SCREENING DRUGS FROM

K. FOETIDISSIMA
(Jacq.) Cogn.

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M. Sc. Thesis in Medical Pharmacology,
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Thesis Outline

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Screening for New Drugs from Medicinal Plants: (I) *Kedrostis foetidissima* (Jacq.) Cogn., Cucurbitaceae

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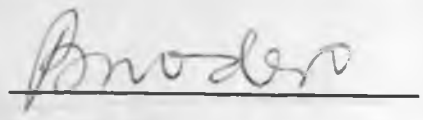
A thesis submitted in fulfilment for the Master of Science Degree, in Medical Pharmacology, in the University of Nairobi.

1984

DECLARATION

This thesis is my original work and it has not been presented for a degree in any other University.

Signature: 

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DECLARATION

This thesis has been submitted for examination with my approval as University Supervisor.

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List of Abbreviation

1.	anhyd.	anhydrous
2.	aq.	aqueous
3.	ave.	average
4.	^{o}C	degree cent-igrade
5.	CC	Col-umn Chromatography
6.	Cm	centimetre
7.	compd	compound
8.	calcd	calculated
9.	cf	confer (compare)
10.	CPE	Cytopathogenic Effect
11.	conc	concentration
	DEET	Diethyltoluamide
12.	dil.	dilute
13.	dist.	distilled
14.	ed.	edition
15.	Ed.	Editor
16.	Ed 50	Effective dose-fifty
17.	EtoAc	Ethylacetate
18.	et al;	et alii (and others)
19.	etc.	et cetera (and so on)
20.	e.g.	exempli gratia (for example)
21.	expt	experiment
22.	exptl	experimental

GIT Gastrointestinal Tract

23. hr(s) hour(s)

24. hplc high performance liquid chromatography

25. ibid. ibidem

26. i.e. id est (that is)

27. ID 50 Infective dose-fifty

28. IP Intraperitoneal injection

29. IM Intramuscular injection

30. IR Infra-red radiation

31. IV Intravenous injection

32. KF Kedrostis foetidissima

33. kg Kilogramme

KNH - Kenyatta National Hospital

34. KOL Kenya Orchads Ltd., Machakos

35. LD50 Lethal dose-fifty (median lethal dose)

36. Max. Maximum

37. MAR Minimum Allowable Range

37. MBC Minimum Bactericide Concentration

38. MED Minimum Effective Dose (PC90, LD90, DD90, RD 90 etc.).

39. Min. Minimum, Minute

40. m metre

41. MIC Minimum Inhibitory Concentration

42. mg milligramme

43. mm millimetre

44. ms Mass Spectrometer

45.	MTD	Maximum tolerated dose (see LD10)
46.	nmr	nuclear magnetic resonance
47.	NNN	Novy McNeal and Nicolla Medium
48.	pH	Hydrogen ion concentration
49.	ppm	parts per million (µg/ml, g/L, mg/kg)
50.	prepn	preparation
51.	Ps.	Pseudomonas
52A.	R.H.	Relative humidity
52B.	RF (No 78)	Resolution Factor
53.	RPMI 1640	Rosewell Park Memorial Institute 1640
54.	S.	Second
55.	SC	Standard Control
56.	So.	Solvent
57.	sol.	soluble, solute
58.	soln	solution
59.	SSPE	Subacute Sclerosing Panencephalitis
60.	Std	Standard
61.	Staph.	Staphylococcus
62.	TCID50	Tissue Cell Infective Dose-fifty
63.	Temp.	Temperature
64.	TI	Therapeutic index
65.	TLC	Thin layer chromatography
66.	µg	microgramme (mcg)

67.	µl	microlitre (mcl)
68.	µm	micrometre
69.	µv	ultra violet
70.	VRC	Virus Research Centre
71.	USA	United States of America
72.	Vacuo	Vacuum
73.	vs.	versus
74.	vol.	volume,
75.	wt.	weight
76.	%	percent (per hundred)
77.	ø	diameter
78.	Rf	Resolution factor
79.	ρ	Density

A B S T R A C T

Abstract

A substance with a broadspectrum antimicrobial activity has been isolated from the African medicinal herb, Kedrostis foetidissima (Jacq.) Cogn., Cucurbitaceae. The leafy parts of K. foetidissima have been used traditionally, in Kenya to treat children infectious diseases caused by viruses (measles, chicken-pox, cowpox etc.). However the intended and claimed efficacy on the diseases; and the safety to the patients treated with the herb, were never demonstrated by scientific experiments. Here experiments, in vitro with agar diffusion, serial dilutions and plate spray, against pathogenic microbial agents; and other techniques, (dipping, leaf discs and climbing tests), against parasitic arthropods, were conducted with Kedrostis extracts. to investigate efficacy of the herb. The Kedrostis oil (Fig. 1) (organosoluble portion) was found to be active on microbial, bacteria (14), virus (1), fungi (2) and Leishmania spp (2) and on insects (4) and ticks. It's toxicity was hence tested in vivo by intraperitoneal administration to mice, and found to be low (e.g., LD 50, of $\geq$ 1600 mg/kg and MTD or LD10 of $\geq$ 600 mg/kg) as shown in Fig 19.

Further fractionation of the oil by solvent extraction and chromatographic seperations (TLC, CC, HPLC), yielded an almost pure active component (141.1mg; 0.00023%) with constant R_f (0.695) in silicagel TLC and petroleum ether:chloroform (1:1) solvent system antimicrobial activity, characteristic HPLC peaks (Fig 15) and uv fluorescence (Fig 14). The antibiotic activities against different organisms and pests were also compared, in vitro, with standard antibiotics and pesticides currently used, such as penicillin, streptomycin, rifampicin, cotrimoxazole, erythromycin, minocycline, imidazole (clotrimazole), sodium stibogluconate, diethyltoluamide, and found to be almost equal in activities, as shown in the text.

The results of the experiments are illustrated in Tables 3,5,9,10,11,12,13 and in Figure 14. The findings of the studies indicated that the crude Kedrostis oil had significant antimicrobial activities as shown by its minimum inhibitory concentrations (MIC) against bacteria, ($\leq$ 50 ppm), measle virus (1 ppm), fungi, (0.5 ppm) Leishmania donovani ($\leq$ 100 ppm), and moderately high activities against insects and ticks. The minimum effective concentrations (LD 90's) against the arthropods were: larvicidal on Aedes aegypti (15.00%) and Maruca testulalis

(50.00%); feeding deterrent on Spodoptera exempta, (0.05%) and repellent on Rhipicephalus appendiculatus ticks, (18%). The foregoing observations, suggested that:

(a) the plant K. foetidissima (Jacq.) Cogn., would be important medically and agriculturally, because it contains active components, that might be used to control microbial pathogens and parasitic arthropods of public health and agricultural importance;

(b) the crude oil was moderately safe to mice, (LD 50; 1602.40±20.0 mg/kg, compared with pyrethrins LD 50; 1000 mg/kg) and hopefully to man;

(c) the oil exhibited broad spectrum of antimicrobial activity against several species of gram-positive and gram-negative bacteria, fungi, viruses, protozoa, and showed activity on insects and ticks (as a larvicide, an antifeedant and a repellent);

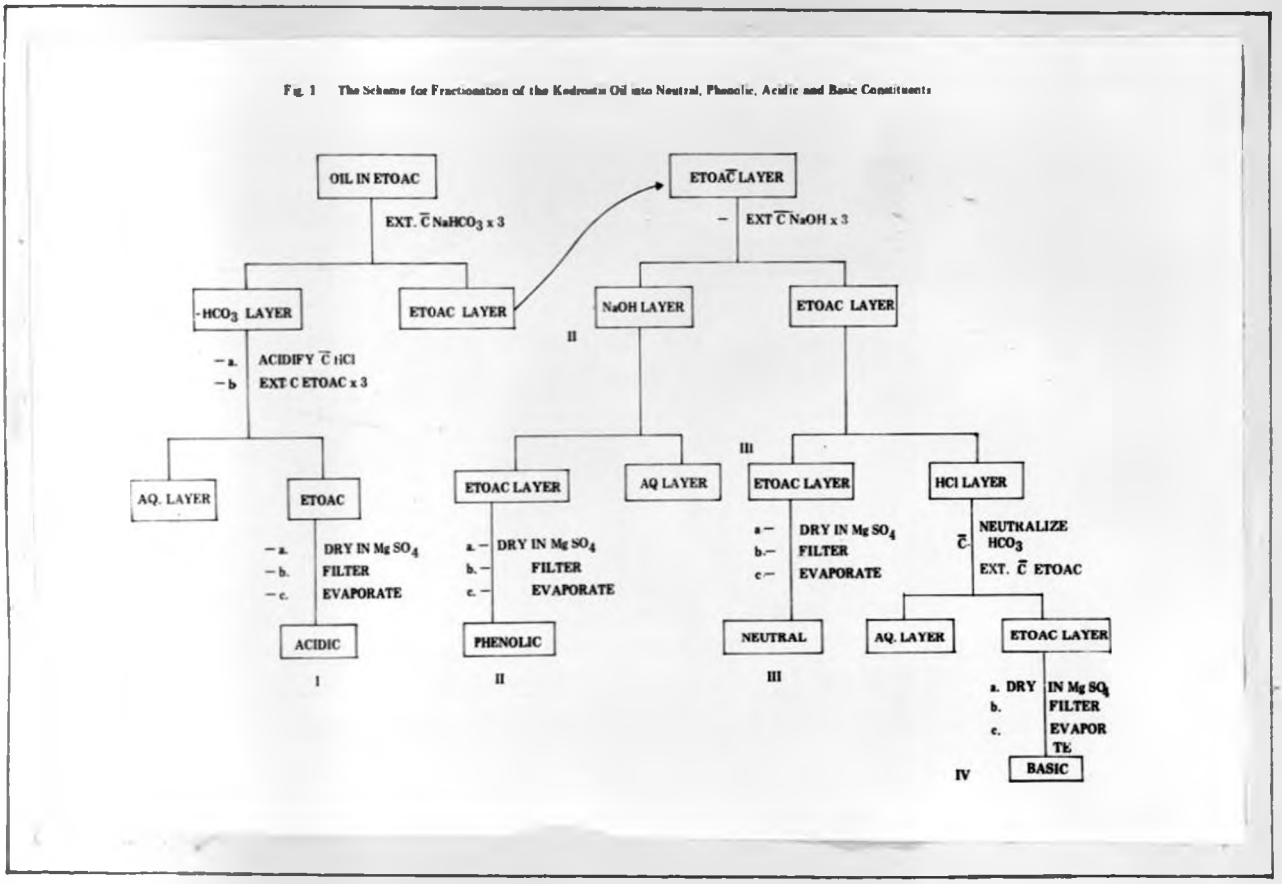
(d) it showed bactericidal mode of action.

Although the antibacterial effects were almost equal to activities of other antibiotics (Table 4 & 5), the insecticidal effects were inferior to the activities of some products from several African plants. These included products from Clausena anisata, Warburgia spp., Ajuga remota, Harrisonia spp., etc. But the pesticidal activities were about equal to the levels of pyrethrum and DEET, sold commercially and used for pest controls.

Owing to the significant positive results obtained from in vitro studies with KF oil, further in vivo investigations are urgently indicated, to establish chemotherapeutic efficacy, pharmacological properties, chronic toxicological effects in animals and the chemistry. These could increase the confidence and so help in the decision whether or not to conduct clinical trials with the new active agent from Kedrostis foetidissima.

Figure 1

Fractionation of KF Oil



Note 1. KF oil was partitioned between 25 ml of 10% solutions of: $NaHCO_3$, HCl or NaOH, and ethylacetate (EtOAC), and fractions dried in anhyd. $MgSO_4$ and evaporated in vacuo at 40^oC

Chapter I

I N T R O D U C T I O N

I N T R O D U C T I O N

Historical Note

Modern drugs are made of products from plants, animals, minerals and synthetic materials. Apart from the synthetics, this has been true from time immemorial, Goth (1966). Not only did ancient man learn by trial and error, to select from among the abundant flora, the plants that were useful as food and medicines and left out the poisonous ones, but he also took advantage of some toxic plants, to trap mammals, birds and fish for his food. This conditions prevailed also in Africa, where many plants are still used today as medicines and for rituals, Trease and Evans (1966); 1978); Kokwaro (1972; 1978); Watt and Breyer - Brandwijk (1962). In Africa however, very little if any, scientific investigations have been conducted with the indegenous plants, to discover and develop new drugs; or to verify the claims by traditional practitioners about the therapeutic merits in their herbal plants. Such studies would additionally reveal, the plants with deleterious effects, i.e., those that are teratogenic, carcinogenic, mutagenic, poisonous etc.

Taxonomy:

In the present project plant extracts were screened for biological activities against pathogenic microorganisms and parasitic arthropods, to discover better drugs or pesticides, than the currently used chemicals. The investigations started with *Kedrostis foetidissima* (Jacq) Cogn., in the family cucurbitaceae, with 110 genera and 640 species, that are abundant in the tropics. Most of the plants in the family

are woody or herbaceous and have climbing stems which bear tendrils and often grow from tuberous root-stock, Trease and Evans (1978). Jeffery (1967), described 23 species in the genus Kedrostis, from the flora of East Africa. All the species in the genus are small to medium sized, trailing herbs with simple leaves and tendrils; the flowers are usually greenish yellow, or greenish-white and the fruits, solitary, paired and contain small timid seeds. The most common Kedrostis species in the East African regions are: K. hirtella (Naud.) Cogn., K. leloja (J.F.Gmel.), C. Jeffrey., K. pseudogijef (Gilg.) C. Jeffrey; K. gijef (J.F. Gemel.), C. Jeffrey. Type; Arabia, Yemen; K. heterophylla, A Zimm, K. midbraedii (Cogn.), C. Jeffrey and K. foetidissima (Jacq.) Cogn. Most of these have been collected in Kenya, Uganda, Tanzania, Ethiopia and Arabian region and from Southern Africa region. The species Kedrostis foetidissima (Jacq.) Cogn., was first reported in 1791 in Medik. Phil. Bot. 2:69. DC. (1881) reported it as the plant collected in West Africa and cultivated in Vienna.

Synonyms:- Between 1789 and 1962, it was described under several synonyms as: Trichosanthes foetidissima (Jacq.), Bryonica rostrata, Rottl., B. perrottetiana, ser., DC.,

Rhynchocarpa foetida (Schrad) linn., Cyrtonema divergens, A. Rich; Zehneria obtusiloba, sonds, Kedrostis foetidissima (Jacq.) Cogn. var. perrottetiana (ser.) Cogn., K. foetidissima (Jacq.) Cogn. var microcarpa, Cogn., K. rostrata (Rottl.) Cogn; K. obtusiloba (sond.) Cogn., K. foetidissima (Jacq.) Cogn. var. glandulifera; Melothria foetidissima (Jacq.) Roberty and K. foetidissima (Jacq.) Cogn. subsp. obtusiloba (sond.). It was finally agreed that the species be named, Kedrostis foetidissima (Jacq.) Cogn., Jeffrey (1967).

Description

Kedrostis foetidissima, is a perennial herb growing rapidly during rainy seasons and disappears in drought. It has been described by Kokwaro (1976) and Jeffrey (1967), as: glabrous to densely glandular hairy and climbs by simple tendrils to a height about 2 metres; the whole plant when disturbed produces strong repelling odour, resembling putrified animal tissue or human stool. The stems get thickened at the base when older and originate from tuberous rootstocks. The leaves are orbicular, cordate and simple; the male flowers bear yellow petals; the female ones stand on solitary long stalks and bear broad petals; the fruits are usually beaked, hairy, scarlet and fleshy; with cherry black colour when ripe and contain small smooth

seeds with narrow gelatinous marginal wings of 4 - 6 x 2 - 4 x 1.5 - 2 cm. Figures 3 - 11 show varieties of Kedrostis foetidissima species collected from different regions of Eastern, Central and Southern Africa.

Distribution

The K. foetidissima is widely distributed, and has been collected all over tropical Africa, Sou-thern Africa and tropical Asia, from India to Burma, (see figure 3-11). It is usually found in the rain forest margins, in bushy river banks, along undisturbed fences, in woodlands of semi-evergreen and deciduous bushlands and in wooded grasslands at the heights of 0 - 1830 metres above the sea level.

Figure 2

The East African Herbarium, Nairobi, Viewing of the Preserved Plants Specimens

Figure 3

Kedrostis foetidissima (Jacq.) Cogn., Collected in Gibraltar Mediterranean Region, Preserved in Natal Herbarium, South Africa and East African Herbarium, Nairobi, 24th October 1970.

Figure 4

Kedrostis foetidissima (Jacq.) Cogn., Burundi Variety, Collected on 10 May 1971.

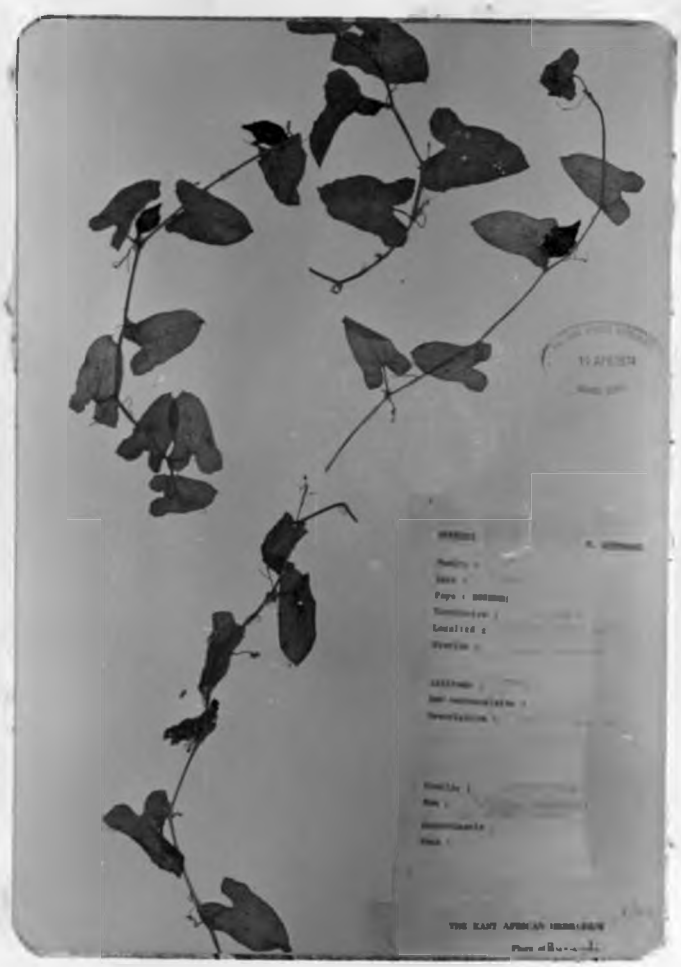


Figure 5

Kedrostis foetidissima, Zaire, Collection.

15 January 1974

Figure 6

Kedrostis foetidissima (Jacq.) Cogn.,
Collected from Tana River, Kenya.

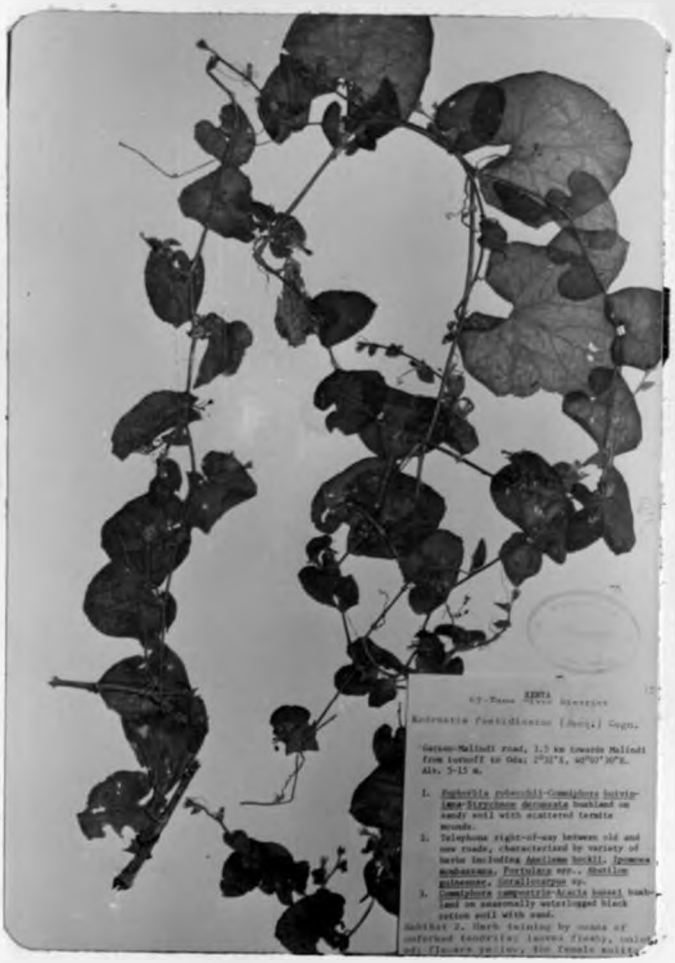


Figure 7

Tanzanian Kedrostis foetidissima. Collected 9 January 1971.

Figure 8

The K. foetidissima (Jacq.) Cogn.; from Chiromo River, Nairobi, Picture Taken 15 February 1984.

Figure 9

Another Chiromo Variety of K. foetidissima (Jacq.) Cogn., Collected, 21 January 1965.

Figure 10

Specimens of K. foetidissima (Jacq.) Cogn., from Olelkisalic National Park, Kajiado District. Collected, 8 January 1964.

Figure 11

Kedrostis foetidissima (Jacq.) Cogn., Variation microcarpa Cogn., Collected in Uganda, 5 January 1951.

Traditional medicinal Uses

Many species in the family cucurbitaceae have been reported to possess poisons (e.g., Colocynthis citrullus. O. Kitz); insecticides (e.g., Colocynthis vulgaris. Schrad); and medicines for treating various infections and infestations. These included the use of Melothria punctata. Cogn., as an emetic and a purgative by the Nyanja of Malawi, Colocynthis citrullus as a carthatic, and an anthelmintic for treating tapeworms and roundworms diseases in cats in South Africa and in St. Thomas and Prince Islands. Other species were used as enemas, to relieve lumbago, for treating horse sickness, as piscides, pot herbs, for cough relief, as arbotifacients; for charms, as dog medicines, and as antitumour remedies, etc., in various parts of the world, including Australia, Tanzania, India, Angola, South Africa, North Africa, Congo, Brazil, Philippines, Pueto-Rico, Mauritius, Surinam, etc., Watt and Breyer - Brandwijk (1967).

Within the genus Kedrostis, K. africana, Cogn., has been used as purgative and an emetic, when infused in wine or brandy, in South Africa; K. nana produced strong odour of carbon disulphide, which caused irritation in sheep and rabbits; its roots were also used as a purgative and an emetic; and that K. natalensis, A. Meeuse, caused a fatal

poisoning in the Transkei, Lugard (1909). Kedrostis foetidissima, the subject of the present screen, has been used traditionally in Kenya, for treating diseases caused by viruses (e.g., measles, chicken pox, septic wounds, etc.), Kokwaro (1976).

Previous Scientific Studies

Exhaustive search through the chemical and biological abstracts locally in East Africa and by computer screen in North America, with the help of Professor John Law (1981), revealed that no previous scientific studies had been conducted on K. foetidissima (Jacq.) Cogn. However Watt et al., (1967), mentioned the presence of cucurbitacin - like compound with $C_{32}H_{44}O_8$ from the roots of a strain of K. foetidissima. The source of this information was not given.

Present work

Because of its widespread use as medicine in Kenya, Kedrostis foetidissima (Jacq.) Cogn., was used in preliminary experiments in vitro and in vivo, against species of microbial agents (e.g., bacteria, fungi, viruses, protozoa and helminthes) and some parasitic arthropod

vectors and pests. The immediate aim was to test for the effectiveness and the safety of the crude medicine from K. foetidissima.

Objectives

The major objectives of this screening project were:

(a) to verify (confirm or disprove), the claims of the presence of any bioactivity in the KF extracts by testing in vitro against microbial pathogens and parasitic arthropod pests;

(b) to determine the levels and significance of the activity if found in "a" above, by dose - response and dose-effect relationship studies, on various organisms;
(c) to study the spectrum of the activity(s) on several species of parasites; (d) to study the nature of the activity, whether growth inhibition or lethal to the organisms by the measurements of the minimum inhibitory concentrations (MIC) and the minimum lethal concentrations (MLC) of the extract (e) to search for model molecule which could lead chemists to synthetic work; and (g) to determine the margin of safety by comparing the effective doses against various parasitic agents with the toxic doses of the extract to mammals. Thus the safety margin was estimated by the therapeutic indices and by the comparisons of the

test substance with some known standard drugs or pesticides in current use.

Approach:

To achieve the objectives, tests were conducted *in vitro* with extracts of *K. foetidissima* against: bacteria by the methods of Pagano (1964) & Bondi (1964); viruses by the tissue culture test of Parker (1961) and Grumberg (1979); fungi by the TLC spray test of Bowers (1980); protozoa (*Leishmania donovani*) by the serial dilution technique of Collier and Lourie (1946), and helminthes (hookworm) by the filter paper dipping test of Standem (1964) and Brown et al., (1961). Due to very significant effects shown in the antimicrobial screen, the preliminary studies with KF oil were extended to the search for pesticidal activities.

Here the Kedrostis oil was also screened for pesticidal activities against; mosquitoes (*Aedes aegypti*) the vector of yellow fever and filariasis; the brown ear tick (*Rhipicephalus appendiculatus*), the vector of east coast fever (ECF), a serious cattle disease in East Africa; the cowpea podborer (*Maruca testulalis*) another serious pest of cowpea in Kenya, Okeyo - Owuor et al., (1981)

and the African armyworm (Spodoptera exempta), a very serious pest of grassland pastures and cereal crops, Odiyo (1981); Brown (1980); and Yarrow (1981). Various bioassay methods were used to tests pesticidal activities of the extracts against these pests. Thus the "dipping bioassay" was used to test the larvicidal effects on A. aegypti and M. testulalis; the "leaf disk test" for the antifeedant effects on S. exempta, and the climbing test for testing the tick repellent activity.

All the tests aimed at estimating the minimum effective dose (MED) of the active extract, that would control 90 per cent of the pest population or protect 90 percent of the victims (animal or plants) from attack by the vector or pests.

The MEDs or the lethal dose-ninety (LD90), the feeding deterrent dose-ninety (DD90) or the repellent dose - ninety (RD 90), were calculated in each case from a linear curve produced by plotting the probability units (Probits) of the percentage responses against the logarithms of the corresponding doses. The MEDs of the KF oil were compared with those of diethyltoluamide (DEET., an antifeedant and a repellent), and pyrethrum mixture (a larvicide and a repellent).

The toxicity of the active extract (KF oil) was finally investigated in mice, to determine the acute median lethal dose (LD 50) and the maximum tolerated dose (MTD or LD10). The KF oil was thus given to mice intraperitoneally in corn oil (elianto) and mortality rates noted.

Rationale of the Study

The rationale of the screening programme was to use, simple, rapid, and less expensive battery of tests, employing three dose levels of each test substance (extract) per species of the specimen organism; and to establish the dose - response (or dose - effect) profile, the toxic effects, and the pattern specificity of the test substance. Thus the screen progressed from general observation in the field to the specific objective testing and recording in the laboratory; and to the use of sophisticated instruments after the active fractions had been detected and partially purified. For the active portions, the quantitative pharmacological measurements (LD 90, LD 50 & LD 10) were also calculated from the probit vs. log - dose linear curves from the relevant tests.

Experimental Controls.

Any possible biases and individual observer subjectivities were checked by the incorporation into each experiments, both positive and negative controls. In addition, each dose-level of each test substance was replicated at least three times and the number of samples (insects and mammals used per test), were always equal to or more than ten. Occas ionally, double-blind studies were introduced to check the precision and the accuracy of the results and to exclude unnecessary variables.

During the preliminary screening bioassay, both quantal and graded responses were recorded, although only the quantal (or quantitative) observations were used to calculate the percentage responses. The MIC and the MLC (MBC for bacteria) were determined from the serial dilution assays on microbial pathogens. All the values, (MED, LD50, LD10, MLC and TI), were useful because they revealed the significance and the nature of the effects and so possibly the modes of action of the active compounds in the crude extracts, in different organisms. The organosoluble oil of Kedrostis exhibited high antimicrobial effects against bacteria, viruses, fungi, and leishmania. The details of methods and results are reported here.

CHAPTER II

PHARMACOLOGICAL STUDIES

(Material & Methods)

MATERIAL AND METHODS

A. Sources of Material

1. Chemicals
2. Reagents and Media
3. Plant Material
4. Microbial Agents
5. Arthropods Pests
6. Mammals

B. Methods

Antimicrobial Experiments, *in Vitro*

(1,2,3,4, & 5)

Pesticidal Experiments *in Vitro*

(6,7,8, & 9)

C. Toxicity Experiment *in Vivo*, (10)

A. Sources of Material

1. Chemicals

All the chemicals used were pure and analytical grades. Those obtained from the British Drug Houses (BDH) included: sodium hydroxide (NAOH), sodium sulphate anhydrous ($NaSO_4$), sodium biocarbonate($NaHCO_3$), hydrochloric acid (HCL), and sulphuric acid (H_2SO_4). The chemicals from E. Merck, Darmstadt, Germany, were: methanol, ethanol, ethylacetate (EtOAC), acetone, petroleum ether (40 - 60) 60 - 80, B.P.), and hexane.

2. Reagents and Media

Many reagents were used for cultivating microorganisms; these included: Novy McNeal and Nicolls (NNN), Rosewell Park Memorial Institute (RPMI) 1640, Eagles Minimum Essential Medium (MEM) 199, Potato Dextrose Agar (PDA), Tryptone Soy Agar (TSA), Tryptone Soy Broth (TSB) and Peptone Water (PW). The NNN was prepared from peptone, beef extract, agar and sodium chloride, all bought from Oxoid in United Kingdom (UK). The RPMI 1640 and MEM 199 were specially formulated by Flow Laboratories, U.S.A. They were supplemented by Hepes (20 mM). Sodium biocarbonate (10 mM), glutamine (20 mM), Sodium chloride (0.85%), and bovine foetal calf serum (10-20%); Antibiotics: penicillin (5 U), Streptomycin (5 µg) and nystatin (5 µg for viral culture) or 5-fluorocytosine (5 µg per ml for leishmanial culture), were added to control bacterial and fungal contaminations.

The PDA, TSA and TSB, were also prepared with ingredients obtained from Oxoid. Vero tissue cultures were prepared in the Virus Research Centre, (VRC) Nairobi. Several drugs used for comparative studies against microbial pathogen included; antibacterial; penicillin streptomycin, rifampicin, cotrimoxazole erythromycin and minocycline; fungicide, bis-phenyl - (2 - chlorophenyl) - 1 imidazole (clotrimazole); antileishmania, sodium stibogluconate (pentosam); and pesticides ; pyrethrum mixture and diethyltoluamide. They were used as reference standards. All these drugs were bought from chemists shops in Nairobi. Corn oil (Elianto) served as a solvent and a negative control during toxicity studies on mice, whereas the tomato juice (KOL) was used to suspend the fungal spores for the spray test.

3. Plant Material

The aerial parts of K. foetidissima used in this investigation were collected in the surroundings of Nairobi and in Western Kenya. The samples were botanically identified in the East African Herbarium, in Nairobi, where samples were deposited. Figures 3 - 11 show various vareities of K. foetidissima (Jacq.) Cogn, collected from different regions of Africa. The preserved specimens were supplied by E.A. Herberium.

For extraction, sample (61.876 kg) of the plant material was mecerated with analytical grade, redistilled methanol (70 litres), using Atomix or Waring blender, and the mixture kept for seven days at room temperature, in 15 litre glass-stoppered jars.

Each jar was agitated once a day during the extraction period. The mixture was then filtered through buchner funnel using whatman filter paper No. 40 and an aspirator a - 2 (Tokyo Rihakikai Co. Ltd.). The combined filtrate was concentrated in vacuo at 40°C, to leave only aqueous suspensions which was exhaustively partitioned with ethylacetate (EtOAC). The EtOAC portion was reduced in vacuo to two litres, decolourized with activated charcoal for 30 minutes, filtered and then dried with anhydrous sodium sulphate to remove traces of water. The filtrate was again concentrated in vacuo at 40°C to yield a golden oil (31.47 gm; 0.051%). Exploratory tests in the early stages of the study had indicated that freeze-dried aqueous portion had no bioactivity on microbial agents (bacterial and fungi). Hence the subsequent fractions were discarded while the organosoluble oil was screened for active compounds.

The active oil was fractionated further into acidic, phenolic, neutral and basic components whose percentage yields are shown in Table 1b After bioassaying all the portions, the active (neutral) fraction, was separated in silicagel CC, into among others, an active component (141.1 mg;0.00023% which is expected to be structurally determined.

Table 1 a

Percentage Yields from KF Oil by Solvent Extraction

Subject	Wt(g)	Yield (%)
Raw Plant Sections	61500.00	100.00
KF Oil	31.1000	0.051
Neutral Portion	19.5900	0.0321
Active Compound	0.1410	0.00023
	(141.10 mg)	

Table 1 b

The Percentage Yield from
Solvent Extraction of KF Oil

Fractions	Percent Yield
Oil	100.00
Neutral	67.50 ± 3.6
Phenolic	11.03 ± 2.2
Acidic	8.58 ± 0.80
Basic	2.17 ± 1.30
Active compound	0.71 ± 0.05

NOTE: The percentage yield was calculated from means of three separations of each portion. The oil was 0.051% of the crude plant extract, and the active components was 0.71% of the neutral fraction, but 0.00023% of the overal plant parts extracted. Active compound was monitored by the antibacterial agar diffusion and the fungicidal TLC plate spray.

4. Microbial Agents

(a) The standard bacterial stocks obtained from KNH were Staphylococcus aureus, Streptococcus pneumoniae S. pyogenes, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, and Klebsiella pneumoniae. Those from New Nyanza General Hospital were: Streptococcus fecalis, Shigella flexneri, Salmonella typhosa, and Vibrio comma (inaba strain) The Aerobacter aerogenes strain was typed in ICIPE Microbiology Laboratory. All were grown in TSB for 18 - 24 hours and stored at 4°C. Other strains were obtained from the M.P. Shah Hospital.

(b) Rubeola (Measles) Virus: The measles virus was obtained from the Virus Research Centre (VRC), where it had been stored at - 70°C in MEM-199. It was originally isolated from patients' urine sediments and propagated in Vero cells suspended in Eagles growth MEM. These Virus-infected cells were used for chemotherapy tests against measles.

(c) Fungi: A plant pathogen, Cladosporium cucumerinum and the human pathogen, Trichophyton mentagrophytes, were tested for fungicidal effect of the KF oil. The former was obtained from Professor W.S. Bowers of Cornell University, New York, and the later was from the KNH Mycology Laboratory. Both species were propagated and maintained in PDA, enriched with

20% tomato juice from KOL. For the bioassay 20 days old fungal spores were suspended in tomato juice filtrate.

(d) Leishmania donovani: The L. donovani isolates for the chemotherapeutic test, was acquired from ICIPE cryobank. It originated from a patient in Makueni Hospital, Machakos District, during kala-azar epedemic in 1979. The promastigotes were cultivated in NNN medium, then preserved at - 196°C under liquid nitrogen. Before chemotherapeutic assay, the flagellates were sub-cultured in NNN then in RPMI 1640 media.

(e) Hookworm: The ova and cysts of Ancylostoma duodenale, isolated from infested stools in the KNH routine laboratories, were incubated at 37°C, according to Brown et al (1961), and used in experiments in vitro, employing the "Filter Paper Dipping Test".

(f) The Vero Tissue Culture Cells: The trypsinized African green monkey kidney cells, grown in MEM-199, were incubated to form monolayers on the side of test tube before being infected with measles virus. The cells were prepared at VRC, Nairobi.

5. Arthropods:

(a) Mosquitoes. The Aedes aegypti (Lin), the yellow fever mosquitoes, which also transmit filaria worms, were obtained from ICIPE insectary. They had earlier (1979), been supplied by the National Public Health Laboratory Services (NPHLS). There, the stocks were kept since 1940s during the anti-yellow fever campaigns. The mosquito larvae were reared on wheatgran and ground bovine liver. The adult females were maintained on rabbit blood, while the males were fed on 2% sucrose solution. The third instar larvae were used for larvicidal screening tests.

(b) The Podborers: Maruca testulalis (Geyer), obtained from Mbita Point Field Station, South Nyanza District, were bred in the ICIPE Insectary. The larvae were maintained on cowpea flowers, their natural food, Okeyo - Owuor and Ochieng (1981) and Ochieng, et al., (1981). The second and third larval instars selected according to Odebiyi (1981), were used to test the larvicidal activities of the plant extracts.

(c) S. exempta: The last (6th) instar larvae of the armyworm were used for the feeding deterrent test. The old stocks originated in 1975, from the former East African Forestry Research Organisation (EAFRO) and were bred in ICIPE insectary under controlled conditions at 25.00 ± 0.5°C with 85% RH. Occasionally the colony was strengthened with field collections during invasion seasons. In the laboratory, the armyworms were maintained on maize leaves, but in the wild they thrived on the plants as shown in the introduction. For the antifeedant bioassay, many larvae were crowded in Kilner jars to produce, for bioassay, the black gregerious types, resembling wild stocks, Ma and Kubo (1977).

(d) The Brown Ear Ticks: The ticks Rhipicephalus appendiculatus (Neuman) and related species, which transmits the ECF disease in cattle, Cheng (1973), were used for acaricide activities of KF oil. The ticks were bred and reared in the ICIPE Tick Research Programme Laboratories in Muguga, Kenya, by the method of Bailey (1960), modified by Hadani (1961). The starved mature nymphs or adult ticks were used in the "Climbing bioassay" for tick repellent activities of the plant extracts.

6. Mammal:

(a) Mice: The albino mice of mixed sexes, maintained on pellets and tap water, were used for toxicity experiments in vivo. They were kept under standard conditions according to Dhew (1963) and those about 6-8 weeks old, of equal weights (18-21gm), from the same breeding batches and in good health, were used for the studies.

(b) Rabbits: The white laboratory rabbits maintained on pellets , cabbage, carrots, and tap water ad libitum, supplied the blood for feeding female mosquitoes and for the preparation of NNN medium.

METHODS

Antimicrobial Studies in Vitro (1.2.3.4. & 5).

With the exception of the agar diffusion and the TLC plate spray tests, most of the techniques used for screening tests involved serial dilutions. The agar diffusion was used to detect the bacterial susceptibilities, whereas the TLC spray test detected fungicidal activities of the extract. Thus the serial dilution was used to screen the activities of the extracts against bacteria, virus, protozoa and helminthes.

1. Antibacterial Test, in Vitro:

Past experiences have indicated close correlation between *in vitro* and *in vivo* antibiotic activities, with only a few exceptions, Hawking (1964). Here Kedrostis extracts (aqueous and organosoluble), were screened *in vitro* for antibacterial effectiveness, by the agar diffusion for sensitivity and the spectrum of activities; and by serial dilution to study the nature of the effect, (i.e., MIC and MBC).

(a) Agar Diffusion: This technique employed two modes of applications, (the direct and the sensitivity disc), against 13 different pathogenic species of bacteria (four gram-positive *cocci*, 8 gram - negative *bacilli* and one *Vibrio comma* (*cholera*).

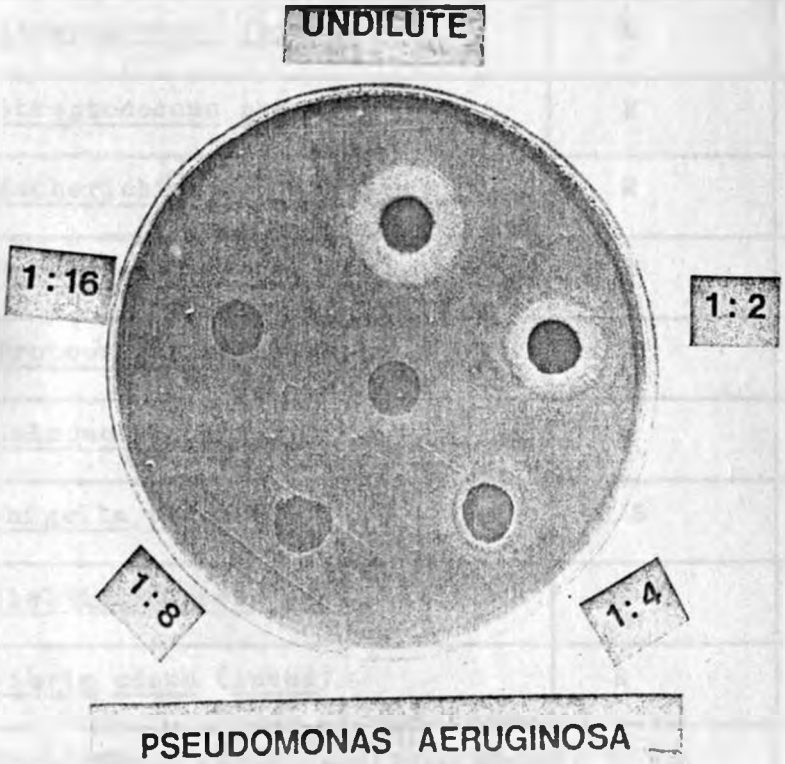
Procedure: The direct application consisted of placing the extract on designated spots on the surface of TSA plate previously seeded with standard bacterial concentrations. (3×10^8 cells/ml). The sensitivity disc method involved placing on the agar, small filter paper discs (6 mm ø), previously punched out with cork-borer, sterilized for 30 minutes at 121°C and impregnated with 5 µl of KF oil or 2mg of the freezer-dried aqueous soluble extract. The cultures were all incubated for 24 hours at 37°C. The sizes of the zone of growth inhibitions around the treated spots or the sensitivity discs, reflected the ability of the components in the extracts to diffuse through the agar and to stop bacterial growth. The observations were related to the antibacterial activity of the extract.

Antibacterial Test in Vitro:

(b) Serial Dilution Test (S D T). The SDT used tube or plate dilutions which were performed as follows: a loopful of each bacterial culture isolate was transferred into 5ml of fresh sterile tryptone soy broth and incubated for 24 hours at 37°C. Three hours before, the antibacterial test, another loopful from the overnight culture was subcultured to a fresh 5ml broth and incubated. After 2 - 3 hours the new sub-cultures were diluted with fresh broth to final bacterial concentrations of 3×10^8/ml each, by matching them with Mcfarlands barium sulphate nephlometer standard, Balley

Figure 12

Sensitivity Disc Test for Antibacterial Activity



Note 2. Various quantities of the extracts on filter paper discs (6 mm ∅), placed on TS A, previously seeded with bacterial cultures, were incubated for 24 hrs., at 37°C. The activities were assessed by the sizes of inhibition zones around the discs.

Table 2

KF Oil Antibacterial Activity - Direct Application

Organism spp.	Aq. Extract 2mg/spot	Organic solvent Extract 5 µl/spot
Staphylococcus aureus	R	H/S
Streptococcus pneumoniae	R	H/S
Streptococcus fecalis	R	H/S
Streptococcus pyogenes	R	H/S
Escherichia coli	R	H/S
Pseudomonas aeruginosa	R	H/S
Proteus vulgaris	R	H/S
Salmonella typhosa	R	H/S
Shigella flexneri	S/S	H/S
Klebsiella pneumoniae	R	H/S
Vibrio comma (inaba)	R	H/S
Pseudomonas aeruginosa (pyocyanea)	R	H/S
Aerobacter aerogenes	R	H/S
Neo-staph. sp	R	H/S

NOTE: The extracts were applied directly on the surface of TSA (blood agar), seeded with standard (3×10^8 cell/ml) bacterial culture, incubated 24 hours at 37°C. The reactions were scored as R, S/S, S & H/S for resistant, slightly sensitive, sensitive and highly sensitive.

Table 3

Antibacterial Activity of KF Oil - Sensitivity Disc.

Organism(genus & species)	Isolate from Diff. Sources	Water Extract (ZI,mm)	Oily Fraction (ZI, mm)
Staphylococcus aureus	13	R	18.65
Streptococcus pneumoniae	2	R	19.50
Streptococcus fecalis	1	R	13.00
Streptococcus pyogenes	2	R	17.00
Escherichia coli	9	R	17.75
Pseudomonas aeruginosa	10	R	20.67
Proteus vulgaris	10	RR	18.00
Salmonella typhosa	1	R	15.00
Shigella flexneri	4	S/S	15.00
Klebsiella pneumoniae	5	R	20.00
Vibrio comma (inaba)	2	R	14.00
Pseudomona (pyocyaneae aeruginosa)	1	R	20.00
Aerobacter aerogenes	1	R	16.50
Staphylococcus sp (Neo)*	1	R	18.00
Total No. Strains	62		

NOTE: Similar quantities as in table 2 of the aq. and organo-soluble extracts were applied on filter paper discs (5 mm Ø), dried and placed on TSA, seeded with std bacteria culture (3×10^8 cell/ml.). The bacterial sensitivities were measured by the diameter (Ø) of the zone of inhibition (ZI) in mm. The Neo-Staph was a recent urethral swab isolate from the hospital (NNPGH)*.

Table 4

The Antibacterial Activity of KF Oil Compared with other Antibiotics.

The Antibacterial activities were measured by the sizes of ZI in diameter (Ø).

Organism	Drugs				
	KF Oil	Rifampicin	Bactrim	Erthro-mycin	Mino-cyclin
Staph. aureus	18.65	15.00	0.00	-	-
E. coli	17.75	15.00	0.00	-	-
Ps. aeruginosa	20.67	0.00	0.00	-	-
Prot. vulgaris	18.00	20.00	25.00	-	-
Kleb. pneumoniae	20.00	15.00	0.00	-	-
Sh. flexneri - 5	15.00	10.00	spd	-	-
Neo staph. spp	18.00	18.00	18.00	18.00	20.00

Spd = Spread evenly without clear inhibition area.

ZI = Zone of Inhibition

Ø = Diameter

- = Not Tested

and Scott (1970). For chemotherapeutic assay, a volume (0.05 ml each) of the standard bacterial suspensions, was transferred to 1.95 ml of broth, containing different concentrations of the drug, (e.g., 2000, 1,500, 1,000, 500, 100, 50, 10 and 1 µg/ml), in screw-capped flasks.

Minimum inhibitory concentration (MIC):

The MIC: In the broth test, the tube with the lowest concentration showing complete macrosopic inhibition of the bacterial growth (as judged by the degree of turbidity when compared with the negative control), after 18-24 hours incubation, at 37°C, was interpreted as containing the MIC. The MIC was thus the bacteriostatic end-point. The tubes containing MIC and above were clear and those below were cloudy. Samples taken from the clear tubes were also examined microscopically to ensure there were no live bacteria.

Minimum Bactericide Concentration (MBC):

From the visually clear (non - turbid) assay tubes of the treated cultures, 50 µl volumes were removed and well mixed with sterile 25 ml of melted and cooled (45°C) tryptone soy agar (TSA). The TSA tubes with various inocula, were then poured aseptically into sterile plates which were subsequently incubated at 37°C. The plates were examined for the presence of the originally cultured bacterial colonies, after 24 and 48 hours of incubation.

The number of colonies of the bacteria in the plates were correlated to the number of viable bacterial cells in the 50 µl inocula, from the clear samples. The concentration of the drug, which reduced the number of colonies to fewer than 10 in a plate subculture, was designated as the minimum bactericide concentration (MBC), which was also the bactericidal end-point. The MIC+MBC of the KF oil were determined simulteneously, under similar experimental conditions. Other assay tubes were similarly run but with bacteria alone and equal number with the reference drug (or extract) alone in the broth tubes and agar plates. These provided comparative controls (Table 5). The gram-positive cocci were subcultured in TSA with 5 percent, fresh rabbit blood, whereas the gram-negative bacilli were tested on tryptone soy agar alone. The results of MIC & MBC are shown, whereas the comparisons of the (AD and SD) techniques were also shown in Table 6.

Table 5

Comparison of the Bacterial sensitivities by Serial Dilution Techniques.

Bacterial spp	MIC & MBC KF Oil (µg/ml= ppm)	MIC & MBC Pencillin (units/ml)	MBC & MIC Streptomycin (µg/ml=ppm)
Staph. aureus	50.00	1600.00	-
E. coli	50.00	800.00	2000.00
PS. aeruginosa	250.00	-	2000.00
Prot. vulgaris	2000.00	-	2000.00
Kleb. Pneumoniae	100-125	-	2000.00
Sh. flexneri - 5	100-125	-	1000.00

MIC = Minimum inhibitory Concentration

MBC = The Minimum Bactericide Concentration as tested by agar dilution with subculture of 50µl from clear tubes.

\- = Not Tested

Table 6

Comparison of Bacterial Susceptibilities by the Agar Diffusion (AD) and the Serial Dilution (SD).

Organism	A D		S D	
Bact. ssp	ZI Ø (mm)	Rank No.	MIC (µg/ml)	Rank No.
Staph. aureus	18.65	(3)	50.00	(1)
E. Coli	17.75	(5)	50.00	(1)
Ps. aeruginosa	20.67	(1)	100-250	(5)
Prot. vulgaris	18.00	(4)	2000.00	(6)
Kleb pneumoniae	20.00	(2)	100(100-125)	(3)
Sh. flexneri	15.00	(6)	100(100-125)	(3)

NOTE: The measurements were extracted from tables 4 & 5.

AD = Agar Diffusion

SD = Serial Dilution

2. Studies of the Antiviral Effects:

The multiplication of measles virus in animal cells usually cause abnormal changes called cytopathic effects (CPE) in the cells, Enders (1954). Thus measles infection in human body also causes eruption in the buccal mucosal cavities, whose secretions often show multinucleated giant cells. The reticular endothelial syncytial masses can be observed in the tonsils, lymph nodes, spleen, lymphatic tissues of the intestine and appendix. The masses usually contain cytoplasmic and nuclear inclusions. Such CPEs changes were used in this project to grade the degrees of *in vitro* cell damages caused by viral multiplications. When the vero cells were infected with rubeola virus in vitro, three categories of CPE were observed, which included:

(a) the changes consisting of cytoplasmic or nuclear inclusions;

(b) the formation of giant cells or coalescence of cells into syncytial masses; and

(c) the changes consisting of rounding and bizarre outline formation, cytoplasmic granulations, cellular or nuclear swelling, pyknosis and nuclear chromatin granulation. These changes invariably lead to cell deaths and to the disruption of the cell monolayers as demonstrated by clear areas (plaques). All these CPE groups were used to grade the changes caused by viral infection and also the effects of the drug to reduce CPE,

according to Standem (1963), Table 7.

The present antiviral chemotherapy incorporated a contact test and the test for inhibition of viral multiplication. By this method, measles virus particles growing in monkey kidney tissue culture cells, (vero), were incubated in the presence of known concentrations of the test substance, (extract), at 25°C for fixed periods varying from minutes to hours. After each incubation period, the level of residual (or surviving) virus was estimated by removing fixed volumes from the treated vials to fresh tissue culture tubes. The subcultures were again incubated at 37°C for 7 days, examined and compared with both negative and positive control treated under similar conditions. The titre of the virus-infected cells used for the assay was equal to or more than 10 tissue culture infective dose-fifty ($\geq$ 10 TCID 50), also expressed as the lethal dose fifty (LD 50). The tissue cultures were thus infected with constant virus inoculum (0.1ml $\geq$ 10 TCID 50), and treated with various concentrations of KF oil. The dose at which virus caused CPE was reduced by at least 50 percent in the cell culture was the MIC. But before the virus-infected cells were treated with the extracts, vero cells were tested only with the extracts to determine the cell tolerance to it.

Table 7

Grades of the Microbial Sensitivities

CPE Rank	% Production of CPE
-	0.00
+1	20.00
+2	40.00
+3	60.00
+4	80.00
+5	100.00

NOTE: The method of grading CPE was adopted from Standem (1963).

From such test, the maximum tolerated dose (MTD) of the extract to the cells was determined. The ratio of the MTD and the MIC gave the therapeutic index of the new drugs in treating measles in vitro in culture cells (i.e., MTD/MIC = TI).

Tolerance of Vero Cells to KF Oil:

Procedure Five drops of the vero cells suspensions were added to various concentrations of kedrostis oil (extract) in 1.75ml of MEM-199 medium, and incubated horizontally at 37°C for 48 hours, then examined for CPE production against blank control. The results are shown in Table 8, whereas the degree of CPEs were graded by the method of Standem (1963) as shown in Table 7. (see Table 8 under result section).

Table 8

Tolerance of Vero to KF Oil

Test No.	Conc. µg/ml	CPE %
1	0.00	0.00
2	0.125	0.00
3	1.250	0 - 20
4	12.50	$\leq$ 20.00 *
5	125.00	80.00
6	1250.00	100.00

NOTE: To 1.75ml tissue culture vero suspension in MED - 199, were added 0.25mls each, of various KF concentrations (1, 10, 100, 1000, 10000 µg/ml) in MEM. The well mixed cultures were incubated horizontally together with positive and negative control for 48 hours then examined for the extents of CPE development.

*(MTD.= LD10)

Studies of the Antiviral Effects of KF Oil:

Procedure:

Into three screw-capped vials containing 0.8 ml each of MEM-199 mediums, were added volumes (0.2 ml each) of MEM-199 containing various concentrations of KF Oil. ($\leq$ MTD). Next, 0.1 ml volumes of virus-infected vero cell suspensions (equivalent to about 10 TCID 50) were added to each vial, mixed well and incubated at 25.0 $\pm$ 0.5°C. After exactly 1 minute ($1_{/60}$) 1,6 and 24 hours, subcultures of 1 drop (25 µl) each were transferred from each vial to fresh cell suspension in tubes. The subcultured tubes were incubated horizontally at 37°C for 8 days then examined for the presence and the grades of virus-caused CPEs. Each dose-level of KF oil and control was replicated three times and the means of the percentage CPE reductions reported in Table 9. The final concentrations of the KF oil in the assay tubes were 0.20, 2.00, and 20.00 µg/ml.

These were used to challenge the virus in the cells.

Table 9

Antirubeola (Measles) Activity of KF Oil.

Test No.	Dose (µg/ml)	CPE Reduction %
1	0.0	0.00
2	0.20	40.00
3	2.00	60.00
4	20.00	100.00

NOTE: The kidney cells, (vero monolayer), cultured in MEM - 199 (Eagles) only (control), and MEM - 199 plus KF oil (20.000, 2.00 and 0.20 µg/ml), were inoculated with measle-infected vero cells (0.1 ml $\geq$ 10 TCID 50). After 24 hours, at 25.00 $\pm$ 0.5°C, volumes (25µl/each) were transferred to vero suspension in MEM - 199 (5 ml). They were then microscopically examined after seven days of horizontal incubation at 37°C. The presence and the extent of CPEs were ranked according to Table 7.

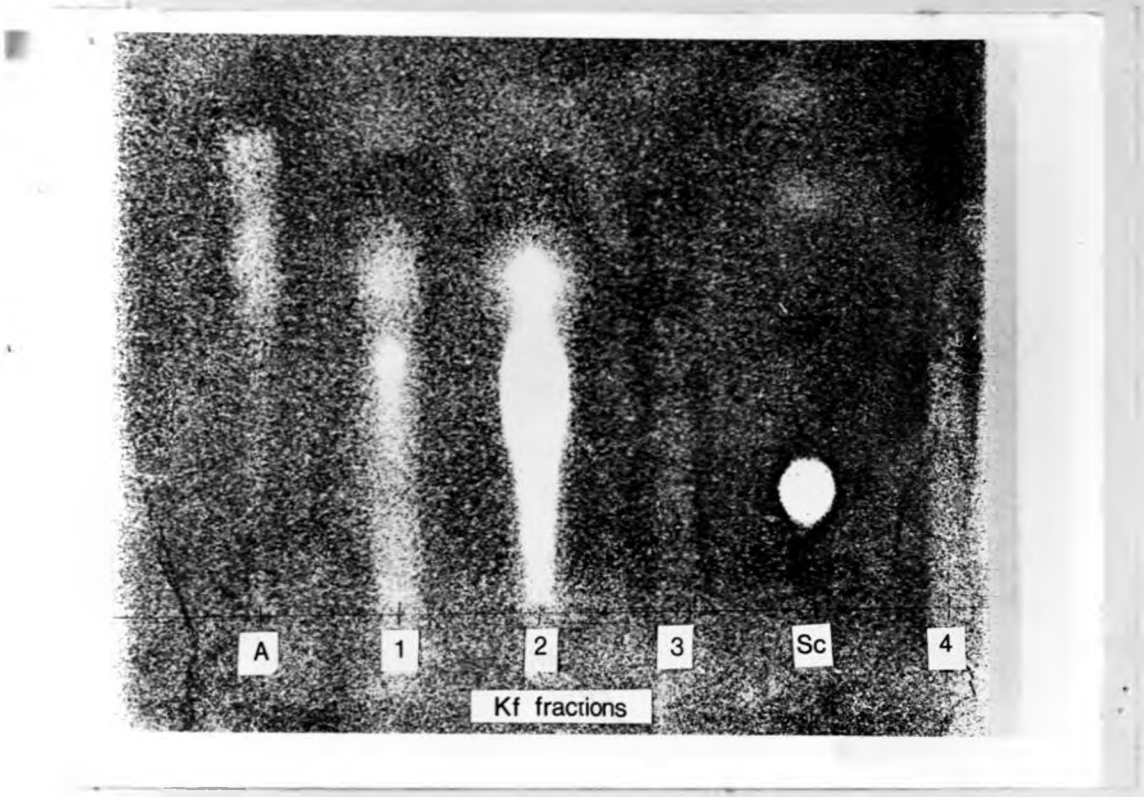
3. Antifungal Test in Vitro:

Twenty day old fungal spores on PDA plates were suspended into tomato juice filtrate and applied on extracts spotted on silicagel coated TLC plates to study the fungicidal effects. Thus tomato juice filtrates seperated by centrifugation of the juice at 1000 rpm for 10 minutes, were poured, (20ml), onto fungal cultures in plates and loosened with a rubber policeman, then strained through gauze and used for spraying. But before spraying the TLC plate, extract (2 mg aq. soluble, or 5µl/KF oil), were spotted onto the silicagel coated (0.5 mm thick) TLC plates (20 x 20 cm) and developed in (1:9) methanol: chloroform, in a tank containing filter paper wick to maintain saturation in the chamber. The development was stopped when the solvent front reached within 2 cm from the top of the plate. Along with Kedrostis extracts, a solution of imidazole-based drug, (2mg of 10% cotrimoxazole), was inoculated on the plate and developed in the same solvent system as the extract. The fungal spores were sprayed, (inside a hood), from an atomizer driven by nitrogen gas. Just sufficient spores were applied on the silicagel without making it flow. The treated plates were incubated in horizontal positions (with fungal spores upwards) in a TLC plate holder, housed in a polythene bag containing wet towels, to keep the inner chamber humid.

After 48 hours, the spores had grown across the plate which then looked dark (*C. cucumerinum*) or yellow. (*T. mentagrophytes*); except for the spots with the active component, which remained white, owing to fungal growth inhibition. The results are depicted in Figs. 13 and 14. Fraction No. 2 from KF oil and the standard control (SC) showed fungicidal activities. The active compound was also demonstrated by HPLC, No. 1 (Fig. 15).

Figure 13

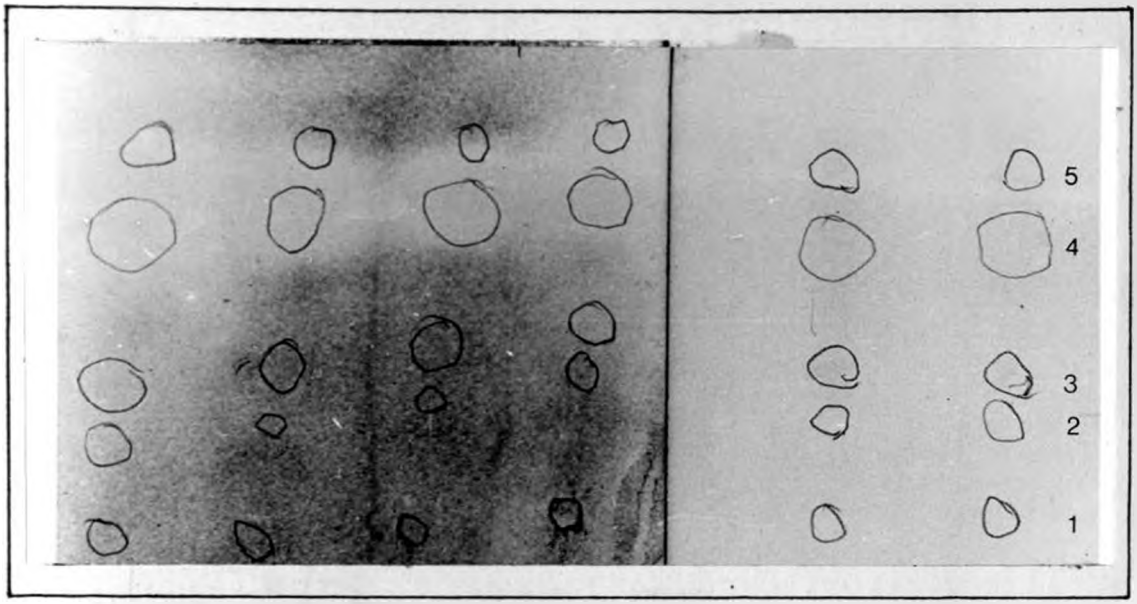
Antifungal Assays with Fracitons of KF Oil



Note 3. Fractions of the oil (A, 1, 2, 3 & 4), and a fungicide standard control (SC), spotted and developed on silicagel TLC plate (20 x 20 cm Ø), in methanol: chloroform (5:95); were dried and incubated with fungal spores at 25°C for 48 hrs., then observed for growth inhibition. Fraction no. 2, showed higher fungicidal effects than SC.

Figure 14

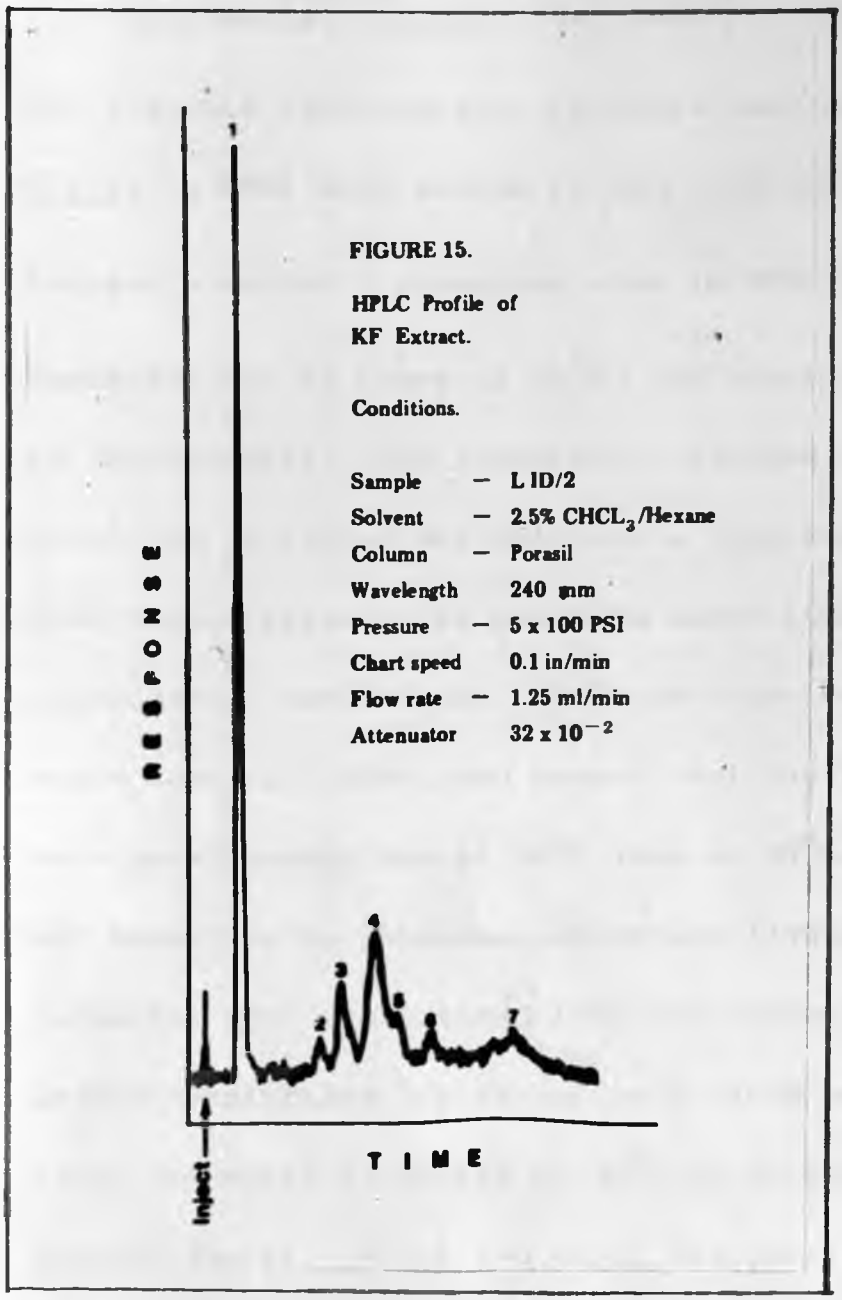
Identification of the Active Components



Note 4. Compound no. 4 isolated by TLC; detected by H_2SO_4 incineration, and uv radiation showed antifungal activity.

Figure 15

HPLC Profile of the Active Components



Note 5. Different components from fraction no. 2, were screened for antimicrobial activities.
No. 1 showed antifungal and antibacterial activities

4. Antileishmania Test; *in Vitro*

General Principle:

Leishmania donovani, the causative organism for visceral leishmaniasis in Kenya, was tested *in Vitro*, in RPMI 1640 medium (5 ml), with various doses of Kedrostis extracts dissolved also in RPMI 1640 and incubated for 48 hours at 34°C, for susceptibilities to the extracts. For comparative studies, various dilutions of sodium stibogluconate (pentosam) were also tested against the parasite under similar experimental conditions. Previous experience elsewhere, Hawking (1964), had shown that the flagellates were more susceptible at 34°C than at 25°C, and not at all sensitive to pentosam, Beveridge (1963). The parasites were subcultured into 5ml volumes RPMI 1640 medium containing 15% foetal calf serum and antibiotics, and again incubated at 25°C in screw-capped plastic flasks. After 6-8 days, the parasites in a number of flasks were pooled centrifuged at 1000rpm for 10 minutes and the pellet suspended in a little (0.5 ml) fresh RPMI 1640 medium. The number of parasites per mm^3 (1 µl) were counted in a haemocytometer.

The parasites suspension was then diluted so that each mm^3 had between 1,400-1,700 flegallates, (equal) to $1.5\text{-}2x10^6$ cells/ml). Various concentrations, (1,10,100, 200,400 and 800 µg/ml of the test material (kedrostis oil and standard drugs), were made up in 1.5 ml of medium and mixed with 0.5 ml of standard parasites suspension ($1.5x10^6$ cells/ml) in flasks. For negative control 1.5 ml RPMI was mixed with 0.5 ml of the parasites. All the cultures were incubated at 34^oC for 48 hours, then assessed under inverted microscope and finally counted in haemocytometer to determine the survival rates of the parasites.

Table 10

Antileishmanial Effect of KF Oil, in Vitro.

(The scores were based on the number of surviving parasites).

Test No.	Dose µg/ml	Survival	% Reduction
1	0.00	(+)	0.0
2	1.00	(+)	50.0
3	10.000	(+)	80.00
4	100.00	(-)	100.00
5	200.00	(-)	100.00
6	400.00	(-)	100.00
7	800.00	(-)	100.00

NOTES: Various concentrations (1,10,100,200,400 and 800 µg/ml) of KF oil in 1.5ml RPMI 1640 medium, were mixed with standard (1.5×10^6 promastigotes/ml) parasite suspension in 0.5ml of the medium. After incubation at $34^{\circ}C$ for 48 hours, the number of surviving flagellates were counted in a cell counter and used to calculate the percent cell reductions caused by the drug. The experiment was repeated three times for each concentration; so were the controls with parasites (c-) and without the parasites (c+).

5. Anthelmintic Test, *in Vitro*

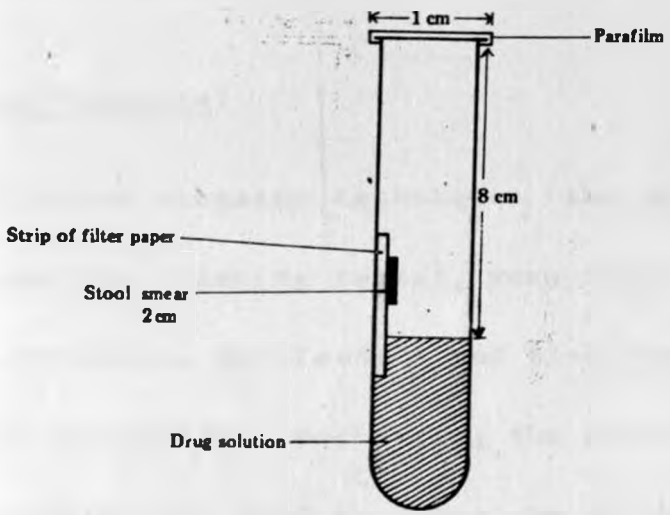
The anthelmintic effects of the kedrostis oil was tested against the old world hookworm (*Ancylostoma duodenale*) ova and cysts.

Procedure: The assay consisted of applying 5 gm of heavily infested stool in the middle of a strip of filter paper (8x1cm), and dipping the paper into 5ml KF extract suspension in normal saline (N/S) in a test tube (12x2cm), so that the stool was exactly 2 cm above the liquid.(Fig. 16) Three tubes were treated per dose-level of (1, 10, 100, 1000, µg/ml) of the extract and three for control containing only N/S. All the tubes were covered with parafilm and incubated at 37°C. After 8 days the solutions and the solid smears were screened for the presence and survival of the larvae (in solution) and ova and cysts (in the smear).

Figure 16

Apparatus for Anthelmuntic Experiement

Figure 16. The apparatus for the Anthelmintic Assay



A test tube containing drug solution, with filter paper strip holding 5 gm infested stool.

Note 6. Stool (5 mg), heavily infested with hookworm ova and cysts, were incubated with KF oil at 37°C for 8 days as shown in Fig. 16; to assess the ovicidal and larvicidal effects of the oil.

Pesticidal Experiments in Vitro, (6, 7, 8 and 9)

General Comments:

Various bioassay techniques, (the dipping, the leaf disk and the climbing tests), were used to test the extract for larvicidal, antifeedant and tick repellent effects; against mosquitoes, podborers, the armyworm and the ticks. The results were used to calculate the MED's for the reaction of each arthropod species.

6. Aedes aegypti Larvicidal Effect.

Twenty mosquito larvae of same age, weights and from the same batch were introduced into 100ml distilled and aerated water, containing 250 mg/ml of KF oil, plus wheat gran (food). The insects were maintained at 25°C ± 0.5 and 75% RH for 24 hours. The mean positive effects of the extracts, moribund (M) and dead (D) states observed, was 86.0 ± 16.0% (6), compared with 8.0 ± 3.0% (6) for the control. Subsequent serial dilutions of the KF extract were prepared and used to dip groups of 20 each of 2nd and 3rd stage A. aegypti larvae, under similar experimental conditions as above. The results from the serial dilutions tests are shown in Table 11.

Table 11 a

Larvicidal Effect on Aedes aegypti.

Conc mg/ml	% Response	mg/ml
0.00	6.63	
20.00	40.00	
40.00	80.00	
80.00	85.00	
160.00	100.00	

NOTE: The LD50 was 22.40 mg/ml (or 2.24%) and LD90 was 151.30 mg/ml or (15.13%).

7. Maruca Larvicidal Activity:

Into 10 test tubes TI - T10 of 6 x 1 cm, were introduced single flowers previously soaked in standard solution (0.50 g/ml) of the KF extracts. Another 10 tubes C_1 - C_{10} contained flowers soaked only in the solvent (ethylacetate 1ml in 9ml water). Maruca testulalis larvae of similar instars (2nd or 3rd) were individually introduced into each flower and left to feed; at 25°C and 75% RH. After 24 hours the treated flowers were removed and fresh ones replenished. The reactions of the larvae after eating the extract treated flowers were scored as normal (N), moribund (M) and dead (D), daily, till emergence to the adult stages. The moribund and dead states were regarded as positive activities. The results of the larvicidal effects of 0.5 g/ml on M. testulalis was 90.0 ± 10.0%(6), compared with 16.70 ± 9.0%(6) for the control.

Subsequent dilutions of the extract were similarly bioassayed against other groups of 10 larvae each (test) and 10 (control). The results from the serial dilutions are reported in Table 12.

Table 12a

The Effect on Maruca testulalis - Larvae

conc. g/ml	% Response	LD 50 g/ml
0.0	0.00	
0.00042	20.00	
0.0042	20.00	
0.042	30.00	
0.42	80.00	0.106
0.50	90.00	

NOTE: The LD90 was 50%, whereas the LD50 was 10.60%.

8. Armyworm Antifeedant Test.

Four leaf discs (10 mm ø), from young (70 cm high) maize (Zea mays) plants, treated with acetone (20 µl on each side), containing various concentrations of the extract, were arranged around a glass petri dish (9 cm ø). Four other discs treated with acetone alone (control), were arranged alternatively in the same plate. The last (6th) instar larvae of S. exempta previously starved (two hours) were released into the dish and left to eat the leaf discs for 120 minutes.

The left over leaves were collected into treated and controlled groups, dried at 110°C for 60 minutes, and weighed. The percentage of the eaten leaves were calculated by the formula % DD = (1 - T/C) 100, where the T = consumed treated-disc and C=the leaf disc, untreated, but dried and weighed without offering for food to the armyworm to eat.

The results are shown in Table 13 whereas the experimental set up is shown in Fig 17.

Figure 17

Leaf Disc Test for Armyworm Antifeedant Activity

Note 7. Ten 6th instar) S. exempta larvae offered choice of fresh maize leaf-discs: 4 — treated (test) and 4 acetone treated (control), in plate (9 cm Ø), were left to eat for 120 min. The weights of consumed leaves (test and control) found, were used to compute percentage feeding deterrent doses (Table 13).

Table 13a

The Armyworm Antifeedant Effects of KF Oil.

Conc. µg/ml	Response %	DD 50 µg/ml	DD 90 µg/ml
0.00	0.00		
4.00	10.20		
40.00	31.28		
400.00	88.45	100.00	500.00

NOTE: The feeding deterrent dose fifty (DD50) was 0.01% while the DD90 was 0.05%.

9. The Tick Repellent Test:

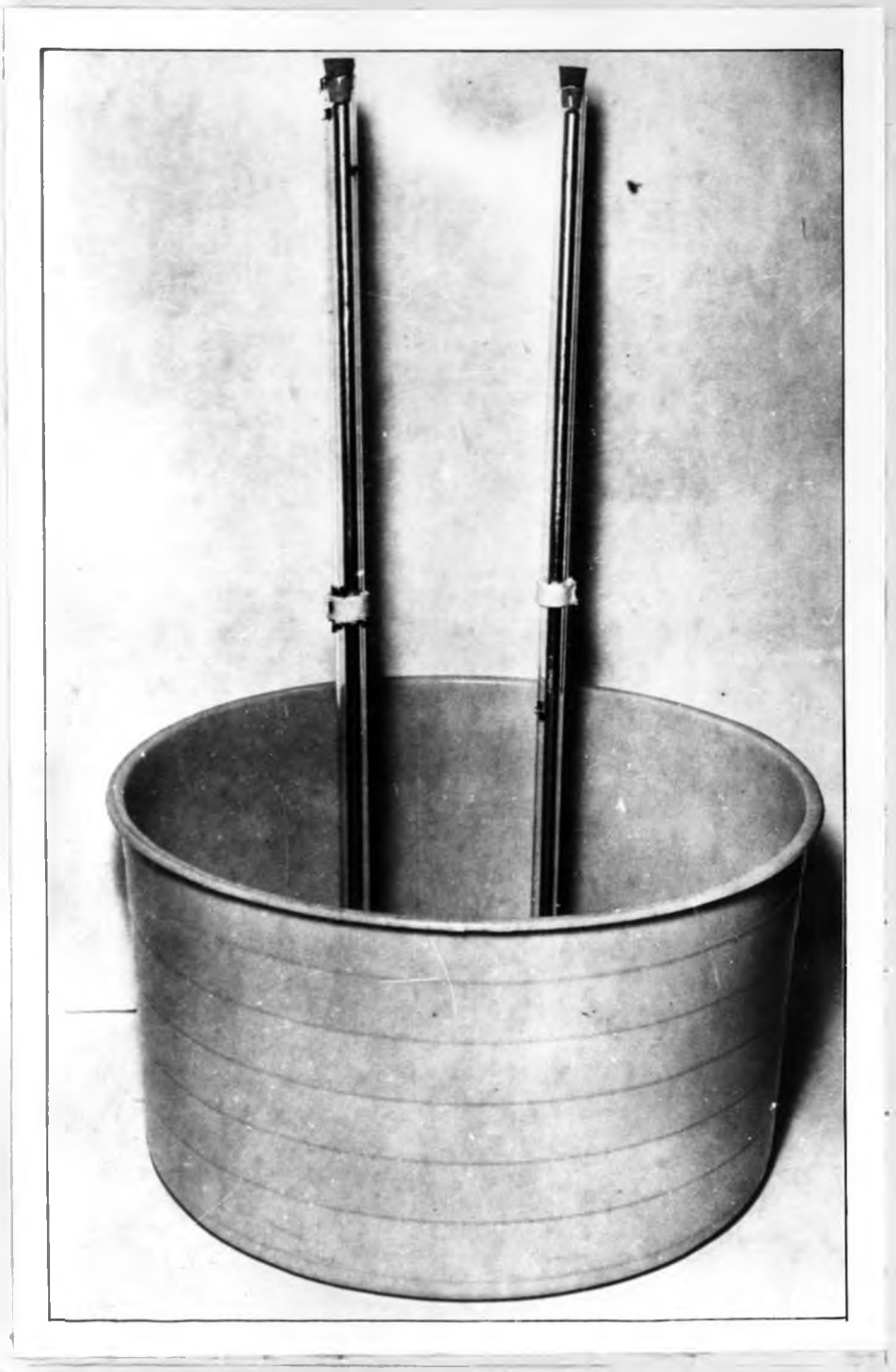
The climbing test consisted of releasing thirty starved (6-8 weeks), fully hydrated adults or mature nymphs of R. appendiculatus, on a metal platform (14 x 14 cm), set in a basin (25 cm ø), containing water at 30°C. On two plastic rods (45 cm/long and 1 cm ø), fixed upright on the platform (8 cm apart), were fitted cotton bands (1 cm wide and 6.5 µg/ml ρ), previously dipped for 5 minutes in a standard solution of KF extract and in acetone solvent (control). Figure 18 depicts the climbing test apparatus.

Observation: The positions of all the 30 ticks were monitored for 90 minutes. The number of ticks that were above, on and below the bands (treated and controlled) were noted. Various concentrations of the KF Oil were run similarly and repeated at least 3 times. The mean percentage repellency were then calculated, according to the formula % RD = (1-T/C) 100, using the passes at the control band as C. The results are tabulated in No. 14. There were free up-and-down movements of ticks at the control band, whereas the ticks that ventured past the band treated with higher doses of KF oil never came down.

The bands dipped in acetone were dried at 30°C before they were fitted onto the rods.

Figure 18

Climbing Test for Tick Repellent Activities



Note 8. Thirty starved (6 — 8 weeks) R. appendiculatus ticks released on warm (30°C) metal platform, were assessed for repellent effect of the oil applied on cloth band, tied on a rod; and compared with a control band (Fig 18). The number of ticks passing both bands were recorded for 90 min., and used to calculate percentage repellent doses (Table 14).

Table 14 a

Tick Repellent Activity of KF Oil.

Conc mg/ml	Response	RD 50 mg/ml	RD 90 mg/ml
0.00	0.00	1.12	1.82
0.50	32.76		
2.50	72.78		
5.00	100.00		

Note: The RD 50 was 0.11% while the RD90 was 0.18%.

Toxicity Experiment in Vivo

10. Determination of LD 50 and MTD in Mice:

Various concentrations of KF oil dissolved in corn oil were administered IP, in single doses (0.2 ml per/mouse), to groups (of 10 mice each), of both sexes (4-6), in the following series of tests (1-3): In test (1), doses (0.025, 0.25, 25, 250 and 2500 mg/kg body weight), were given to different groups; in test (2), dose range of 500, 1000 2000 mg/kg was used, and in test (3), 315.0 630.0 and 1,260 mg/kg, were administered. Each series of tests were given on the same day, together with two other control groups (one group of 10 receiving 0.2 ml, corn oil and the other receiving no treatment). All the groups were allowed pellets and water and were maintained at 25°C in plastic cages kept in a clean room under similar experimental conditions.

All the experimental animals were put under observation for at least 7 days before the treatment with the test drug. During the observation period, records were kept of each mouse. Daily examinations of the external organs and systems were recorded as shown in Table 15. Two days before the treatment, two mice, selected at random from any group, were sacrificed and internal organs grossly examined as shown in Table 16. During the experiments, many toxic signs were checked for and marked in the record sheet for every mouse as shown in Table 17. The probit-log curve of the dose - response - experiments is shown in Figure 19.

Table 15

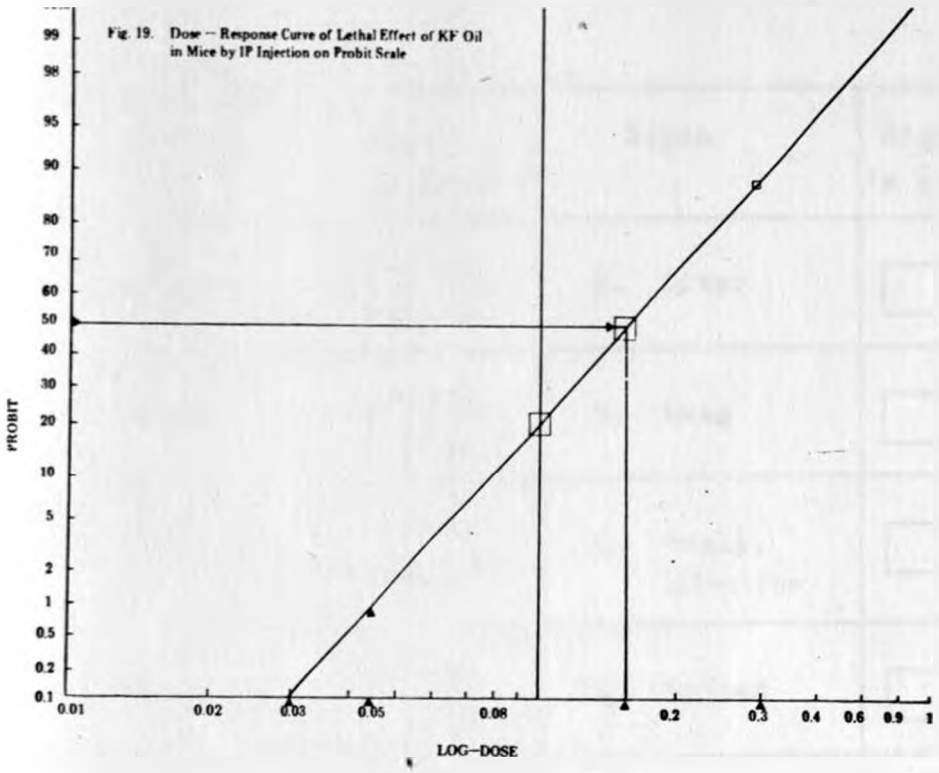
Observations made before Administration of Test Substance

Observation on	Sign	Observation on	Sign
1. Anal Region		7. Eating habits	
2. Anorexia		8. Hair coat	
3. Cardiac Function (pulse rate)		9. Mouth	
4. Activity		10. Nostril	
5. Ears		11. Respiration	
6. Claws		12. Stool	
7. Eyes		13. Teeth	

NOTE: The records of N or P, for normal or pathological were marked in appropriate positions.

Figure 19

Dose — Response Curve for Toxicity of KF Oil in Mice



Note 9. Groups of 10 mice each, of mixed sexes, weighing 19.0 ± 5.0 gm, were treated intraperitoneally (ip), with various doses of KF Oil in corn oil (test) and corn oil alone (control); and the mortality rates recorded for 7 days. The probit vs. log-dose plot produced a linear curve for computing LD50 and LD10 of the KF oil.

Table 16

and Samples

Internal Organs Inspected for Gross Changes

before and after Treatment with Toxic Substance

Organ	Sign (N or P)	Organ	Sign (N or P)
1. Bladder		8. Liver	
2. Blood		9. Lung	
3. Brain		10. Small, intestine	
4. Esophagus		11. Spleen	
5. Heart		12. Stomach	
6. Large, intestine		13. Stool	
7. Kidney		14. Urine	

NOTE: Two mice selected at random from any group were sacrificed and their internal organs removed and examined grossly. The Pathological organs were studied histopathologically to decide on the nature of the abnormality.

Table: 17

Responses Observed during Toxicity Studies in Mice.

1. Physical Activity	10. G.I. Effects
2. Tremors	Vomiting
3. Writhing	Salivation
4. Convulsion	Defecation
5. Respiration	Bleeding
6. Muscle Spasm	11. Skin colour
7. Eye sight	Blanching
8. C.N.S. Effect	Cyanosis
Sedation	Vasodilation
Analgesia	12. Cardiac collapse
Hypnosis	13. Insomnia
Anaesthesia	14. Anorexia
Reflex	15. Malaise
9. Motor activity	16. Micturition

CHAPTER III

RESULTS

RESULTS

Antimicrobial Effects of KF Oil in Vitro

Susceptibility of Bacteria Determined by the Agar Diffusion Test. The reactions of the standard gram-positive cocci and the gram-negative bacilli ~~bacterial~~ to Kedrostis extracts are summarized in Tables 2 and 3. They tabulate the sizes of the zones (in mm) of inhibition for 14 bacteria species. The results show that the KF oil (organosoluble) was antibacterial, whereas the water-soluble extract was not. Table 4 summarized the comparison of the bacterial sensitivities between the active extract and 4 other currently used antibacterial agents (i.e., cotrimoxazole, erythromycin, minocycline and rifampicin). It was clear that the 5 µl (5mg) of the KF oil applied on filter disc was about equal in effectiveness to the commercially used sensitivity discs of the named agents when their inhibition zones were compared.

Susceptibilities of Six Bacteria Species Tested by Serial Dilution. The minimum inhibitory concentration (MIC) and the minimum bactericidal (MBC) concentrations of the KF oil, against the equal inocula (2×10^8 cells/ml), of six bacterial species, tested in TSB and TSA dilutions, are summarized in Table 5. The same table shows the activities of penicillin and streptomycin on the bacteria. It was observed that the MIC and MBC for each species were almost equal. These findings tended to indicate, that the KF oil had bactericidal mode of action. Past experience has shown that to be considered bactericidal, the concentration of the antibiotic drug required to kill, had to be less than 2-4 times the dose needed to inhibit the bacterial growth, Bondi (1964). In the present observation the MBC was less than 2 times the MIC for almost all species of bcteria tested.

The tests were conducted with crude extracts, hence antibacterial activities were expected to improve considerably with pure compounds; except perhaps, if the activities depended on synergism, (potentiation or summation) with one or more components in the crude oil.

Comparison of Bacterial Susceptibilities to KF Oil by the Disc, Agar - Diffusion and Broth Dilution Tests. Inhibitory zones about the disc containing 5µl (5mg) of KF were measured and ranked for the six species of bacteria tested. The ranks were compared with those resulting from the broth dilution studies. In general, there was no correlation between the agar-disc and the serial dilution results. Certain strains with the largest dose-sizes of inhibition had also the highest MIC and MBC values. The observations are shown in Table 6. The technique of agar diffusion for quantitating antibacterial compounds was based on the theory that the compounds could diffuse through the TSA seeded with bacteria. But the rate and extent of diffusion is a complex function involving the chemical characteristics of the compound including its molecular size, charge, solubility, as well as the composition of the agar medium. Some compounds may diffuse easily while others poorly in agar. Hence it would not be very accurate to compare two antibiotics, although the method is used in hospitals routinely.

The serial dilution technique either in broth or in agar, however permits precise and accurate measurements of the MIC and MBC of the active compounds against bacteria. The results from the serial dilution methods provided accurate quantities for comparing various antibiotic drugs, and has been used by other workers, Pagano (1964), Atlas and Turck (1968), Bondi (1964) and McCabe & Lorian (1968). The poor correlation of activity of the extracts between the agar diffusion and the broth dilutions methods shows that there might be more than one active compound in the KF oil. Apparently, proper comparison of a new drug (extract) with other antibiotics seemed untainable by the agar diffusion sensitivity test. For further investigations, the pure active component of the KF oil should be retested only by the in vitro serial dilution assays for MIC, MBC, development of resistance, and its activity in combination with other antibiotics, susceptibility to bacterial enzymes such as penicillinase and its efficacy in vivo in infected animals. The completion of these tests would allow the new compound to be tested clinically.

Susceptibility of Measles Virus by Tissue Culture

Serial Dilution Test. The results of the drug-induced CPE and the percentage of viral-caused CPE reductions in the vero cells are shown in Tables 8 and 9. The degree of CPEs were graded according to Standem (1963) (see Table 7), and used to calculate the percentage responses of the measles viral multiplication to the drug (KF oil). The observation recorded in Table 8 had shown that the maximum tolerated dose (MTD or LD 10) of KF oil in vero cells was 12.5 µg/ml. The results of the antiviral activity shown in Table 9 were used to produce on probit scale, a linear curve from which the lethal dose fifty (LD 50 or MIC) of KF oil on measles virus was found to be 1 µg/ml.

These values permitted the therapeutic index (TI) of 12.5 to be calculated, by the formula MTD/MIC or (12.5 µg/ml x ml/1 µg) equals 12.5. Following the observations of Bushby (1963) and Grunberg (1979), the present level of TI of 12.5 is within the minimum allowable range (MAR) (usually ≥ 7).

Hence K_F oil as it is, could very well be used to protect tissue culture cells from measles viral infection (or contamination) *in vitro*. Some of the reasons advanced by the traditional users for giving water extracts of Kedrostis herb to people sick with viruses were to attract the disease (Yamo)* from the internal organs so that this would masquerade as rashes on the skin and eventually dissipate from the body. So the stronger the repelling oduor of the drug, the more likely it was to cure the disease. But the effect could be combined antiviral and antibacterial activity; since the KF oil was found to be.

These theories need to be investigated with the pure active compounds from *K. foetidissima*. Hence the *in vivo* chemotherapeutic tests need to be undertaken soon.

*(Yamo, Luo)

Fungicidal Effect of KF Extract by the Spray Test in Vitro

During the field surveys to observe the traditional use of K. foetidissima as medicine in Western Kenya, it was noticed that the measles-infected children, who were washed with suspensions of KF extracts had no fungal infection anywhere on their skins. In the present screening studies, extracts (water soluble and ethylacetate soluble) were spotted, developed on TLC plates and sprayed with fungal spores. The organosoluble oil exhibited high antifungal effects on C. cucmerinum and T. mentagrophytes species in vitro. The fractions (A, 1,2,3, & 4) of the oil from column chromatography, together with a standard fungicide (SC) were further tested against spores. Fraction No. 2 of the oil showed stronger antifungal activity than equivalent amount of the reference drug (SC), (Fig. 13). The active component of fraction No. 2, separated on TLC plate was identified by further antifungal activity (Fig. 14) as compound no 4 from the bottom of the plate,

Kedrostis oil was also used to cure superficial fungal infection caused by the Trichophyton group on the investigators hand, by applying the oil topically on the infected site. It was tempting to use the oil on people with other seemingly resistant fungal infections, who requested it, but this could not be agreed to, because further tests (pharmacological and toxicological) were needed to be completed before any attempt at clinical work could be considered.

But since *in vitro* antifungal results usually vary considerably from the *in vivo* effects, Hildick - Smith (1967), the topical application in this reports was a useful proof of efficacy on actual infection on skin.

Effects of KF Oil on L. donovani Promastigotes in Vitro

Standard concentrations (2 x 10^6 cells/ml) of flagellates incubated with serial dilutions of KF oil in RPMI 1640 medium, at 34°C for 48 hours, resulted in antileishmanial activities, shown in Table 10. The MIC was 100 µg/ml. Such a level of activity, in vitro, against promastigotes was considered significant , especially, since the Leishmania promastigotes have been reported to be resistant to drugs, Beveridge (1963). The KF is thus likely to be more active against amastigotes. No reference drug was available for comparative studies, since sodium stibogluconate was ineffective.

Anthelmintic Effects.

The following findings were recorded:

(a) no larvae were recovered from any treated tubes but four were got from the control tubes;

(b) ova were recovered from all the stool smears; including the control even though 8 days had passed.

Fo example, 3,4,5,3 and 0, ova were obtained from the control, and the tubes with 1,10, 100 and 1000 µg/ml of the extract. Similar general trend was observed in the replicate experiments. The viability of the ova could not be decided because they failed to develop into larvae.

Efforts to establish the anthelmintic effects of the KF oil against the hookworm larvae were not successful and are expected to be planned better in the near future.

Overall, the antimicrobial screening of the KF extracts have demonstrated that the plant contains components with strong antimicrobial effects, against bacteria, virus, fungi and protozoa. But the anthelmintic test were inconclusive, owing partly to non-availability of specimen and also perhaps, because the method had not been standardized.

Pesticidal Experiments in Vitro

The Larvicidal Effects on A. aegypti and M. Testulalis. The test of KF Oil on the larvae of mosquitoes, A. aegypti and the cowpea podborers, M. Testulalis yielded the results in Table 11 and 12. The percentage responses were based on the positive reactions of moribund and dead larval states. These values were plotted vs. logarithms of the doses, to produce a linear curve.

From the curves, the values of LD 50 and LD 90 of the KF Oil on each insect were calculated. The technique had also been used successfully by Maradufu, et al; (1978).

Table 11b

Larvicidal Effect on Aedes aegypti.

Test No.	Dose (mg/ml)	Response % ± SD
1	0.0	6.63
2	20.00	40.00
3	40.00	80.00
4	80.00	85.00
5	160.00	100.00

NOTE: Twenty 2nd and 3rd stage A. aegypti larvae were released in a beaker (250 ml) with KF oil (20,40,80 & 160 mg/ml) solution, and kept at 25°C for 24 and 48 hours. The health status of the larvae were recorded as normal (N), moribund (M) and dead (D). The M and D (positive responses) were used to calculate percentage responses. The LD50 was 22.40 mg/ml, (or 2.24%), and the LD90 was 151.30 mg/ml (or 15.13%).

Table 12b

The Effect on Maruca testulalis - Larvae

Test No.	Dose (g/ml)	Response % ± SD
1	0.0	0.0
2	0.00042	20.00
3	0.0042	20.0
4	0.042	30.00
5	0.42	80.00
6	0.50	90.00 ± 10.0(6)

NOTES: Ten, 2nd and 3rd instar larvae of M. testulalis, were individually offered cowpea flowers, previously soaked in various concentrations of KF Oil (0.42, 0.042, 0.0042,& 0.00042 g/ml.). Similar No. of flowers were dipped in solvent alone (control). After 24 hours, fresh flowers were replenished. The toxic signs were recorded as M and D and used to calculate percentage responses. The LD50 was 10.6% and LD90 was 50%y.

The Armyworm Antifeedant Activity. Using the apparatus depicted in Figure 17, the 10 S. exempta larvae, given a choice of 4 treated and 4 untreated leaf discs to eat, responded as is shown in Table 13. The percentage of the feeding deterrent doses plotted on probit vs. log-dose, gave the values of the feeding deterrent dose-fifty (DD 50) and the feeding deterrent dose-ninety (DD 90) as 0.013% and 0.05% respectively. Melnikov (1971); and Jacobson (1976), reported similar values for pyrethrins as between 0.1 - 0.4%. Hence the antifeedant effects of the KF Oil were about equal to those of pyrethrum products being used commercially to control insects.

Tick Repellent Effect of KF Oil. The adult brown ear tick screened by the experiment shown in Fig. 18, against Kedrostis oil for repellent activity, reacted as shown in Table 14. Again the values of the MED (RD 90) and the RD 50), were calculated from the curve produced by the log-dose vs., probit plot. These values were 0.18% for RD 90 and 0.11% for RD 50. Thus the dose of the oil that gave 90% repellency was about 2.0%. This was again about equivalent to quantities of DEET that were being used practically.

Table 13b

The Armyworm Antifeedant Effects of KF Oil.

Test No.	Dose (µg/ml)	Response (SD) % ±
1	0.00	0.00
2	4.00	10.20
3	40.00	31.28
4	400.00	88.45

NOTES: Various concentrations of KF Oil in acetone (50 µl) were used to treat four leaf discs. Acetone alone volumes (50 µl) were used to treat another four leaf discs (control). The eight discs were arranged alternatively in a glass petri dish and, 10, 6th stage, starved S. exempta larvae were released into the dish to eat for 120 minutes. The consumed discs of treated and control leaves, calculated after drying and weighing the left-overs, were used to calculate the percentage feeding deterrency as shown in table 13b.
The DD 50 was 105.50 µg/ml (or 0.01%) while the DD 90 was 501.20 µg/ml (or 0.05%).

Table 14b

Tick Repellent Activity of KF Oil.

Test No.	Concentration mg/ml	Response (% Repellency) (± SD)
1	0.00	0.00
2	0.50	32.76 ±
3	2.50	72.78 ±
4	5.00	100.00

NOTES: Thirty starved adult ticks were made to climb past treated cloth band tied (20 cm) on stem of a standing rod (45cm long). Similar solvent treated band was fitted on control rod. Every 15 minutes the number of ticks passing the treated and the control bands were counted for 90 minutes and used to calculate % repellency using the control disc as a baseline. From the dose-response curve, the RD 50 was calculated as 1.12 mg/ml (or 0.11%) and the RD 90 as 1.82 mg/ml (or 0.18%).

Results

Toxic Effects on Mice.

Observation: Dead mice were counted daily for exactly seven days. Other symptoms were also recorded. The results were: in test (1); 100% of the mice which received the 2,500 mg/kg, died within 48 hours. None died within 7 days from among the groups which received other doses (250,25,2.5,0.25 or 0.025 mg/kg), and none from the control groups. In the test series (2); 7/8 (or 87.50%) of the mice that received 2,000 mg/kg, of the extract died; and only 1/8 (or 12.50%), died from receiving 1,000 mg/kg within 7 days. No mortalities occured among the mice which received 500 mg/kg and none in the control groups.

In test (3), 2/10 (or 20%) of the mice receiving 1260 mg/kg of the oil died, and no deaths resulted from other doses (630 and 315 mg/kg); and none from control groups, within 7 days. The probits Vs. log-doses of the positive (% mortality) responses were plotted on probit graph paper, (Fig 19). The linear curve so produced was used to calculate the LD 50, and MTD (or LD 10) by interpolating the 50 and 10 probits of the % responses to the logs of the corresponding doses.

The median lethal dose (LD 50) and the maximum tolerated dose (LD 10) were 1602 ± 20.40 and 625.40 ± 62.0 mg/kg body weight respectively.

Many toxic symptoms, observed in the mice which had received high doses of the oil (above 2000 mg/kg were; increased motor activity, muscle spasm, writhing, respiratory and cardiac stimulation (as demonstrated by high pulse rates), which invariably lead to circulatory collapse, and death of the animals. The doses (2500 mg/kg and above), produced immediate (5 minutes) onset of toxic signs. Lower doses (≤ 2000 mg/kg) were slow to produce toxic symptoms. Most of the toxic reactions lasted upto 6 hours. Any animal which escaped death up to 48 hours recovered.

Other observed toxic signs included convulsion, insomnia, anorexia, cyanosis around treated area showing tissue necrosis.

Table 18

Toxic Effect of KF Oil in Mice

Dose (mg/kg)	Response (%) ± SD
315.00	0.00
630.00	0.00
1000.00	12.50
1260.00	20.00
2000.00	87.00
2500.00	100.00

NOTE: KF oil dissolved in corn oil (elianto) was injected intraperitoneally in 0.2 ml volumes into groups of 10 mice each which were observed for mortality effectsfor 7 days.

C H A P T E R IV

D I S C U S S I O N

DISCUSSION

Extracts of *K. fostidissima* were screend *in vitro* by a scanning process to select the medically useful from non-useful components, against pathogenic micro-organisms and parasitic arthropods. The protocols adopted, used simple, rapid, comprehensive and non-expensive methods, making no prior assumptions about activities of any fractions.

During the experimental demonstrations, objective effects were noted against microbial agents (bacteria, viruses, fungi, protozoa, helminthes); and arthropods (mosquitoes, podborers, armyworm and ticks).

Certain standard methods were used with slight modifications, while other methods had to be developed and standardized in the laboratory. Except for toxicity studies in mice, all the preliminary experiments were based on *in vitro* tests.

Chemotherapeutic Effect of KF Oil.

Initial tests had shown that the aqueous-soluble KF fractions were inactive. Subsequently, the organo-soluble fraction (oil), was screened exclusively, for chemotherapeutic, and pesticidal activities.

The results shown in Tables 3, 9 and 10; and in Figures 13 & 19 indicated, that the kedrostis oil was an effective, broadspectrum, antimicrobial drug, with bactericide mode of action and with a wide margin of safety.

The findings agreed with the traditional medicinal uses of the herb (*K. foetidissima*) as was reported by Kokwaro (1972 & 1976), nor were the observations unusual, because plants have been known from ancient times to produce chemotherapeutic remedies, Laurence (1966) and Cutting (1964). Thus the Greeks, the Aztecs, the Hindus and the South Americans used: male fern and chenopodium oil as anthelmintics; chaulmoogra to treat leprosy; quinine and other alkaloids from Cinchona bark to cure malaria. Moreover the bulk of the African traditional medicinal practice was based on the use of crude plant products. The active doses of the Kedrostis oil were compared with, and found to be equal to and at times higher than those of most currently used antimicrobial drugs. Since there was no reference standard drug for antiviral assay, the blank controls provided the baseline. Attempts to test the anthelmintic activity of the new drug against hookworm larvae were unsuccessful, owing to insufficient specimens and the lack of standard *in vitro* culture method for the nematode. The results were hence inconclusive.

Being a general survey, it was not feasible within the period allowed, to extend the studies to: (1) the factors related to the resistance of microbial pathogens to the drugs; (2) its activity in combination with other drugs; (3) its stability under various environmental conditions

during extractions, hydrolysis by microbial enzymes, and degradation during storage; and (4) its ability to protect animals artificially pre-infected with relevant susceptible microorganisms

Despite these limitations, the present results have clearly and sufficiently demonstrated that Kedrostis oil was a candidate drug which might produce new antimicrobial agent, against several fastidious pathogens, now not amenable to control by current antimicrobial agents

Pesticidal Experiments in Vitro, General Comments:

The preliminary screening for new drugs from K. foetidissima plants gave positive effects against microbial agents. Hence the survey was extended to pesticidal screen, partly, because the pests were readily available within the centre (ICIPE), and also, because the arthropod vectors and pests were equally important to public health.

The pesticide chemicals are necessary for the production of adequate food and fibre, and for continued maintenance of public health. Today, many (over 1000 in more than 100,000 formulations), bioactive chemicals are being used, to protect plants from pests, diseases and weeds, and to control the vectors of animal and human diseases, Melnikov et al., (1971). Since 1940's the use of pesticides in public health has helped to control most of the

vectors of human diseases. As a result many people all over the world have benefitted socially and economically.

Resistance:

The emergence of resistance by the pests to most of the original pesticide groups, has spoilt the previous hopes of er-adicating such diseases as malaria, plague, trypanosomiasis onchorcerciasis, filariasis, schistosomiasis, etc. The only alternative left to the agricultural and public health communities has therefore been to intensify the search for more new pesticides to replace the previous ones. In addition, many current pesticides contain toxic and non-degradable residues, which present danger to environment and lives of non target organisms. Alternative control methods e.g., biological and genetics, which have been tried, never reached developmental or application phases. Hence it is now realized that it may not be feasible to er-adicate the pests after all. So the aim is to attempt reducing the pest populations below their economic injury and coexistent levels.

In the present work the insecticidal and acaricidal activities, illustrated in Tables 11, 12, 13 & 14 and Fig. 19, tended to suggest that the Kedrostis oil was effective and safe

pesticide , comparable to pyrethrins and DEET. Thus it's effective concentrations against the arthropods ranged between 0.05% and 15.0%; those of pyrethrins were 0.1% to 2%, and of DEET were 5% to 10%. For the last two, these doses were the commercial formulations being sold and used in the field: in sprays, creams, and in dusting powders, for larvicidal, feeding deterrent and repellent activities, Melnikov (1971). The results also showed that the effects of the oil against arthropods were species specific (see result section).

The toxic doses of the oil made the larvae moribund (sick) or killed them. The larvae were considered "moribund" if they did not respond to strong stimuli such as bright light, air and water currents, or touch pressures. The responses expected consisted of movement (e.g., rotational or translational changes of whole body).

The KF oil was an antifeedant to the larvae of S. exempta. When tasted by the insect, an antifeedant substance caused cessation of feeding, temporarily or permanently, depending on the concentration and the potency of the active component, Nakanishi (1976). The feeding deterrent dose (DD90) of the oil was 500ppm. This was significant. Other African plants have also been reported, Kubo & Nakanishi (1979) to produce armyworm antifeedant compounds. These are: ajugarin,

from Ajuga remota (Labiatae), effective concentration, 100 ppm; harrisonin from Harrisonia abyssinica, Oliv (Simarubaceae) (Pedo, Luo), (DD90, 20 ppm); polygodial, warburganal and ugendensidial, all from Warburgia Spp. (or Sogomaitha, Luo), (DD90, 0.1 ppm); azadirachtin from Azadirachta indica, the Indian neem tree (or Dwele, Luo); (DD90, 40 ppm).

None of these products have been tested under field situations, nor were their mammalian toxicities mentioned. Kedrostis oil was however expected to produce higher antifeedant activities after the isolation and purification of its active compounds. Moreover, it was safe in mammals.

The oil also exhibited tick repellent activities. Its effective repellent dose (RD 90) being 0.18%. This level of activity was equivent to that of 1% pyrethrins and 5% DEET, which were sold commercially. Other plants with tick repellent activities have been reported from Ghana, West Africa, by Novak (1971). But their effective doses or mammalian toxicities were unknown.

Mammalian Toxicity of the KF Oil.

Mice given single IP doses of the KF oil in corn oil, were carefully observed and manipulated to measure the onset, the peak, the duration, the nature and extent of each toxic effects of the oil.

The percentage responses were related to the doses by a curve which was used to calculate the LD50 and the LD 10. The values obtained were compared with Pyrethrins, (LD50; 960-1050 mg/kg) and DEET (LD50; 2000 mg/kg body weight), Melnikov (1971).

General Comments:

The biological responses to chemical substances could not be predicted accurately, nor explained in terms of physical or chemical laws: Goldwater (1968). Hence the responses, (including antimicrobial, pesticidal and mammalian toxicity), reported here, represented only estimates rather than absolute values. The aim was simply to establish the "dose-effect" and "dose-response " relationships which were important to study efficacy, the health-risk and the safety vs. hazards of the new drug.

In the present context therefore, the "effects" meant individual changes observed in each animal, whereas the "responses" represented the proportions of groups of organisms showing the changes caused by the drug. The individual peak effects, pooled into quantitative percentage responses, were connected to the corresponding doses by the logarithmic method of Miller and Tainter (1944). The chemotherapeutic and the toxicity tests in arthropods and mammals therefore sought to discover the capacities of the

new drug to cause alterations (beneficial or injurious) to the organisms. The results thereof would help in deciding whether or not, (1): the substance was effective, and (2) toxic.

Here, a substance was regarded as toxic if very small dose of it ($\leq$ 50 mg/kg body weight) caused injuries to mammals, and non-toxic if very large doses ($\geq$ 5000 mg/kg body weight) caused injuries to the same animals, Jacobson (1976).

The values of the LD 50 and the minimum lethal dose (MLD or LD10) obtained experimentally in mammals would indicate to the users (potential or actual) of the drug, (in crude or refined form), the risky (or hazardous), doses which should not be exceeded. The acute toxicity observations were limited to 7 days. Again for the present purpose "acute effects were observed over a short period of time (e.g., within seconds, minutes, hours upto 7 days), as compared with chronic toxic effects produced after a long time interval, (e.g.,in days, weeks,months, upto 2 years), Hagan (1959).

Similarly,the margin of safety, as estimated by the therapeutic index, was the ratio of the maximum tolerated dose (MTD) and the minimum effective dose (MED), which cured the infection in vivo, Graham (1966). The therapeutic index could not be calculated until the animal protection studies were completed.

C H A P T E R V

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CONCLUSION

The present screening programme focussed on one medicinal herb, Kedrostis foetidissima (Jacq.) Cogn., cucurbitaceae; to verify the claims of its usefulness as medicine and to estimate its safety versus its hazards in animals and man. Thus kedrostis extracts (aqueous and organosoluble), were evaluated in vitro, against parasites (bacteria, virus, fungi, leishmania, hookworm, insects and ticks). The acute toxic effects of oil was also tested by intraperitoneal (IP) administration to mice in increasing doses and the LD50 and LD10 calculated from the dose-response curve, (e.g., LD50, 1602 mg/kg and LD10, 625 mg/kg body weight).

The major findings of the studies were that:

(a) the plant K. foetidissima, contained active components effective against bacterial Spp., measles virus, fungi (C.cucumerinum and T. mentagrophytes), protozoa, (leishmania donovani flagellates, and tryponosoma congolense not reported here); as shown in the text all, except acute toxicity activities were demonstrated by in vitro tests;

(b) the bioactivity of K. foetidissima was concentrated in the neutral, organosoluble oil, but not in the aqueous portion;

(c) the oil had broadspectrum of activity, against the gram-positive cocci and gram-negative bacilli, and was also active on some viruses, fungi and protozoa;

(d) its anthelmintic effect on hookworm larvae were however negligible; (e) but it was effective against insects and ticks; but exhibited different modes of actions,- e.g., it had larvicidal effects on mosquitoes and podborers feeding deterrent on armyworm (but not larvicide) and repellent on tick (with no lethal-effects shown);

(f) the oil was bactericidal because its MICs and the MBC values were almost equal in serial dilutions tests;

(g) the oil was safe to mice; with a low toxicity (e.g. LD 50 of over 1600 mg/kg body weight) and a high maximum tolerated dose, over 600 mg/kg body weight).

Further, fraction of the oil yielded an active component (ca 140 mg; 0.0002%) which was identified by its Rf characteristic(s) HPLC peaks, uv fluorescence, its antimicrobial activity(s) and by its solubility patterns as were shown in the text, (Fig.1).

The herb K. foetidissima was therefore found to be economically important and had potential for producing drugs and pesticides.

Prospects for the future: Based on preliminary experiments in vitro, and in vivo, the active components of the KF oil are expected to be studied further in order to:-

(a) determine the in vivo efficacy by protection studies in animals infected with various susceptible pathogenic parasites; (b) study the possibility of resistance development by the parasites against the new drug; (c) test the pharmacological kinetics of the active components; (d) evaluate the ability of the new compound to undergo synergism,(potentiation, summation)or inhibition when combined with other drugs; (e) determine further the adverse toxic effects of the oil, including major pharmacodynamic properties by acute, subacute and chronic toxicity studies, in more species of mammals; (f) to identify the active compound(s) by physical spectra, with the possibility of optimization to more active and safer molecules; (g) screen other related plants in the genus Kedrostis and family cucurbitaceae, for new drugs and pesticides, against more fastidious parasites, not currently amen-able to control by the present drugs or methods. These include diseases such as trypanosomiasis, leishmaniasis, filariasis, schistosomiasis, leprosy, malignant neoplasms, etc.

The bioassay methods developed here should prove useful for similar screening project, elsewhere.

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