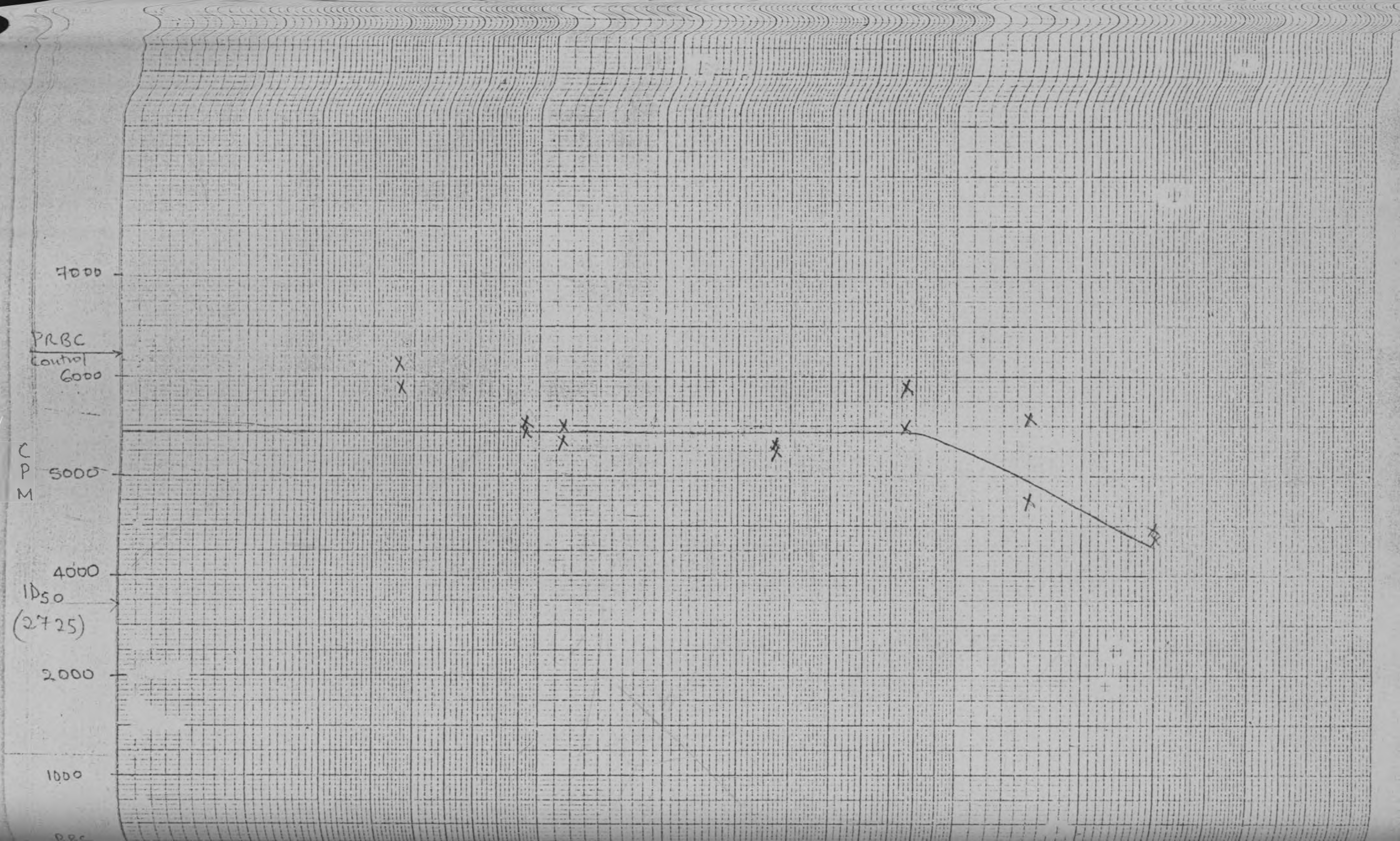


Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

Activity of the aqueous extract of Bidens pilosa against Plasmodium falciparum (K39) in vitro from results in Table (4)

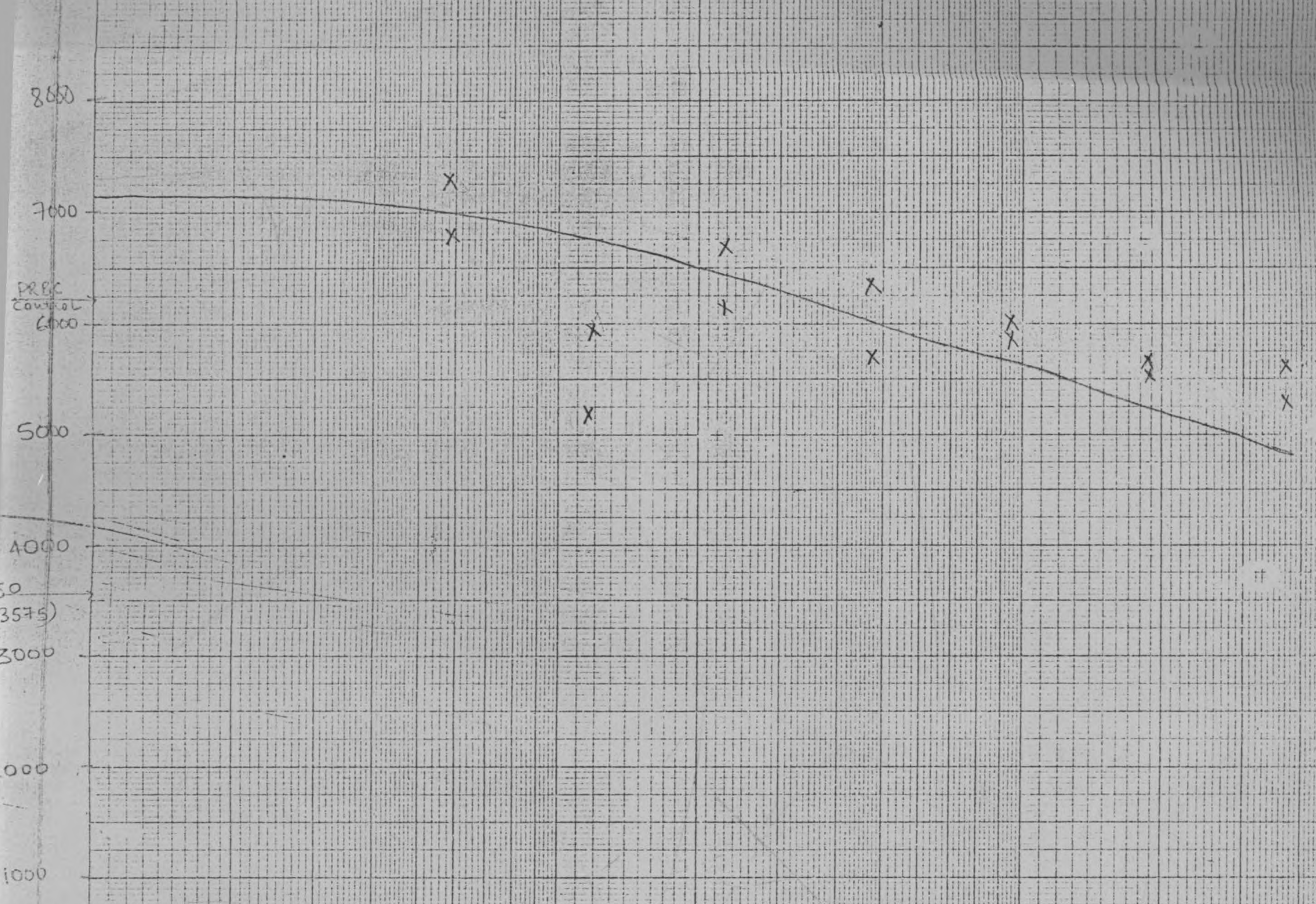
URE (4)



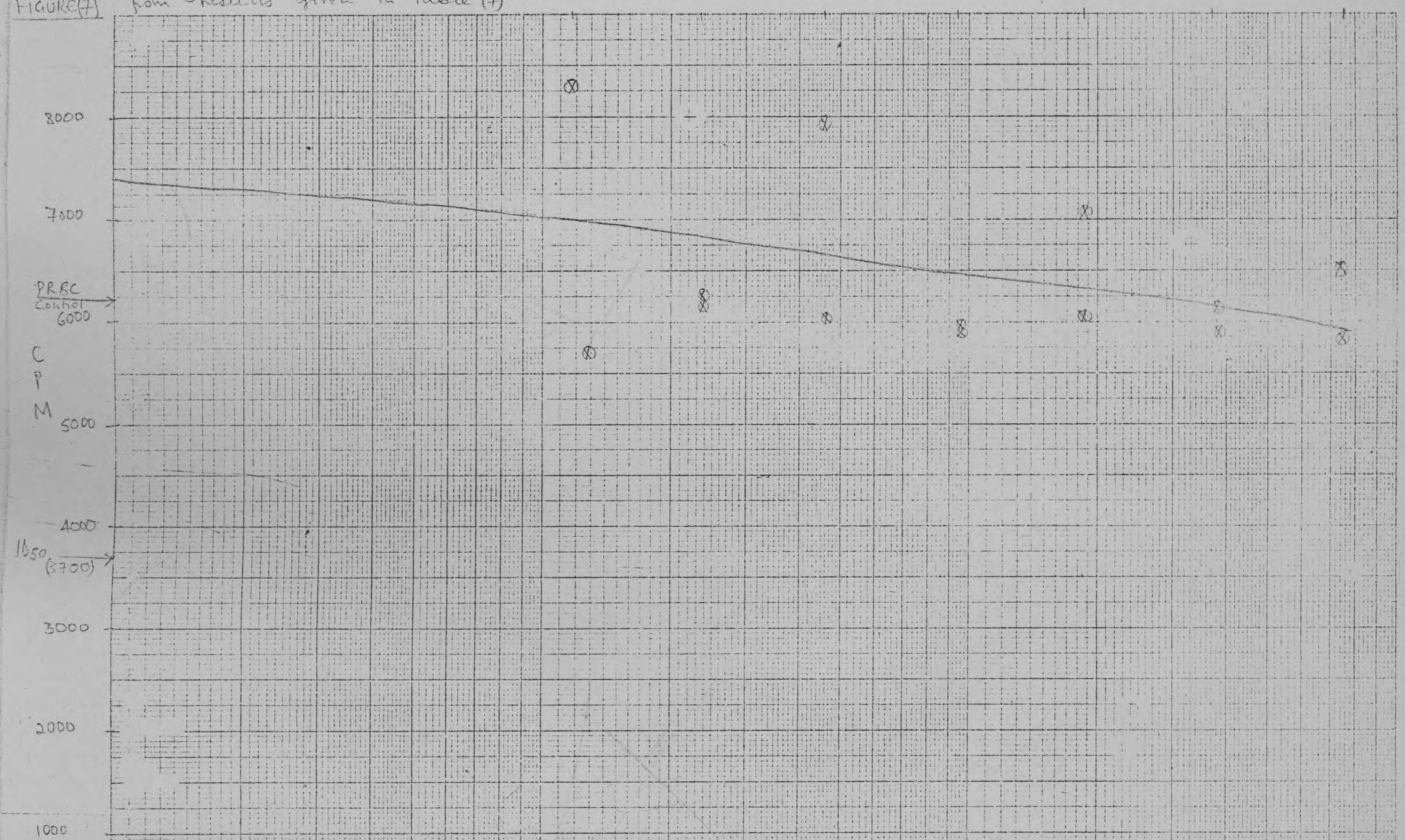


7000
PRBC
control
6000
C
P
M
5000
4000
ID50
(2725)
2000
1000

URE (6) Activity of the ethanolic extract of Bidens pilosa against Plasmodium falciparum (K 39) in vitro from results given in Table (6)

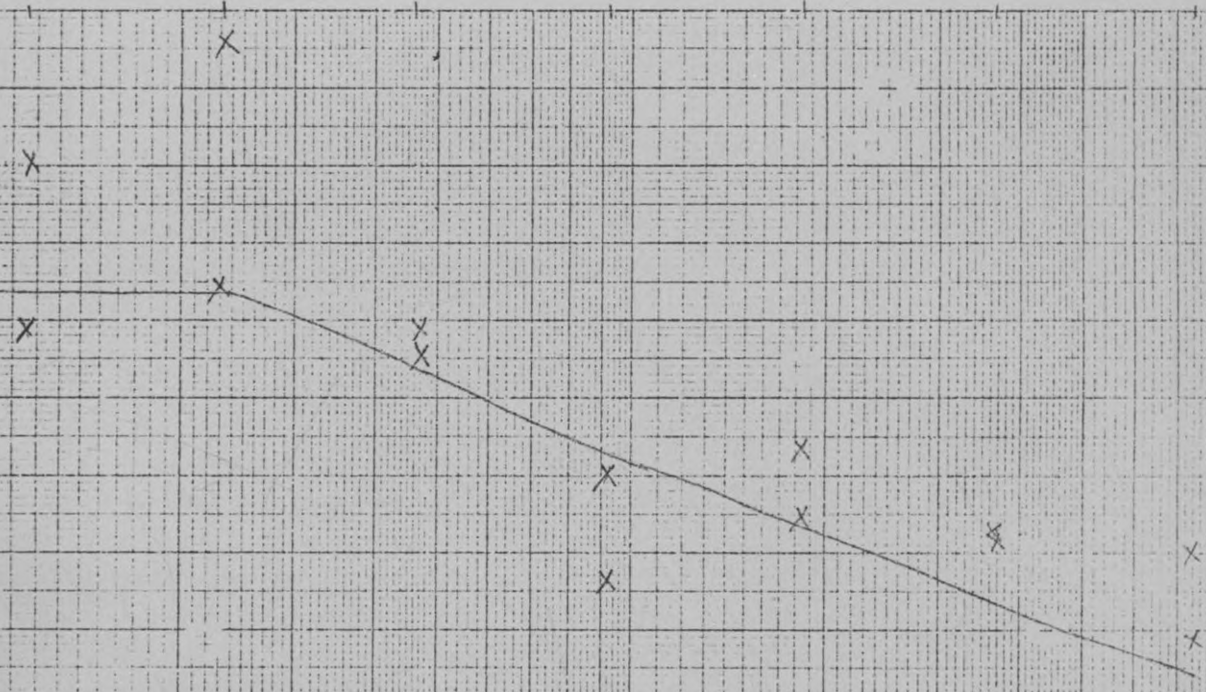


FIGURE(7) Activity of the aqueous extract of Caesalpinia volkensii against Plasmodium falciparum (K33) in vitro from results given in Table (7)



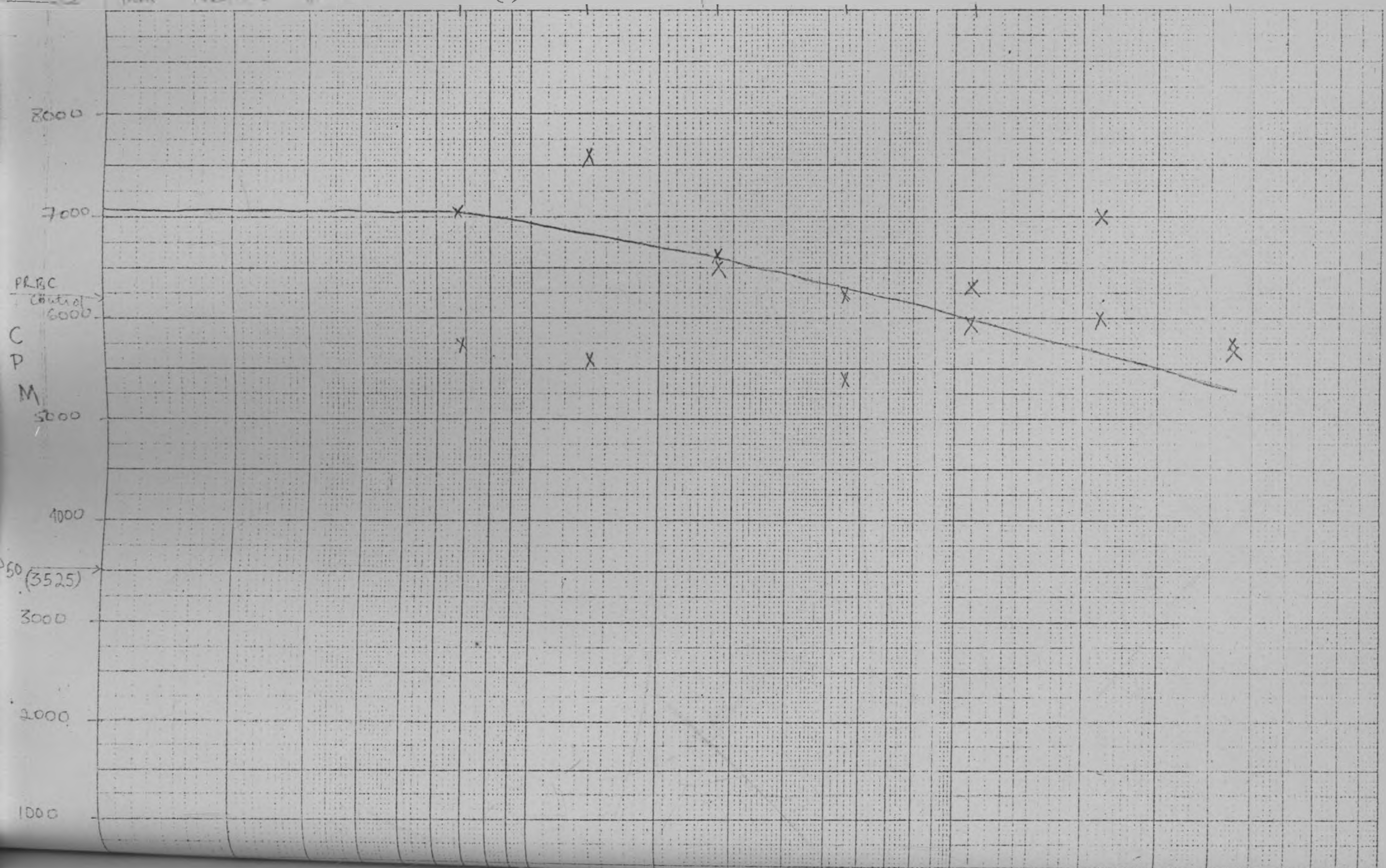
Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

(8). Activity of the aqueous extract of Bidens against P. falciparum (K33) in vitro from results given in Table (8)



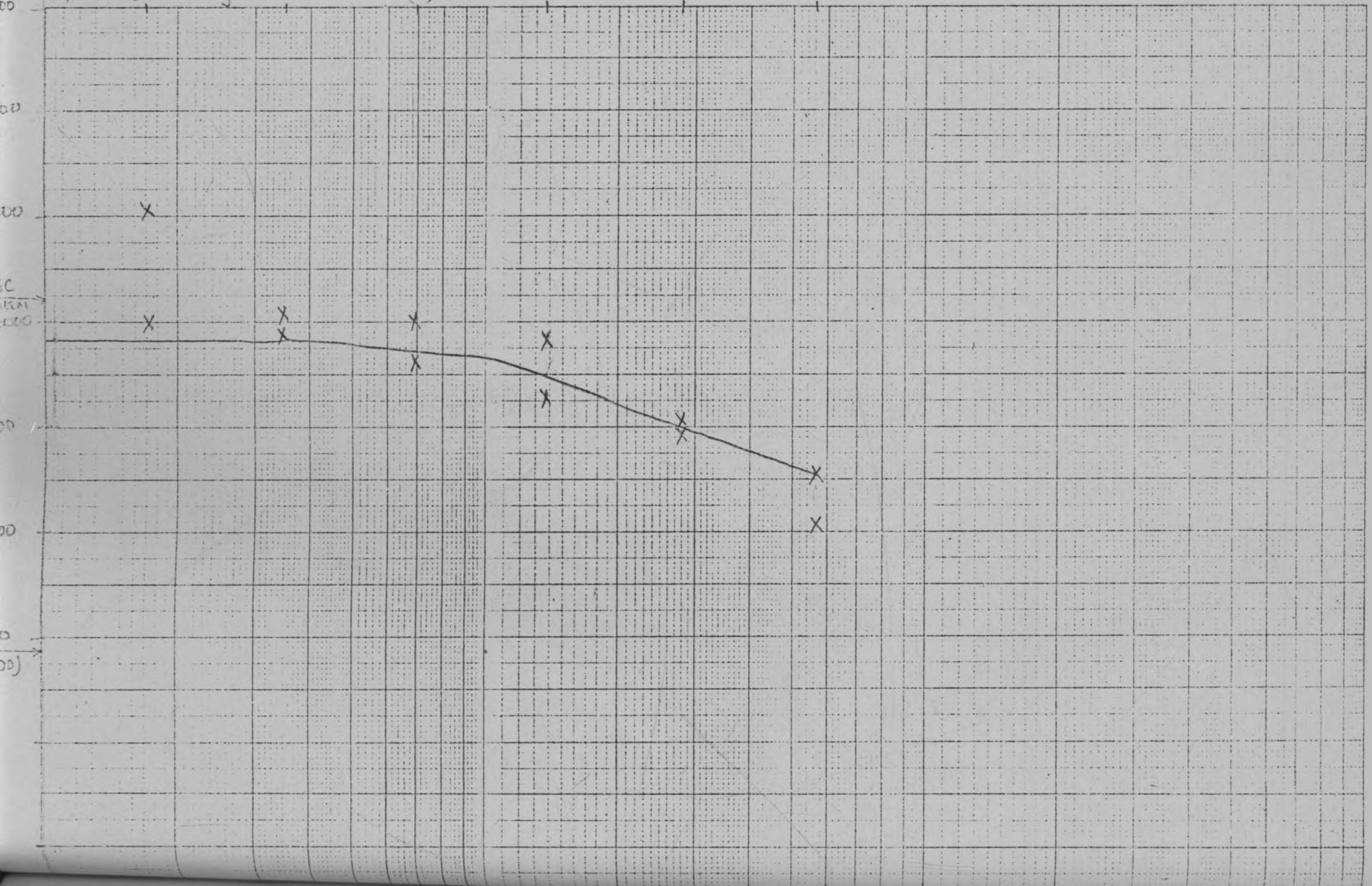
Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

FIGURE (9) Activity of the aqueous extract of Tithonia diversifolia against Plasmodium … from results given in Table (9)



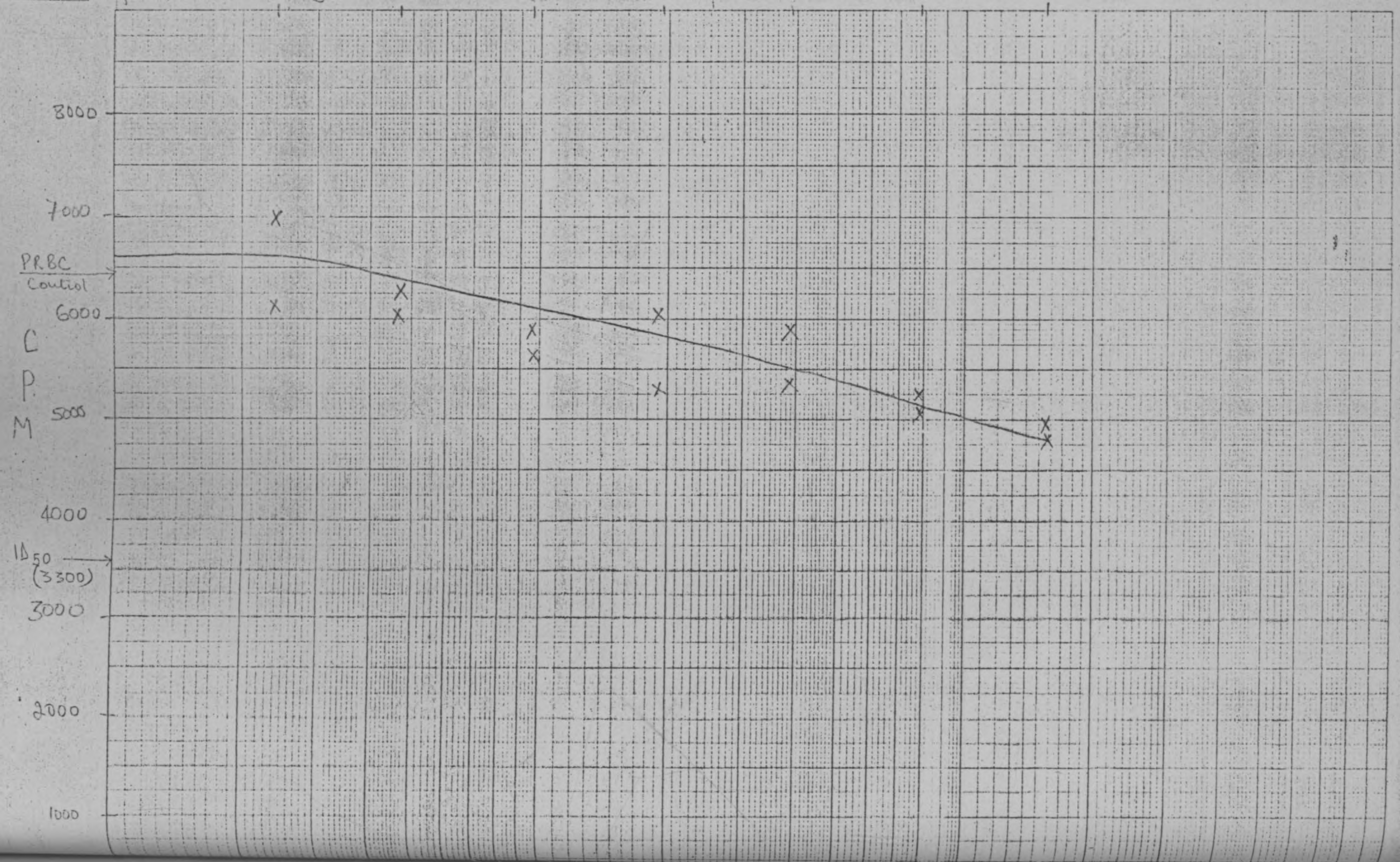
Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

10) Activity of the ethanolic extract of Tithonia diversifolia against Plasmodium falciparum in vitro from results given in Table (10)



Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

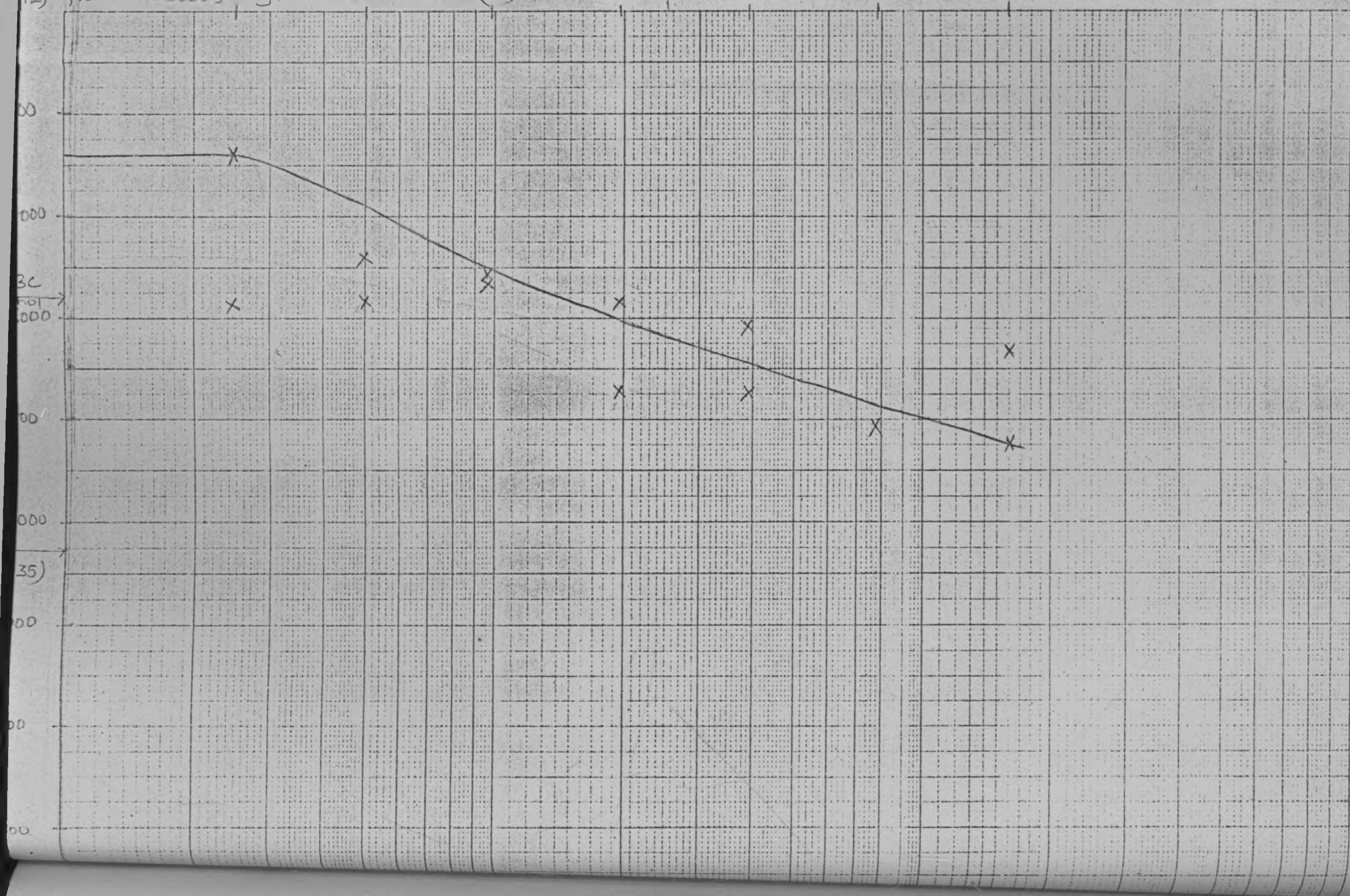
FIGURE (11) Activity of the ethanolic extract of Catha edulis against Plasmodium falciparum (K3 3) in vit from results given in Table (11)



Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, and 1 cm

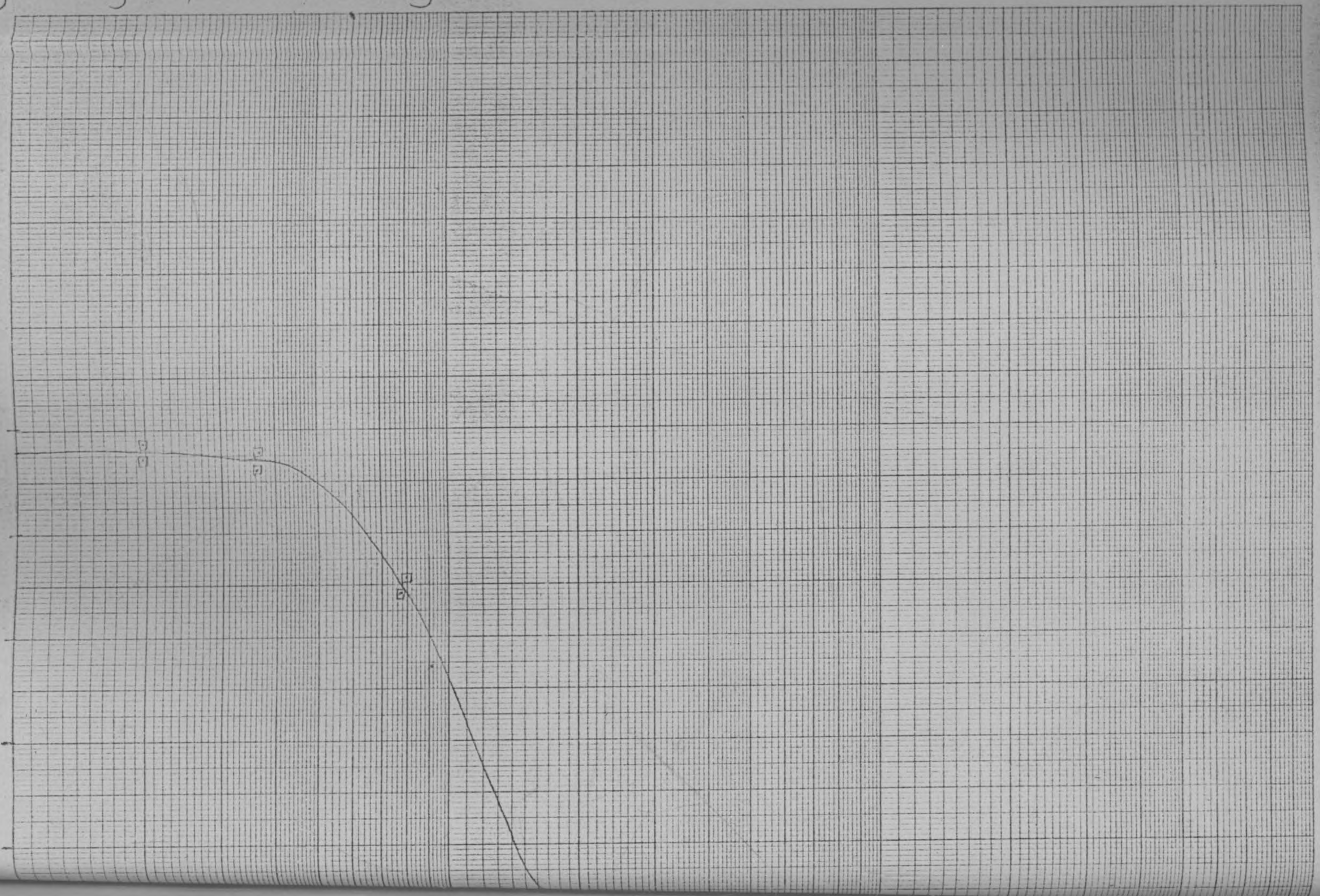
Activity of the aqueous extract of Catha edulis against Plasmodium falciparum (K 33) in vitro

12) from results given in Table (12)



Chartwell Graph Data Ref. 5531 Log 3 Cycles x mm, ½ and 1 cm

(13) Activity of Chloroquine against Plasmodium falciparum (Uganda I) in vitro.

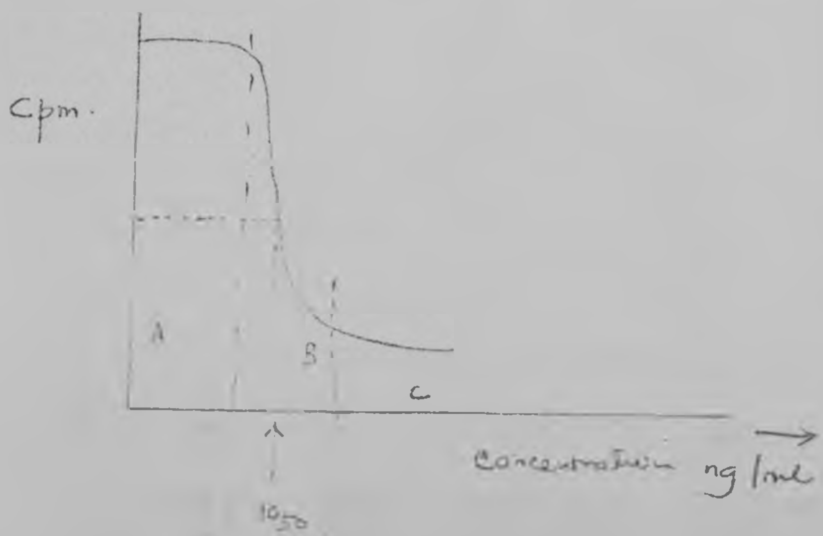


DISCUSSION

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.

Incoporation of (G -^{3}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 incoporated 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 falciparon used are Chloroquine sensitive. One draw back was that the concentrations used were much lower than 1000ng/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 100ng/ml, this was the ethanolic extract of Tidhonia diversifolia (55.9318 ng/ml).. The highest concentration was that of the acqueous extracts of Caesalpinea Volkensii (755.5 ng/ml) IF the concentrations were as high as expected (theonitically 1000ng/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 signoidol curve is expected whereby:-



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. Its in this region that we get the ID_{50}

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

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 viro* method using the owl monkey or man. Such a method thyough 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 falciporum* 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.

References:

(1) Progress in Medicinal Chemistry
Volume 8 Page 299
Edited by Ellis and West
Butterworth and Co. (Publishers) Ltd
London: 88 Kingsway, WC2B 6AB
1971.

(2) Unicef Social Statistics program.

(3) L.J. Bruce-chwatt
Transactions of the Royal Society of Tropical Medicine
and Hygiene.
Volume 64, No 2 Page 210
1970

(4) Loius S. Goodman and Alfred Gilman
The Phermocological basis of Therapeotics
5th Edition Page 1048
Macmillan Publishing Co. INC, Newyork

(5) W. Peters
Chemotherapy and drug resistance in Malaria Academic
Press, London and Newyork.
1970

(6) D.C. Turk and I.A. Porter
A short textbook of Medical Microbiology
3rd Edition Page 208
Hodder and Stoughton
London, Sydney, Aukland, Toronto
1977.

(7) The Merck Manual
13th Edition Pages 159-162
Merck Sharp and Dohme Research Laboratories
Rahway, N.J.
1977.

(8) Blacklock and Southwell
A guide to human parasitology
Edited by T.H. Darey and W. Crewe
9th Edition Page. 53
H.K. Lewis and Co. Ltd. 136 Gower Street, London
1973.

(9) Sir Gordon Correll et all
Chemotherapy of Malaria
Page 32-51
World Health Organization, Geneva
1955

(10) Martidale
27th Edition Pages 343-360
The Pharmaceutical Press
London

UNIVERSITY OF NAIROBI
LIBRARY

(11) R.E. Desjardins, C.F. Confield, J.D Haynes and J.D Cheslay
Quontitatire assessment of antimalarial activity
in vitio by a semi-automated microdilution technique.
Antimicrobia Agents and Chemotherapy 16, 710-718 (1979)

(12) R.L. Bendin and N.D. Pacheco
Cultivation of exo-erythrocytic stages of malaria
parasites - a review.

(13) Benard Vedcourt and E.C. Tromp
Common poisonous plants of East Africa Page 99
Collins, London
1969.

(14) C.K. Maitai
A Toxicological investigation of Cotha edulis forsk(Miraa)
Ph.D Thesis
Nairobi
1973.

(15) Edward P Claus and Varro E. Tyler
Pharmecognosy
6th Edition Page 164
Henry Kimpton London
1970.

(16) M. Watt and M.G. Breyer-Brand wijk
Medicianl and poisonous plants of Southern and Eastern
Africa
2nd Edition, Livingstone, 1962 Page 206

(17) Kokwaro J.C.
Medicinal Plants of East Africa
East Africa Literature Bureau
Nairobi
1976.

(18) D.R. Laurence
Clinical Pharmecology
4th Edition Page 8.27 - 8.33
Churchill Livingstone
Edinburg, London and Newyork
1973.

(19) Report of W.H.O. Scientific group in resistance
Malaria parasites to drugs
Technical Report series W.H.O. No. 296
1965.

(20) Special programme for research and training in
tropical diseases.
Newsletter No. 16
December 1981.