# BIOCHEMISTRY RESEARCH ACTIVITIES COVERING 20 YEARS OF NIMR


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**1: Onchocerciasis**

Major scientific achievements were the discovery of genetic diversity within *Onchocerca volvulus* in Africa: Cameroon, Tanzania and Sierra Leone. Countrywide undertaking studies on the prevalence and experience observed with serodiagnosis of the disease.

The genetic diversity was evaluated by the development and application of molecular tools for the characterisation of the adult *O. volvulus* isolates. Long term objective was to assess the role of strain variation in the epidemiology of the disease.

Adult *O. volvulus* from the different countries were isolated from modules which were surgically removed from infected people. After excision, the nodules were washed with 1% (W/V) sodium dodecyl sulphate for 5 minutes, put in saline (0.9% NaCl) and excess human tissue was removed. Subsquently they were transferred into digestion medium (Eagles medium containing 100 μg/ml gentamycin and 4mg/ml collagenase B) at $37^0$C.

After the human tissue had completely been digested (8-24 hours), the worms were freed from the nodules. Males were washed with saline + 10mM EDTA and

stored in liquid nitrogen. Females were kept in 0.5ml digestion medium for 1-2 hours, washed and stored in a similar mauncer as for males.

Both nuclear and ribosimal DNA were isolated separately for parasite characterisation. The worms were free thawed 3 times. Lysis buffer (50 mM NaCl; 50 mM Tris-HCl pH 7.2; 10 mM EDTA, pH 8.0) was added to the sample to a total volume of 0.5 ml. Triton X-100 and proteinase K were added to final concentration of 1% (W/V) and 400 ug/ml respectively. The mixture was incubated at $60^0$C for 3 hours or overnight. The DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA pellets were resuspended in 50 ul TE buffer (10mM tris-HCl, pH 7.2; 1 mM EDTA, pH 8.0) and stored at - $20^0$C until further use.

With available DNA, five different methods (Meredith, 1989, 1991; 0' Donnell, 1992; Herder, 1996) were used to characterise the parasite: Analysis of the 150 nucleic acid base pairs repeat, internal transcribed spacer; southern blotting and hybridisation using savannah forest specific probes; application of random amplified polymorphic DNA (RAPD) and microsatellite polymeraze chain reaction. There was sequence variation between 150 base pair repeats, but systematic differences were hard to detect, making difficult to develop specific probes and primers to distinguish between worms from different areas. Using RAPD however it was possible to distinguish between *O. volvulus* and other nematodes. With microsatellite PCR significant differences were detected with frequency of alleles in worms were homozygous AA. In Cameroon, different alleles A,B, and B were identified; the allele frequency distribution in the worms from savannah region was quite different from worms of their bioclimates. In Tanzania, a very heterozygous pattern of alleles was found; a total of seven alleles A,B,C1,C2,C3 and D were identified and were not evenly distributed within the different foci. The D type was found in Amani only.

Studies on the prevalence of human onchocerciasis and experience with serodiagnosis, were carried out to Ph.D. level (1992) with the University of Dar es Salaam. Onchocerciasis was found to be on the increase in Bwakira (63.6%) Mahenge (58.6%) and Ruvuma (31%). With a decline in Amani focus (22.4%). Indirect haemagglutination antibody test was sensitive and specific for serological testing using adult *Onchocerca gutturesa* antigen. The serological data was complementary to the skin snip and clinical diagnostic parameters. Sensitivity was highly variable with highest levels in Amani (93.7%), Bwakira (87.7%), Kilosa (81.8%) and Ruvuma (75.0%).

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## 2: Development of Insecticides and Repellents from local herbs

***Citrus Peel Oil as Mosquito Larvae Insecticides***

Experiments (Mwaiko, 1992) showed that *Culex quinquefasciatus* larvae were susceptible to peel oil extracts of orange (*Citrus sinensis*), bitter orange (*Citrus aurantium*) and lemon (*Citrus limon).*

Importance of this investigation was to develop cheap insecticides which will be available locally as an alternative to other insecticides particularly those to which mosquito show resistance. The crude peel oil extracts were tested at different concentrations in 200 ml portions of 2% ethyl alcohol water solution. Objective of this investigation was to develop a cheap insecticide which will be available locally as an alternative to other insecticides to which *C. quinquefasciatus* has shown resistance. With further aim to identify the active components of the oil extracts for subsequent field evaluation tests.

The oils had sweet pungent smell and caused eye irritations. They were acidic with pH of 23-3.0 and insoluble in water but soluble in ethyl alcohol and kerosene like other known insecticide. That is why these oils had to be dissolved in water into which ethyl alcohol was added to 2% during the susceptibility tests. The yield for these produced were 0.16%, 0.19% for bitter orange, lemon and orange peels respectively.

The culicine larvae were susceptible to the peel oil of these three citrus species. Similarity on the susptibility was observed with bitter orange and orange peel oil as indicated by their respective similar regression lines with the values of $LC_{50}$ of 50.11 x $10^3$ug (Table 1).

Lemon peel oil was found to be more effective than bitter orange peel oils, with $LC_{50}$ and $LC_{95}$ of 28 x $10^3$ and 89.12 x $10^3$ ug respectively. A mixture of the peel oils for all three citrus species was much more effective than for the peel oils for the individual species, with $LC_{50}$ and $LC_{95}$ of 15.84 x $10^3$ ug and 56.23 x $10^3$ ug respectively.

The $LC_{50}$ and $LC_{95}$ are of the same order as those obtained by Busherod (1977) for Malathion, 48.00 x $10^3$ and 105.00 x $10^3$ ug respectively, with this mosquito species in Kwale, along the East Africa Coast, Tanga region, Tanzania.

The oil nature of these peel extracts however, may also be an advantage since oiling has been an antilarval technique which has been used successfully in several countries. Tests on lemon peel oil extract as a mosquito larvicide were then carried out (Mwaiko, 1994). Most of the specifications and tests on the suitability described in this investigation, are on the basis that an oil larvicide acts by coming into contact with larvae and entering through spiracles and tracheal system. Desirable properties of the oil therefore, are those which will help to make contact with and penetrate into the larva. The accepted description of an efficient oil larvicide is as follows: a cheap mixture oil of suitable viscosity. When applied on water surface, the oil spreads well on into a thin uniform, persistent stable film which is toxic to larvae and pupae of the mosquito.

The oil was found to be toxic on larvae, pupae and eggs of *Culex quinquefasciatus*. The oil also fulfilled other required specifications like specific gravity, spreading pressure and viscosity. It was toxic at wide pH range, stable to heat and light in terms of chemical change which could alter larvicidal action of the oil ingredients. However it was volatile, did not form a permanent film on water surface for longer periods to effect the larvicidal action.

**3: Testing Tobacco Residues for Larvicidal Activity**

Having shown that citrus peel oils were effective insecticides against larvae of *Culex quinquefasciatus*. Problem as a larvicide, was instability of the product on water surface in hot tropical climate. It was speculated that terpines might be the active ingredients in the oils and subsequently it was suggested that joining the terpenoid units might probably form a stable product. Through literature, it was found that the joint terpenoid units are present in tobacco leaves in nature. From these facts, it was decided to test the factory tobacco residues against the *C. quinquefasciatus* larvae (Mwaiko, 1995).

Products of tobacco residues were obtained from two factories in Dar es Salaam and Morogoro, Tanzania. The tests were carried out under field conditions. The residue particles were found to be fast spreading on water surface, with a visible oily film. Fourth-instar *C.quinquefasciatus* larvae, collected from a natural breeding site, were used. A range of known amounts of tobacco powder were added to 400ml of top water in mud pots. To each pot, 70 larvae were introduced. After 24 hours, the contents of each pot was poured into a white enamel dish and assessment of live, moribund, or dead larvae was made. Larvae mortality was then calculated.

Result showed that approximately 100% larvae mortality occurred when the 70 larvae in the pot containing 14 gm tobacco powder. A more precise experiment showed the corresponding figure of 12gm.

Shrubs of tropical genus *Ocimum Linnaeus* (Family abiatae), are used widely in Africa for their reputed mosquito repellent properties. Flower and leaf extracts have been found to be effective as the commercial repellent Diethyl toluamide, (DEET). *Ocimum salve* grows to a height of over one metre in open bush, in area of eastern Africa. Further toxicity experiments on this plant species against *Culex quinquefasciatus* were undertaken.

Flowers, first and second leaves from the tip of the plant were used to generate fumes using two litre fumigation chamber. Adult mosquitoes to be tested were from a laboratory colony. Lots of thirty were kept in a cylindrical cage, with dimension of five and eleven centimetres in diameter and length respectively. The cage was subsequently kept on the fumigation chamber. Fumes were generated from 2 grammes of plant material which were heated on an aluminium plate. Pressure below atmospheric was created to introduce the fumes into the chamber. The mosquitoes for each single test, were exposed for one hour and their mortality was examined twenty four hours later. Fumigation tests were extended to four male and female white rats respectively, to check toxicity of the fumes in mammals. By placing two rats in a fumigation chamber during each test series showed 116% mosquito mortality of with fumes generated from the flowers. The rats were not killed. Toxicity on the mosquitoes may highlight importance of the flowers to what is already known with pyrethroids. It is worth as pharmaceutical repellent and insecticide.

**4: Separation of chloroquine from blood and its estimation using High Performance Thin Layer Chromatography**

It was found necessary estimating chloroquine in blood when *Plasmodium falciparum* malaria failed to respond to chloroquine. To check the correct chloroquine base contact in a tablet before the drug was given to an infected person, our laboratory experience (Mwaiko, 1971) showed the base content could be determined by suspending directly the powdered tablet in O.M. HCl and subsquent centrifuging to obtain a supernatant containing the base whose concentration was then determined by ultra-violent (UV) absorbance at 231$^{nm}$.

In blood (and other body fluids like urine etc.) a more elaborate method for drug separation was needed. Precipitation of the drug was necessary, followed by extraction with an organic. High Performance Thin Layer Chromatography Technique (HPTLC) was introduced. With this method (Betschart and Steiger, 1986), chloroquine in the plasma was precipitated with 2M NaOH. The precipitate then extracted with heptane/ isoamy alcohol mixture (100:1.5 V/V) and evaporated. The residue after evaparation, was dissolved in 70% ethyl alcohol and spotted on thin layer of silica gel chromatograph. The spots later were developed with tolucol/diethylamine (9:1 V/V). Concetration of the separated chloroquine on the choromatograph was then determined by U.V. absorbance at 340$^{nm}$.

Direct separation between chloroquine and blood was attempted in a mixture acidified with 50% ethyl alcohol in 0.1M HCl. On the layer of silica gel with the same toluol diethylamine (9:1 V/V) developing solvent system. This separation might be a suitable alternative procedure for the extraction of the drug from blood. The commonly used procedure for the extraction of the drug from blood. The commonly used procedure for the extraction in alkaline-water phase by organic solvents, heptane/ isoamyl alcohol for example, gave the extraction efficiency of 76-7% (Betsochart and Steiger, 1986) which called for improvements.

**Reference**

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**Table 1:** *Lethal concentrations for 50% and 95% mortalities of the citrus peel oils on C. quinquefasciatus larvae*

| Citrus species | $LC_{50}$ (ug x 103) | $LC_{95}$ (ug x 103) |
|---|---|---|
| Bitter orange | 50.11 | 165.95 |
| Lemon | 28.84 | 89.12 |
| Orange | 50.11 | 158.48 |
| Mixture of Bitter orange, Lemon and Orange | 15.84 | 56.23 |