# PLANT EXTRACTS AS PYRETHRINS SYNERGISTS FOR THE CONTROL OF HOUSEFLY, *MUSCA DOMESTICA L.* (MUSCIDAE: DIPTERA)



BY

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Chemistry of Egerton University



EGERTON UNIVERSITY

SEPTEMBER, 2005

## DECLARATION

I declare that this thesis is my original work and has not been previously submitted for an award in this or any university for any degree as by my knowledge.

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## DEDICATION

This thesis is dedicated to Mr. and Mrs. Mitei, whose vision and energy brought this work to reality, the entire Mitei's family who provided support.

Dr. Cheplogoi who inspired the work and

Linda who injected the impetus to strive and achieve.

## ACKNOWLEDGEMENTS

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## COPYRIGHT

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## LIST OF ACRONYMS

| | |
|---|---|
| CoA | Coenzyme A |
| $CDCl_3$ | Deuterated chloroform |
| $CHCl_3$ | Chloroform |
| DDT | Dichlorodiphenyltrichloroethane |
| EtOH | Ethanol |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-mass spectrometry |
| IPA | Isopropyl alcohol |
| IR | Infra red |
| $KD^{t}_{50}$ | Time to knockdown 50% of entire test population |
| LSD | Least significant difference |
| MDP | Methylene dioxy phenyl |
| MeOH | Methanol |
| MGK | McLaughlin Gormley King Company |
| MHz | Mega hertz |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Dihydo nicotinamide dinucleotide phosphate |
| NMR | Nuclear magnetic resonance |
| Pbo | Piperonyl butoxide |
| Pys | Pyrethrins |
| SST | Shell sol T (Mineral oil) |
| STD | Standard |
| TLC | Thin layer chromatography |
| TPP | Tetraphenylporphyrin |
| V/V | Volume per volume |
| WHO | World Health Organisation |
| W/V | Weight per volume |

## GENERAL ABSTRACT

Dipterans such as mosquitoes, houseflies, *M. domestica* L. which alone is known to transmit > 20 human diseases and many other household insects breed at prodigious rates and cause vast damage and suffering to mankind. The control of these insects relies heavily on pyrethrum-based insecticides. The cost of production of this highly valued insecticide, which produces rapid knockdown effect on insects, is very high and its competitiveness in a market crowded by synthetic insecticides is due to the boosting influence of synergists with which it is usually formulated. Commercial synergists commonly used include piperonyl butoxide (Pbo) and MGK 264 which are threefold expensive than the production of pyrethrins itself while their toxicity and continuous supply are becoming increasingly unreliable. A few botanicals have shown potency, though not satisfactorily in producing environmentally friendly, cheap and readily available alternative pyrethrins synergists for the control of both outdoor and household insect pests including disease vectors. However, most species of higher plants have never been described, much less surveyed for chemical or biologically active constituents and new sources of synergistic materials remain to be discovered.

This study involved the evaluation of dry fruits, root barks and leaf powders from *Brassica napus, Zanthoxylum chalybea, Vepris uguenensis, Persea americana*, *Sesamum indicum*, *Aloe vera* and *Piper nigrum* as synergists for pyrethrins against housefly, *M. domestica* L. The plants were selected for the study since they were suspected to be containing compounds having the 3, 4-methylenedioxy-phenyl group, the basic framework of chemical structure within which the likelihood of a compound having some synergistic properties might be predicted. The plants parts were collected, air-dried, ground to fine powder and extracted separately using hexane followed by MeOH/EtOH and bioassayed for pyrethrins synergism. EtOH extract of the seeds of *P. nigrum* and $CHCl_3$/MeOH extract of the root bark of *V. uguenensis* exhibited synergism to pyrethrins. Column chromatographic analysis yielded fractions that were subjected to tests to confirm their synergistic activity. One of the two fractions from EtOH extract of *P. nigrum* and two of the six from $CHCl_3$/MeOH extract from the root bark of *V. uguenensis* maintained the activity witnessed in their crude forms. The superior portions were further purified, subjected to NMR, IR and GC-MS spectroscopy and melting point tests revealing that the synergistic compounds to pyrethrins against houseflies were piperine from *P. nigrum* and flindersiamine from *V. uguenensis*.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

More than seventy five percent of the known species of living creatures are insects and about 10,000 of this influence mankind adversely and are, therefore considered to be pests (Metcalf & Metcalf, 1993). Insects destroy over thirty percent of food grown and millions of shillings are spent annually protecting crops from the havoc they cause. Dipterans are vectors of parasites causing malaria, filariasis, trypanosomiasis, and onchocerciasis, which debilitate and kill either people or domestic animals on a vast scale. Household insects such as lice, flies, fleas, bugs and ticks cause much irritation and their bites can spread devastating bacterial diseases such as bubonic plague (Busvine, 1993). To maintain and extend the present standards of civilization insects must be controlled, as benevolently as possible, and with minimum impact on the environment.

Natural plant compounds containing pyrethrins, rotenoids, and alkaloids have been used in household insect control (Matsumura, 1975; Jacobson, 1982). The most economically important plant derived insecticides are the pyrethrins, a group of six closely related esters extracted from pyrethrum flowers. Pyrethrins are botanical insecticides from *C. cineruriaefolium* (Compositae) cultivated mostly in the mountainous regions of Kenya, Rwanda, Tanzania and other parts of the world (Casida & Quistad, 1995).

Pyrethrins have been used as an insecticide since the early 1800's in Persia and Yugoslavia (Crosby, 1966). However, the use of this natural product declined in the early 1950's because of the advent of synthetic analogs such as allethrins, which are both more stable and more effective in the field. Nevertheless when properly formulated with antioxidants or stabilizers and synergists the pyrethrins are still economically viable insecticides (Levy, 1981).

The role of the synergist is to increase the potency of the pyrethrins and speed its reaction time by preventing detoxification within the insects. There are a number of synergists that are available commercially and their activities vary depending on the type of insecticide they are combined with. Piperonyl butoxide and MGK 264 are the most commonly used synergists for pyrethrins and have a unique mode of action. Insects have built-in, complex systems that will

counteract an insecticide once it enters its body. Mixed function oxidases (MFO's) are the one of these types of defense mechanisms, which work by binding with the insecticide, rendering it ineffective. When piperonyl butoxide is present in a compound, it will bind with the MFO's. The insecticide is then available to block the sodium, potassium and perhaps calcium channels in axonal membranes, resulting in nerve excitation, leading to release of neurosecretory hormones such as diuretic hormone which eventually causes death to the insect (Hamilton, 1995).

Plants extracts have been known to have insecticidal synergistic activities. These alternatives could reduce over reliance on expensive, toxic and less abundant synthetic pyrethrins synergists for the control of pests and vectors of diseases causing organisms such as housefly and mosquitoes.

In this study, plants belonging to different families were investigated to establish which among them contains compounds possessing pyrethrins synergistic activity.

## 1.2 Statement of the problem

Household insects, vectors of human and animal diseases causing organisms continue to challenge every home today. The only reliable defense in the battle to control them is the use of pyrethrum-based insecticides as aerosol sprays. Pyrethrins alone at higher doses are effective but very costly. However, a less expensive synergist while retaining an effective level of the insecticidal activity is often employed to boost the toxicity of the expensive pyrethrum. Piperonyl butoxide as a synergist is very costly, toxic and its supply is inadequate. Therefore, search for synergists that are affordable, locally available and environmentally friendly are urgently needed and many higher plants may serve as sources to such synergists.

## 1.3 Objectives

### 1.3.1 General objective

The study aims to identify an alternative pyrethrins synergist(s) from plant sources.

### 1.3.2 Specific objectives

1. To extract crude products from *B. napus* (seeds), *Z. chalybea* (seeds), *P. nigrum* (ripe fruits), *S. indicum* (seeds), *P. americana* (fruits), *A. vera* (leaves) and *V. uguenensis* (root barks) using different organic solvents.

2. To screen for synergistic properties of the crude plant extracts on the pyrethrins against housefflies.

3. To isolate pyrethrins synergists from the active crude extracts.

4. To characterize the isolated compounds.

### 1.4 Hypothesis

Local plants may contain specific compound (s) that may act as synergists for pyrethrins as insecticides.

### 1.5 Significance of the study

The pyrethrum industry should benefit greatly from the possibility of using inexpensive, safer and readily available additives for lowering the required concentration of the expensive pyrethrins, allowing expanded uses at favorable cost. However, piperonyl butoxide the excellent general-purpose synergist is very costly and just as the mercurial nature of the pyrethrins is a cause for concern, the future global supply of piperonyl butoxide is also ambiguous. Deforestation in Brazilian rainforests has led to an environmental and political controversy restricting the harvest of *Ocotea* trees from which the piperonyl butoxide precursor safrole is extracted. Piperonyl butoxide is suspected to be carcinogenic, mutagenic and teratogenic and the lack of a commercially suitable alternative synergist that has the same broad activity profile creates a specter of uncertainty about the viability of currently registered pyrethrins-piperonyl butoxide formulations. Therefore, the scientific rationalization of compounds with synergistic properties in higher plants will add value and make them more acceptable to the pyrethrum industry and public in general once their side effects have been elucidated.

### 1.6 Limitations and contribution of the study

This study was multidisciplinary, whereby aspects from chemistry, biological sciences (Botanical and entomological), formulations and biometrics were intertwined. Therefore a multifaceted approach that required utmost attention, hard work, dedication and time was employed. The study was of its own kind with no recent and related research recorded in the few journals and other publications accessed at the two institutions touching on the study. However, Piperine may partly solve the underlying pest vector management problems as a synergist to pyrethrins to be used as an outdoor pest control agent.

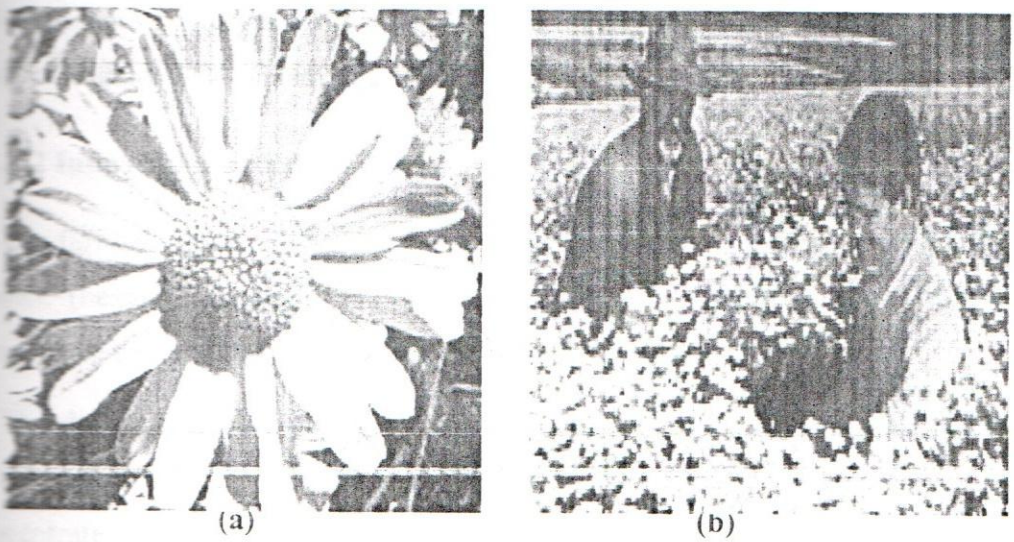
# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Pyrethrum and pyrethrins

### 2.1.1 Botanical aspects of pyrethrum

Pyrethrum, *C. cinerariaefolium* is a perennial plant growing to 45 to 60 cm in height and tending to be woody at the base. It is a member of the Compositae family which produces a large quantity of white, daisy like flowers (Chandler, 1954). The flowers, which are the harvested part of the crop, are produced on branching of fairly rigid stems from a crown of blue-green, deeply lobed leaves (Brewer, 1968). The flower head has an outer ring of white ray florets that are bisexual (Roest, 1976) with a yellow center.



(a) (b)

Plate 1: Pyrethrum flower head (a) and pyrethrum plantation (b)

### 2.1.2 Growing conditions

The climate is of particular importance in the success of commercial cultivation (Serge, 1972). Flowering by the pyrethrum plant is a product of temperate climate (Roest, 1976). A certain degree of chilling is essential to initiate growth and budding (Glover, 1955). Chilling should be essentially followed by long periods of sunshine and ample rainfall (> 1000 mm per month) with a sufficient dry season for weed control (Muturi, 1969). Pyrethrum flowers are successfully grown in deep, well-drained soils (Brandy, 1984) preferably of volcanic origin and double super phosphate fertilizer is applied at planting time.

In Kenya, these conditions are met in highlands > 1800 m above sea level (Muturi, 1969) where rainfall is > 1000 mm a year (Roest, 1976). The main producing zones include the highlands of Kisii district, the east and west of Rift valley province, the foothills of the Aberdares and Mount Kenya, and the higher regions of Kiambu District (Wanjala, 2003). A unique phenomenon in these Kenyan highlands is that the chilling comes with the early rains and as the rains continue so does the flowering and instead of producing one heavy crop, as in Europe and Japan, flowers can be picked up to nine or ten months in Kenya (Roest, 1976).

### 2.1.3 Processing of pyrethrum

#### 2.1.3.1 Harvesting and drying

Time of harvest is critical for obtaining maximum pyrethrins in the flowers and ideally, flowers should be harvested at full development (Roest, 1976). During harvesting, flowers have moisture content of over 80% (w/v) so the flowers are dried in the sun until moisture content is about 10% (Gnadinger, 1945).

#### 2.1.3.2 Industrial extraction

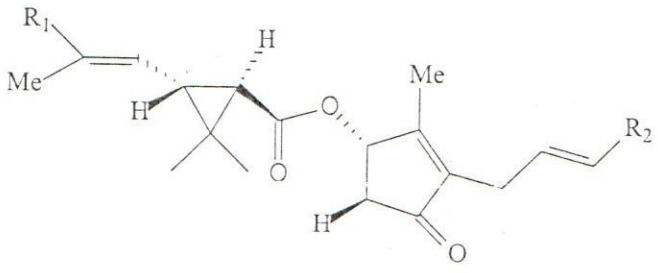
The flowers are then ground to fine powder, which is first extracted with a light petroleum solvent (hexane). Recovery of hexane yields a dark greenish brown viscous oleoresin concentrate containing approximately 30% (w/w) pyrethrins.

The dark viscous oleoresin concentrate is further extracted using methanol. Isoparaffin solvent (shell sol-T) is used in conjunction with activated carbon to give the final pale concentrate containing over 50% (w/w) pyrethrins (Odinga, 1991).

### 2.1.4 Active constituents of pyrethrum

Insecticidal activity in pyrethrum has been shown to be due to the presence of six, structurally related esters termed pyrethrins, sub-divided into two classes: Pyrethrins I and Pyrethrins II. Pyrethrins I comprise of pyrethrin I (**1**), cinerin I (**2**) and jasmolin I (**3**) whereas Pyrethrins II comprises of pyrethrin II (**4**), cinerin II (**5**) and jasmolin II (**6**) (Casida & Quistad, 1995). The insecticidal activity of pyrethrum flowers is believed to have been accidentally discovered in the early 19$^{th}$ century by a German who picked flowers for beauty and threw them into a corner. After they withered several weeks later, the flowers were found surrounded by dead insects stimulating the works by pioneers (Casida, 1973).

The pioneer work of Staudinger and Ruzicka, documented in 1924 (Casida and Quistad, 1995), identified the pyrethrins although their assigned structures were later modified. Twenty years later Laforge and Barthel (1944) identified two new insecticidal components of pyrethrum, the cinerins. Godin *et al.*, (1966) determined the presence of the jasmolins. Due to this historical development, the term pyrethrins became accepted as the collective name of the six components with pyrethrins I and pyrethrins II distinguishing the sub classes.




| | $R_1$ | $R_2$ |
|---|---|---|
| 1 | Me | $CH_2CH_2$ |
| 2 | Me | Me |
| 3 | Me | Et |
| 4 | Me | $MeO_2C$ |
| 5 | $MeO_2C$ | Me |
| 6 | $MeO_2C$ | Et |

(Casida & Quistad, 1995).

## 2.1.5 Mode of action of pyrethrins

Pyrethrins are exceptionally potent as repellent, knockdown and killing agents to a variety of insects. They are extremely toxic, killing mainly by penetration of the integument of insects (Roest, 1976). Insects upon contact with pyrethrins become highly agitated. For instance cockroaches run out from their hiding places while mosquitoes and flies seem to buzz faster in dizzy patterns before dropping over (knocked down) (Serge, 1972). The knockdown activity is associated with polar substituents, pyrethrins II that rapidly penetrate into the integument of the insect. However this substance associated with polarity is rapidly metabolized by the insect enzymes thus permitting later recovery (Casida, 1973).

Studies have shown that pyrethrins act on insects and other arthropods on both sensory, motor nerves and on the central nervous system where sensory nerves are generally most sensitive. Pyrethrins are known to interact with sodium, potassium and calcium channels in axonal membranes leading to nerve excitation and resulting to release of neurosecretory hormones such as diuretic which in turn causes changes that may ultimately result in death (Casida and Quistad, 1995). Thus, insects intoxicated with pyrethrins become behaviourally uncoordinated and hyperactive followed by rapid paralysis and death.

### 2.1.6 Properties of Pyrethrins

Pyrethrins have been extensively used for controlling ectoparasitic arthropods of mammals and birds such as tick, fleas, flies, mites; household and public health pests like flies, cockroaches and mosquitoes; and pests of stored products such as beetles, moths and mites (Roest, 1976).

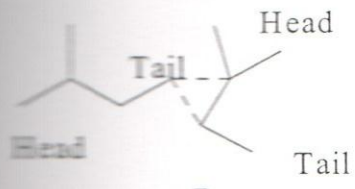
Pyrethrins have been found to be relatively harmless to non-target species and so far considered to be the safest choice in view of their exceptionally low mammalian toxicity (Maciver *et al.*, 1997). They are rapidly degraded in air and by sunlight hence reduction of its bioaccumulation. This short residual effect has led to reduction of environmental hazards and little insect resistance has been recorded (Maciver *et al.*, 1997).

Pyrethrins exhibit an unusual selectivity against target species particularly when used as a mosquito adulticide. Due to its selectivity it has been useful for disinfection of the body, the habitat, and the food for man and his domestic animals (Roest, 1976).

Pyrethrum has a number of disadvantageous properties that in the 1950s saw the advances in synthetic insecticides. Pyrethrins are relatively costly, its supply is limited depending mainly on small-scale farming characterized by non-payments (Roest, 1976). The high cost to some extent is due to synergists and other chemical additives that inhibit photolyisis and oxidation to increase persistence.

### 2.2.7 Biosynthesis of Pyrethrins

Pyrethrins are classified as irregular monoterpenes (7-8) that are derived from isoprene C5 units that do not fit the regular head- to-tail (9) coupling mechanism (Dewick, 2002)

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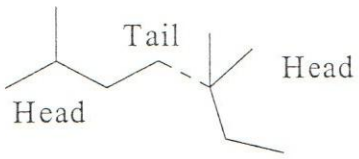


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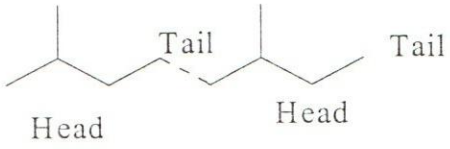


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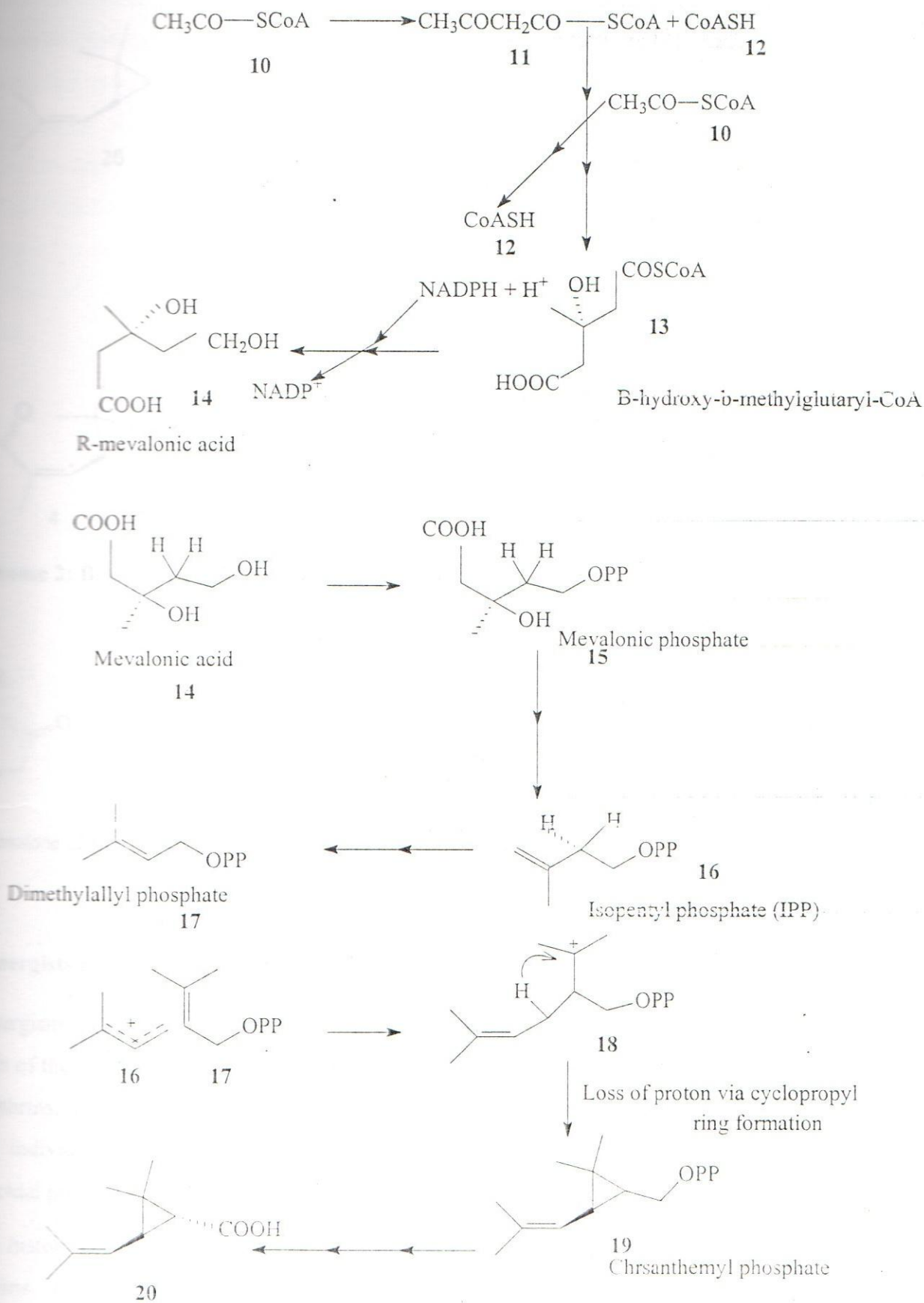
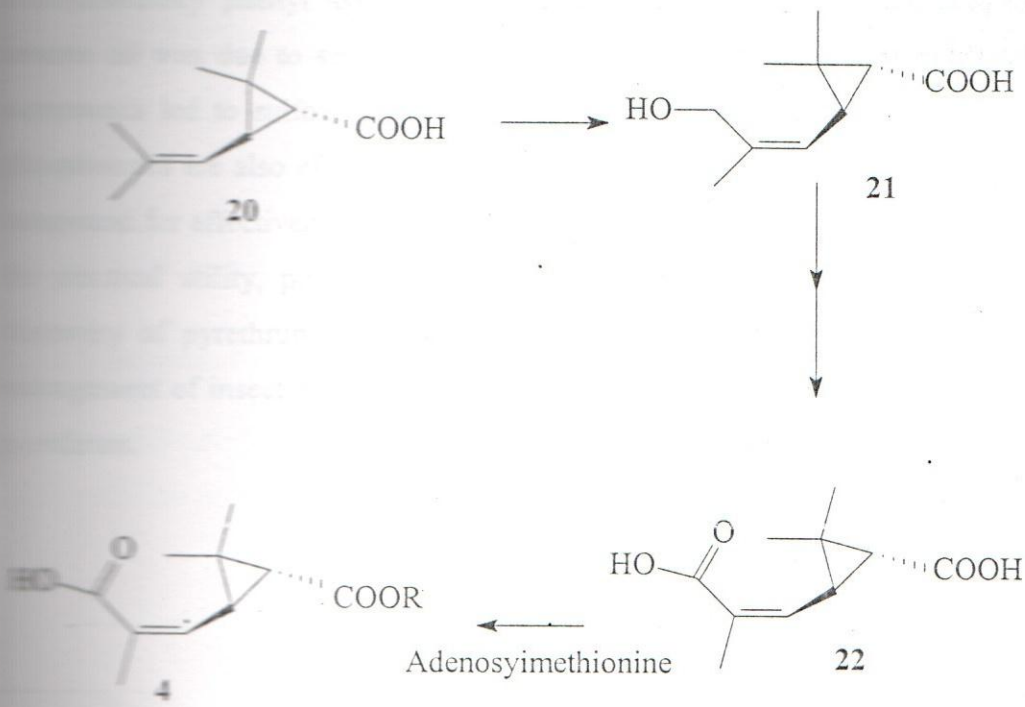
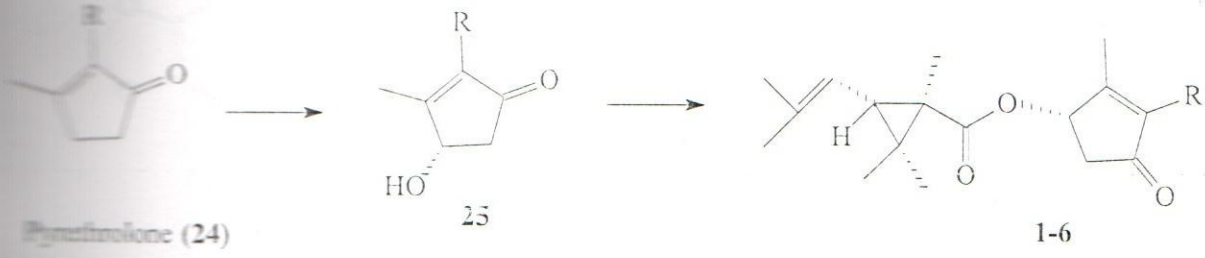
**Scheme 1: Biosynthesis of pyrethrins**




(Casida, 1973; Dewick, 2002)




**Scheme 2: Biosynthesis of alcohol moiety of pyrethrins**

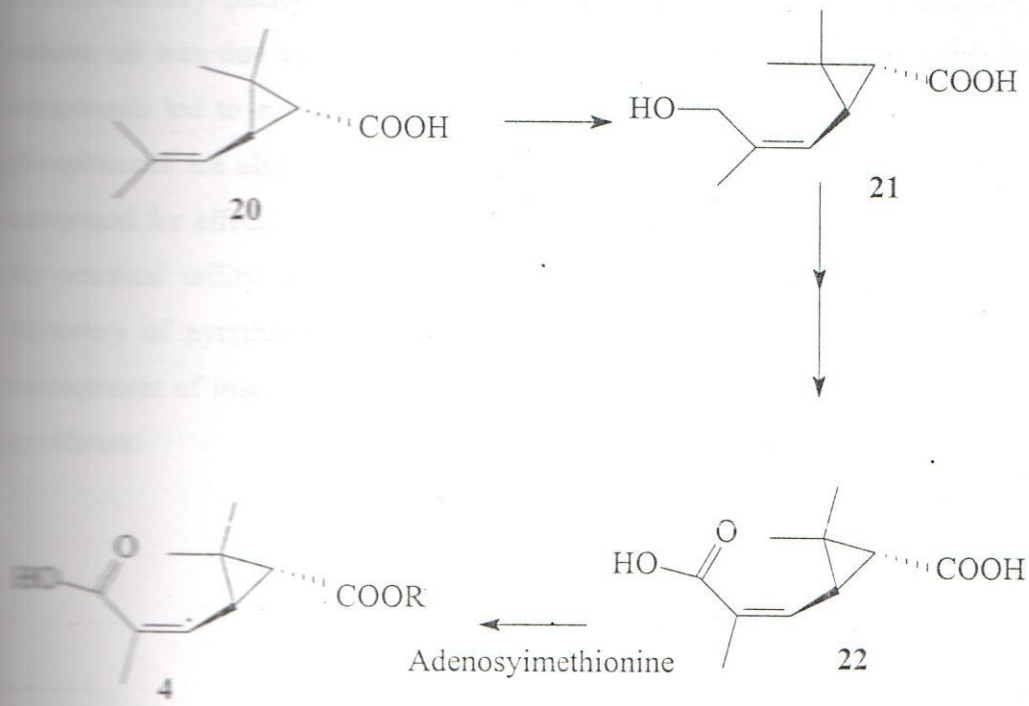



(Casida, 1973; Dewick, 2002)

## 2.2 Synergists and Synergism

Synergism means the cooperation of two agents to produce a total response greater than the sum of their independent effects. Synergists are chemical compounds, which when added to pyrethrins, increase the action of the mixture to a greater degree than the sum of the action of the individual components separately. An insecticidal synergist has marginal or no insecticidal properties of its' own (Hewlett. 1960).

The history of insecticide synergists started with attempts to enhance the potency of the pyrethrins (B-Benard & Philogene, 1993). An observation in 1938 that *N*-isobutylundecylenamide (26) enhanced the insecticidal activity initiated the use of insecticide

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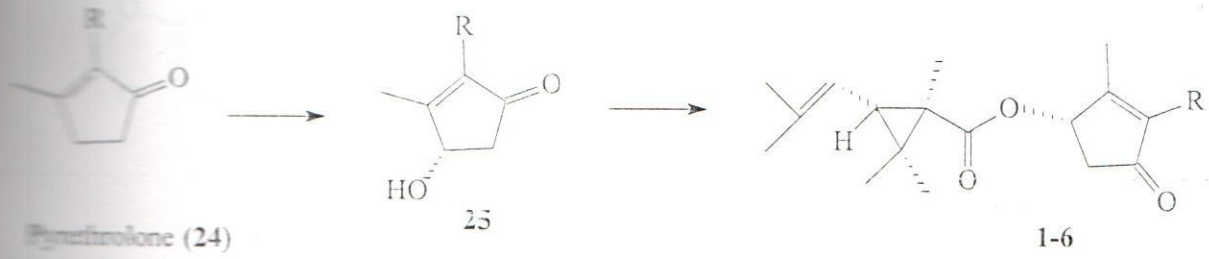


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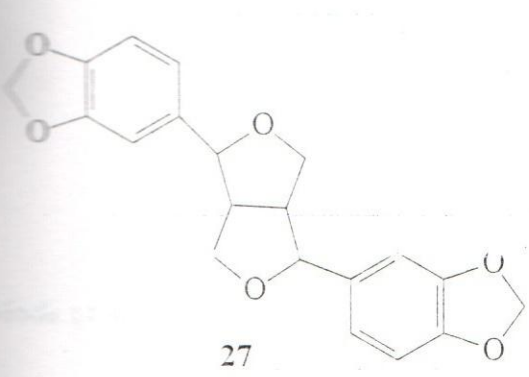
synergists and search for better compounds (Hewlett & Moore, 1958). The discovery of methylenedioxy phenyl synergist started with discovery that the synergistic activity of sesame oil was due to sesamin (**27**) and sesamolin (**28**). Synthesis and testing of related compounds led to sulfoxide, propylisome, tropital and piperonyl butoxide (**29**). Propynyl phosphonates are also effective as are certain amides such as MGK264 (**30**). The sifting of compound for effectiveness, economics and toxicology has led to only two major synergists for practical utility, piperonyl butoxide and MGK264 (Casida & Quistad, 1995). The discovery of pyrethrum synergists has enormously boosted pyrethrum development and management of insect pests since there is greater killing power with more economic use of pyrethrum.

**26**



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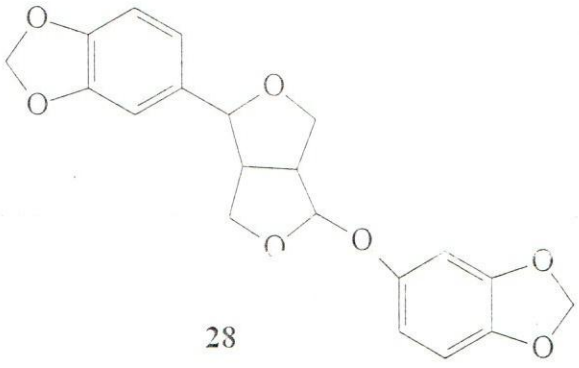

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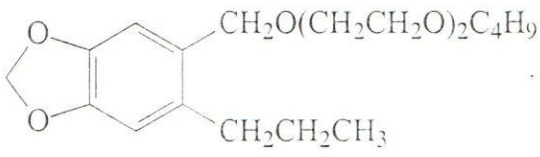

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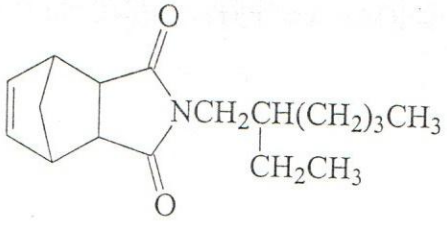


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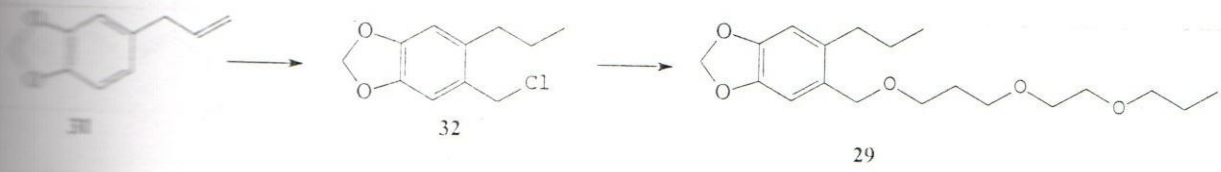
## 2.3 Synthetic commercial Synergist

### 2.3.1 Synthesis of piperonyl butoxide

Piperonyl butoxide is prepared by hydrogenation of safrole (**31**). Chloromethylation, and addition of the butylcarbityl side chain (Scheme 3) (Casida and Quistad, 1995).




31 32 29

**Scheme 3: Synthesis of piperonyl butoxide**

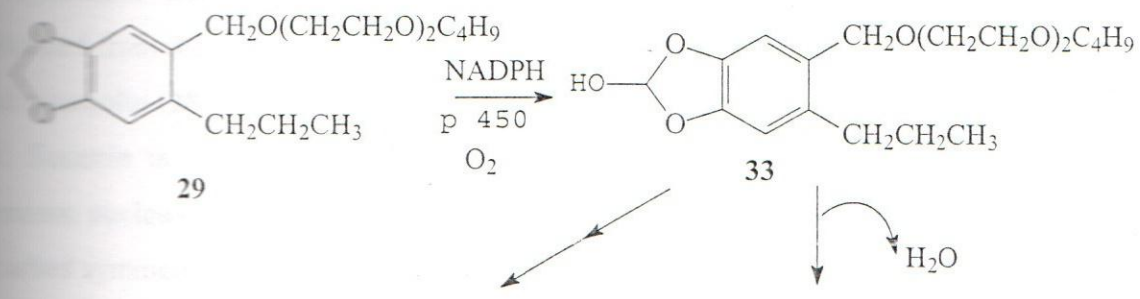
### 2.3.2 Mode of action of piperonyl butoxide

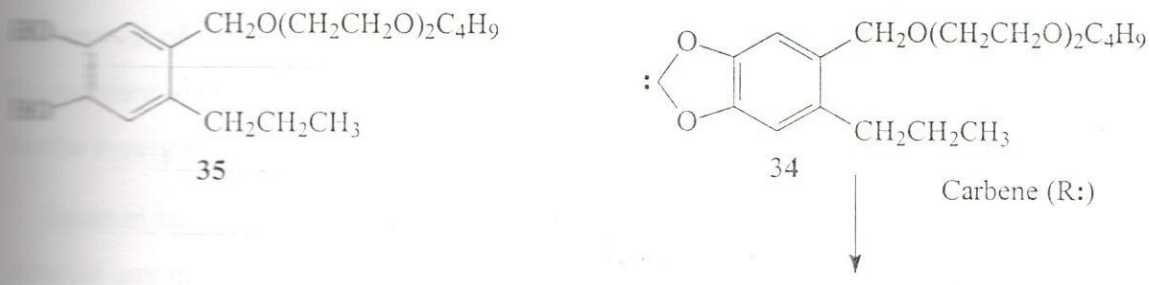
Piperonyl butoxide is a synergist for any insecticide undergoing detoxification by the cytochrome P-450 (**36**) dependent microsomal oxidase (Yamamoto, 1993). It is suggested that metabolism of piperonyl butoxide is significant for synergistic action whereby the methylenedioxy moiety interacts with the iron (Fe) atom of cytochrome P-450 (Metcalf & Metcalf, 1993) forming a metabolite: P-450 complex which blocks the hydroxylating 'OH radicals that detoxify pyrethrins by forming epoxides with double bonds of the side chains and carboxylic acids from methyl groups of the isobutenyl moiety (Scheme 4) (Metcalf & Metcalf, 1993).

The initial rapid inhibition of P-450-mediated metabolic activity is attributed to the formation of a metabolite: P-450 complex (**37**). The best evidence suggests that the

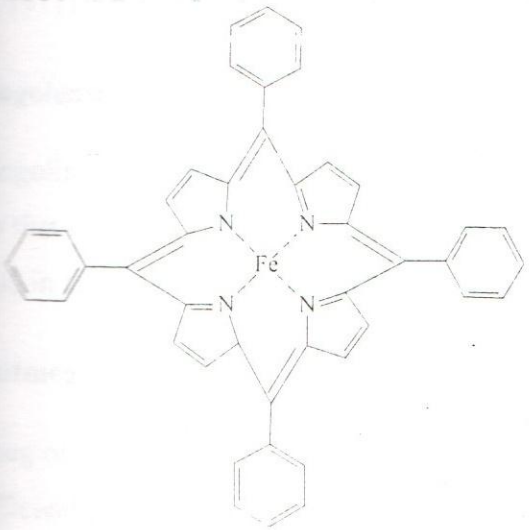
metabolite is a carbene. Since both Oxygen, ($O_2$) and NADPH are required for formation of the complex, oxidation of the methylenedioxy phenyl group probably precedes carbine (**34**) formation. Piperonyl butoxide and other methylenedioxy benzene compounds have been studied and have shown to be both inhibitors and inducers of cytochrome P-450 (Casida and Quistad, 1995).

**Scheme 4: Metabolism of piperonyl butoxide**



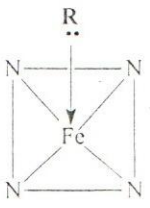




Complex with $Fe^{+}$ of cytochrome P-450




$Fe^{2+}$ —TPP

36




Carbene complex

R⟶ $Fe^{2+}$—Tpp

37

### 2.4 Pyrethrum Synergists from plant sources

These are mostly alkaloids and benzoid extracts (lignans and flavonoids).

#### 2.4.1 Sesame oil

Systematic entomological examination of chromatographic fractions of sesame oil obtained from the seeds of *S. indicum L.*) resulted in two pyrethrum synergists, sesamin (**27**) and sesamolin (**28**), which were found to account for practically all synergistic activity of the oil. Sesamin is a complex substance, which belongs to a class of compounds having a common nucleus composed of two-fused dihydrofuran rings with a substituted phenyl group attached symmetrically to one of the carbon atoms adjacent to each of the other oxygen atoms (lignans) (Budowiski, 1964). Considerable synergistic activity remained in sesame oil after the removal of sesamin and it was found to be due to sesamolin. It is interesting to note that the presence of methylenedioxy phenyl groups in sesamin and sesamolin could be responsible for the synergistic activity (Budowiski, 1964).

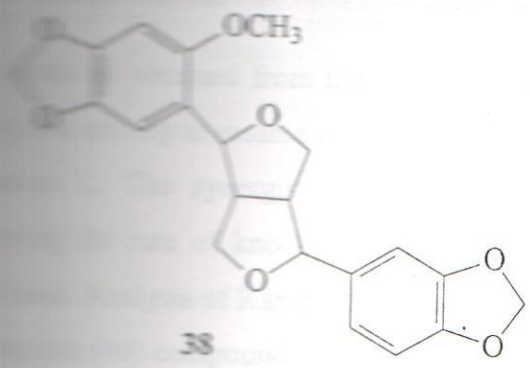
Sesamin has been found to occur in other plants, while sesamolin has not been found to occur in any genus other than *Sesamum*. Sesamin has been isolated from the bark of various *Fagara* species: - *Flindersia pubescens, Chamaecypans obtuse, Ocotea usambarensis*, heartwood of *Ginkgo biloba*, *Evodia micrococca var. pubescens*, and fruit of *Piper guineense* (Weiss, 1971).

#### 2.4.2 Angolense oil

Sesangolin (**38**) has been found in the oil of *Sesamum angolense* has synergistic action equal to that of sesamin and increasing the toxicity of pyrethrins to houseflies approximately three fold in an equiproportional mixture.

#### 2.4.3 Nutmeg oil

Nutmeg oil obtained from the seeds of *Myristica fragrans*, (Myristicaceae) is also known to be efficient as a pyrethrins synergist. The synergistic activity is attributed to the presence of myristicin (**39**) (Dewick, 2002).



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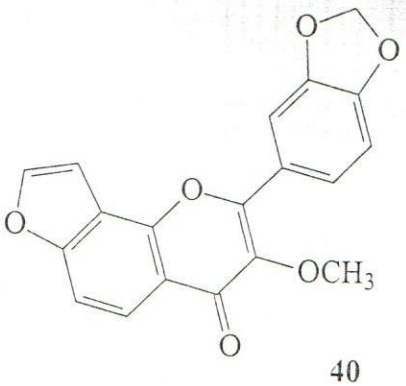

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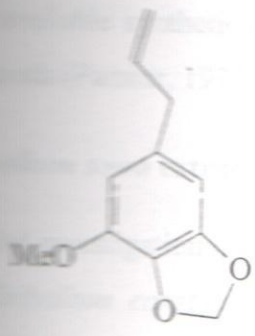

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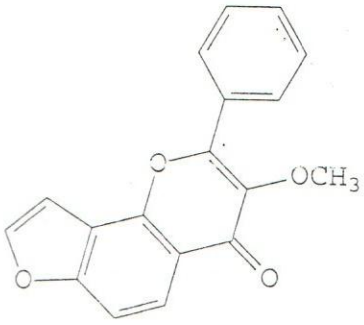

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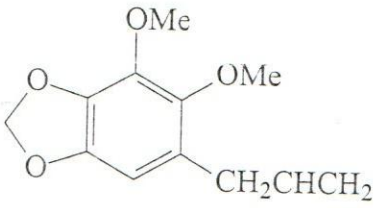

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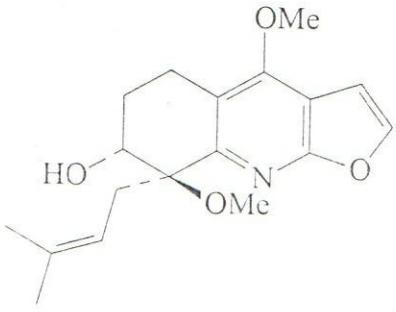


### 2.4.4 Sassafras oil

Safrole (31) a main component of sassafras oil obtained from *Sassafras albidum* (Lauraceae) is known for synergistic activity. Safrole is the precursor for piperonyl butoxide, synthetic synergist commonly used with pyrethrins (Dewick, 2002).

### 2.4.5 Canola oil

Canola oil is an edible vegetable oil obtained from the seeds of *B. napus* and *B. campestris* (Cruciferae). It is used to control insects on a variety of crops. Canola oil is considered safe for human consumption. It is believed to repel insects by altering the outer layer of the leaf surface or by acting as an insect irritant. The specific compound responsible for the synergistic activity has not been discovered (Dewick, 2002).

### Karanja extract

Karanja oil obtained from the seeds of Karanja, *Pongamia glabra* has similar pyrethrins synergistic activity to sesame oil and is more superior to piperonyl butoxide on houseflies, *M. domestica* L. The synergistic activity of karanja oil is equal to piperonyl butoxide in enhancing the rate of knockdown and mortality of the American cockroach, *Periplaneta americana*. Analysis of Karanja oil showed that the synergistic activity is due to the presence of pongapin (40) compound a minor component of oil of *P. glabra*. However, pongapin can become available synthetically in large amounts from karanjin (**41**), a major component of Karanja seeds (Parmar, 1977).

### *Anethum sowa* extract

Detailed investigation of *A. sowa* extract revealed that it exhibits synergism of pyrethrins against *Tribolium constanium*. Dillapiol (**42**) is the compound that synergizes pyrethrins against this test insect (Handa, 1975).

### Hypnotic synergist

Haplophyllidine [**43**] has been used as a hypnotic synergist. The alkaloid has been isolated from the seeds of *Haplophyllum perforatum* and the roots of *Haplophyllum glabrinum* (Rutaceae). Careful analysis of the structure indicates absence of methylenedioxy phenyl group. This confirms the presence of other chemical structures of known pyrethrins synergists, which revealed that besides methylenedioxy phenyl group, a number of other moieties are capable of imparting the property of synergism to various compounds such as pyrethrins (Santos, 1998)

### Other plant extracts

Several plant extracts have been used to synergize pyrethrins against variety of insects (Singh, 1976). These include *Allium sativum* (Liliaceae), *Azadirachta indica* (Meliaceae), *Boswellia serata* (Burseraceae), *Calotropis procera* (Asclepiadaceae), *Citrullus colocynthis* (Cucurbitaceae), *Commiphora mukul* (Burseraceae), *Cymbopogon martini*, *Cymbopogon winterianus* (Gramineae), *Lantana camara* (Verbenaceae), *Mentha aruensis*, *Mentha citrate*, *Mentha piperita*, *Mentha spicata* (Labiatae), *Moringa oleifera* (Moringaceae), *Myristica fragrans* (Myristicaceae), *Nardoa stachys* (Valerianaceae), *Parthenium hysterophorus* (Compositae), *Quisqualis indica* (Combretaceae) and *Salvadora oleoides*, (Salvadoraceae).

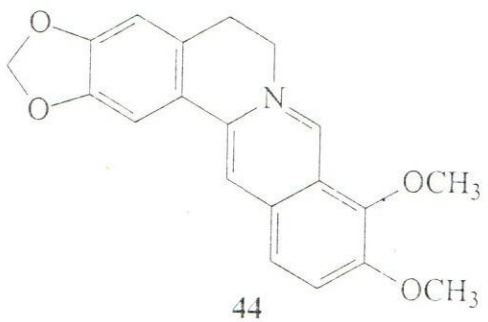
Extracts from *C. mukul*, *C. martini* var *motia*, *Q. indica* and *T. nenifolia* exhibited synergism against *T. castaneum* (Singh *et al.*, 1976)

## The active constituents of plants

### Alkaloids

Alkaloids form a group of low molecular weight nitrogenous natural products that are not simple amines, amides, small peptides and nitrogenous metabolites of primary metabolism (Solomons, 2002). Alkaloids comprise a large group of natural products with diverse structures. Very often alkaloids are abundant in the roots, stem bark, leaves and seeds.

Many alkaloids have been isolated and characterized but only a few have been screened for synergistic activity on common insecticides. However, many alkaloids have structures related to those compounds employed as synergists. Alkaloids contain the methylenedioxy phenyl group, a basic chemical skeleton with the likelihood of having synergistic properties. Berberine [44] occurs in the bark of *Phellodendron amurense* (Rutaceae). Berberine was first isolated from *Berberis vulgaris* (Berberidaceae). Since then, this alkaloid has been isolated from many plants in Papaveraceae among others. Nitidine (**45**) was isolated from the root bark and root of *Z. nitidum* (Rutaceae). The same compound has been isolated from roots of *Z. salicifolia*, *Z. ailanthoides* and stem bark of *Z. americanum* (Rowe, 1989). Piperine (**46**) occurs in the unripe fruit of black pepper, the kernel of the ripe fruit of white pepper (*P. nigrum*) and in the fruit of aschanti (*P. clusii*). It is also found in long pepper (*P. longum*), *P. guineense*, *P. chaba* and the seeds of *Cubeba censii* (Ikan, 1991).

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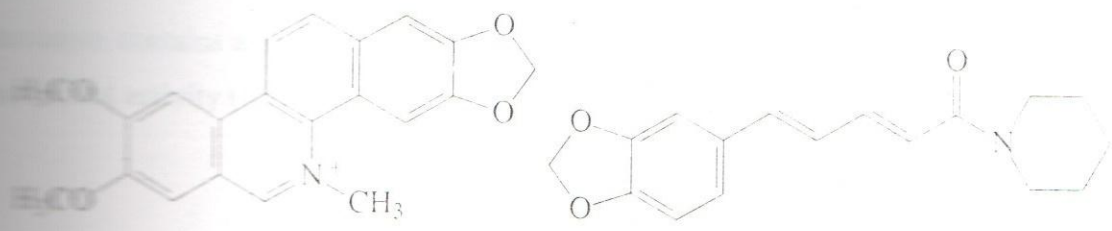
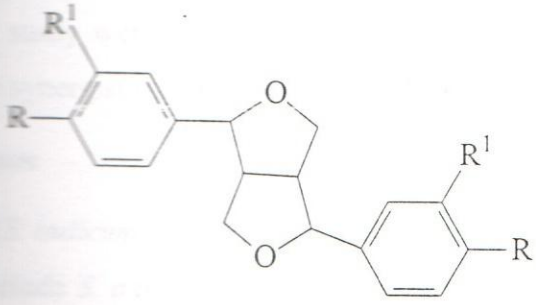


### 2.6.2 Benzenoid extracts

#### 2.6.2.1 Lignans

Lignans and neolignans have varied biological activity. They have been found to act as antifeedants on insects as well as synergists of insecticides (Rowe, 1989). Asarinin, commonly known as xanthoxylin, pinoresinol and eudesamin are examples of lignans that have been investigated as synergists (Haller *et al.*, 1973). Asarinin [**47**] from *Justicia simplex* (Acanthaceae), *Z. alatum* and *Zanthoxylum sp.* (Rutaceae) is related to sesamin, a constituent of sesame seeds. Asarinin is also known to occur in various oriental plants and the bark of American prickly. Pinoresinol [**48**] is a constituent of the exudates of the spruce and related species. However, several epimers are known to occur in various species of plants like *Z. alatum*, *Picea abies* and *P. excelsa*. Eudasamin [**49**] is a constituent of the Kino gum from eucalyptus (Haller *et al.*, 1973).



**47** R, $R^1$ = $O_2CH_2$

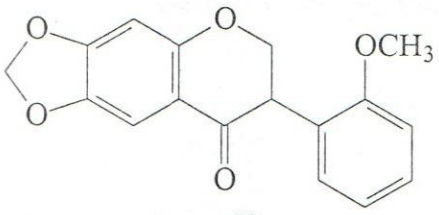
**48** R, $R^1$ = OH

**49** R, $R^1$ = $OCH_3$

#### 2.6.2.2 Flavonoids

Flavonoid compounds are regarded as $C_6$-$C_3$-$C_6$ compounds, in which each $C_6$ moiety is a benzene ring. Flavonoids occur in all parts of plants, including the fruit, pollen roots, and heartwood (Ikan, 1991). There are several classes of flavonoids like flavones, flavonols, flavanones, isoflavones, among others. Examples of compounds that have been known to show insecticidal activity include a tlanlancuayin [**50**] a 3-phenylchromone (isoflavone).

contains a 3,4-methylenedioxy phenyl group that can be suspected to enhance activity (Ikan, 1991).




50

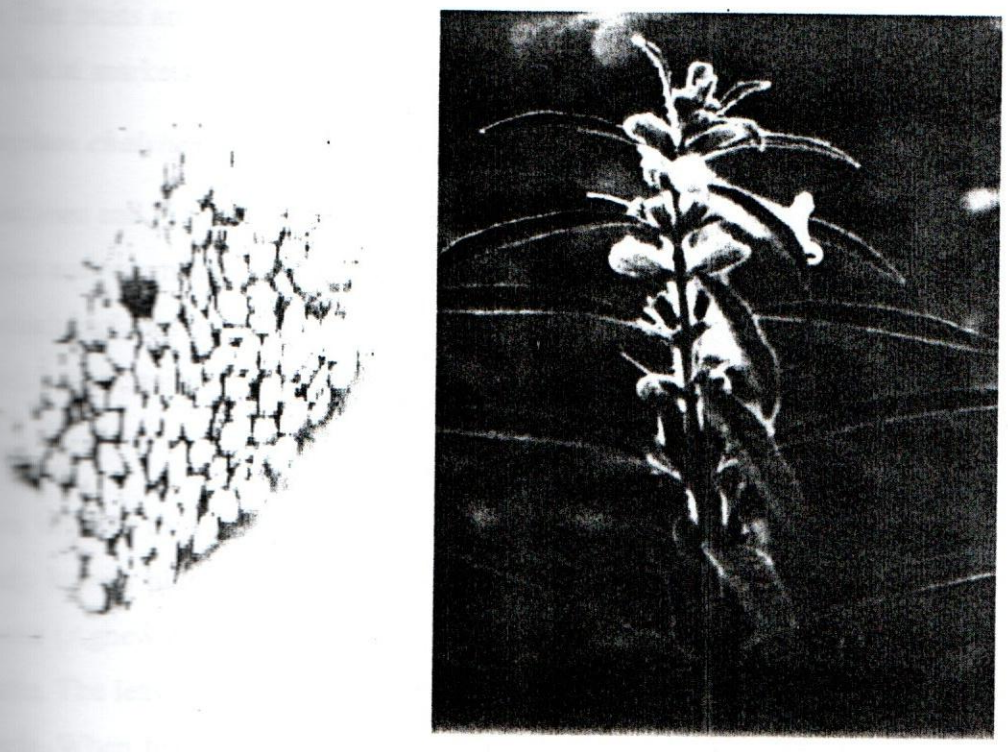
**Plants under study**

Plants have been selected as sources of natural products to be used as pyrethrins synergists. This has been done in view of the compounds employed for synergism. It is evident that plants hold the key in their chemical constitution. An extensive relation between compounds that bear a 3, 4-methylenedioxy phenyl group. The basic framework of chemical structure within which the likelihood of the compound with some synergistic activity is predicted (Elwellett, 1960). Plants that produce these compounds are restricted to some families such as Lamiaceae, Rutaceae, Piperaceae among others (Dewick, 2002). Some plants from the named families were selected for the study with the hope that their extracts contain compounds bearing the 3, 4-methylenedioxy phenyl groups.

Special considerations on the choices of plants for this study were made, bearing in mind the commercialization aspect of the Pyrethrum Board of Kenya's objectives. The plants chosen for this study were locally available and believed to be capable of yielding large quantities of the synergistic compound in question.

***Sesamum indicum***

The sesame (*S. indicum L.*) belongs to the Pedaliaceae family. Other well-known members of the family include *S. angolense* and *Ceratothera sesamoides* (Agnew & Agnew, 1994). It is an annual herb > 1.2 m tall whose leaves are ovate to lancoelate and upto 12 cm long. The upper leaves on the plant are simple whereas the lower leaves are three-lobed. The flowers are purple, pink or whitish, bell shaped, two lipped, upto 3.5 cm angled, about 2.5 cm long and contains whitish, light brown or black seeds. Sesame is planted in warmer parts of East Africa as an oilseed. It is an annual oil crop grown in the coastal and western provinces of Kenya (Schmutterer, 1976).



(a) (b)

Plate 2: Sesame seeds (a) and Sesame plant with flowers (b)

(Katzer/engs/spice photo_html.)

***Brassica napus***

Oil seed rape (*B. napus*) belongs to the Crucifereae (Mustard) family. Other well-known members of the family are *Lunaria annua, Diceratella incana. B. napus* is a hybrid of two species *B. oleäreceae* (kale, cabbage) and *B. campestris* (turnip). A flowering rape has buds at a higher level than the flowers first opened. It's leaves are only half-grasp the stalk. The pod (fruit) is made up of two carpels, which are separated by a false septum, thus providing two chambers. Each pod has 15-40 seeds, at maturity the two carpels are easily split from the false septum thereby shedding the seeds to the ground (Agnew & Agnew, 1994).

***Persea americana***

The avocado (*P. americana*) belongs to the family Lauraceae, a family of mainly tropical trees and shrubs. Other well-known members are laurel, cinnamon, sassafras and greenheart (Samson, 1986). Avocado is a shallow rooted evergreen tree whose leaves are simple, alternate and exstipulate (Dale, 1961). The avocado fruit is generally pear-shaped with a large, round to egg-shaped central seed. The flesh is buttery in texture, contains a high percentage of oil (Samson, 1986). Avocado trees perform well in areas with warm frost-free

largely grown in central highlands. Kenyan farmers produce avocado [illegible] and exports.

**[illegible] and *Vepris uguenensis***

*Z. chalybea* and *V. uguenensis* belong to the Rutaceae family, a large family widespread in [illegible] and warm temperate climates. The Rutaceae family is best known for the genus [illegible], which includes oranges, limes and grapefruit. Glands producing aromatic essential oils [illegible] this family (Noad, 1989)

**[illegible]**

*A. vera* belongs to Aloaceae family, a family that comprises of more than 40 plants. The [illegible] known plants of the Aloe species include *A. tugenensis, A. turkanensis* and *A.* [illegible] (Agnew & Agnew, 1994). *A. vera* is a perennial herb whose leaves are fleshy and [illegible]. The leaves are usually more or less sickle-shaped whose margins are armed with [illegible] teeth. When the leaves are broken a bitter tasting yellow or brown juice is seen. This [illegible] is cited to contain > 200 compounds and it can be interesting to note that one the [illegible] would be synergistically active to pyrethrins.

***Piper nigrum***

*P. nigrum* belongs to the Piperaceae family. It is distributed in the tropical and subtropical [illegible] of the world (Kirtikar & Basu, 1981). It is a shrubby herb often hairless consisting of [illegible] nodes and mainly used as a condiment and medicinal agent (Parmar *et al.*, 1997). The [illegible] of this plant are alternate with stipules joined to the leaf stalk (Agnew & Agnew, [illegible]). Fruits are usually a berry and stalked or sunk in the axis of the spike. The most [illegible] *Piper* species include *P. guineese, P. capense, P. umbellatum, P. longum,* and *P.* [illegible] (Ikan, 1991).

Plate 3: *P. nigrum* plant with unripe fruit
(Katzer/engs/spice photo_html.)

### 2.9 Insects

Insects possess the ability to counteract pyrethrins that would have otherwise killed them. Therefore, it is appropriate that synergists that work by preventing insects from counteracting pyrethrins are required for different species of insects. Adult mosquitoes possess poor ability to counteract pyrethrins and the addition of large quantities of synergist is unnecessary and wasteful. On the other hand, insects such as houseflies have a relatively greater ability to counteract pyrethrins and the addition of a larger quantity of synergist would be appropriate (Sorge, 1972).

### 2.9.1 Houseflies

The common housefly is a thoroughly cosmopolitan insect that is present in nearly every habitation in the world. The housefly, *M. domestica* Linne, belongs to the order Diptera. The dipterans are a well-marked group, which include flies, mosquitoes, gnats and midges. *M. domestica* L. is best known and most important representative of the Muscidae family which comprise of flies that are short, weak-skinned, and not very bristly but never bare with ample wings (Metcalf & Metcalf, 1993).

The housefly's inordinate curiosity leads it to go everywhere and to feed on almost anything, making it dangerous. From manure piles, sewage, garbage, and carcasses of animals food of all kinds in dining room, kitchen, restaurant, and grocery to the lips, eyes,

and nursing bottles of sleeping children is all in the day's work for a busy housefly (Maciver et al., 1997). This promiscuous habits result in polluting their bodies with filth and shedding it on their paths, wherever they go making them ideal agents for the transfer of disease causing organisms. Houseflies are naturally infected with the pathogens of more than twenty human diseases, and are believed to be important vectors of typhoid fever, epidemic or summer diarrhea, amoebic and bacillary dysentery, cholera, poliomyelitis and various parasitic worms. The housefly is a major factor in the spread of the *Trachoma* virus, it is associated with *Haemophilus bacterium* that afflicts eighty million persons and is a major cause of blindness (Metcalf & Metcalf, 1993). However, it must be understood that the housefly is not the sole vector of any of these diseases.

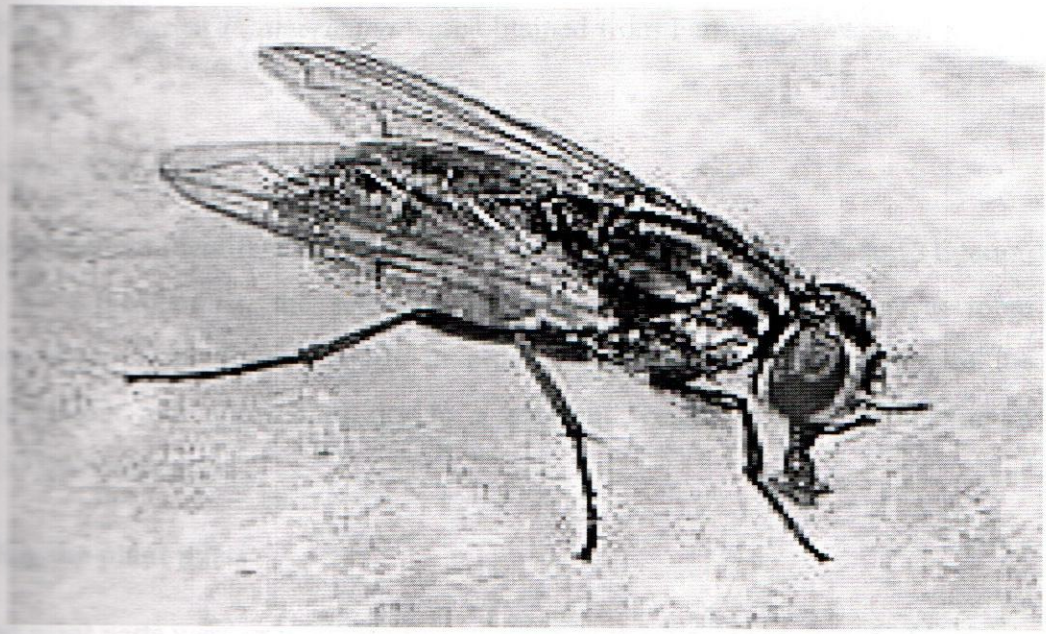


Plate 4: Housefly, *M. domestica* L.

(Plate by Windows picture and fax viewer)

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Collection of plant parts

Seeds of *B. napus* were provided by the Kenya Agricultural Research Institute (K.A.R.I), Njoro. Seeds of *S. indicum*, which originated from Kakamega and those of *Piper nigrum*, which originated from Mombasa were purchased from Nakuru market. Root bark of *V. uguenensis* and seeds of *Z. chalybea* were obtained from Baringo. The Department of Entomology, Pyrethrum Board of Kenya, Nakuru provided leaves of *A.* sp. whereas ripe fruits of *P. americana* were obtained from Farming Systems of Kenya, Nakuru branch.

## 3.2 Drying the plant parts

Following positive identification of the plants in the Department of Botany, Egerton University by a taxonomist, the selected plant parts were allowed to dry at room temperature. Away from direct sunlight in order to avoid any decomposition of the compounds present and allowed to dry to a constant weight to enhance maximum extraction of the compounds.

## 3.3 Grinding the plant parts

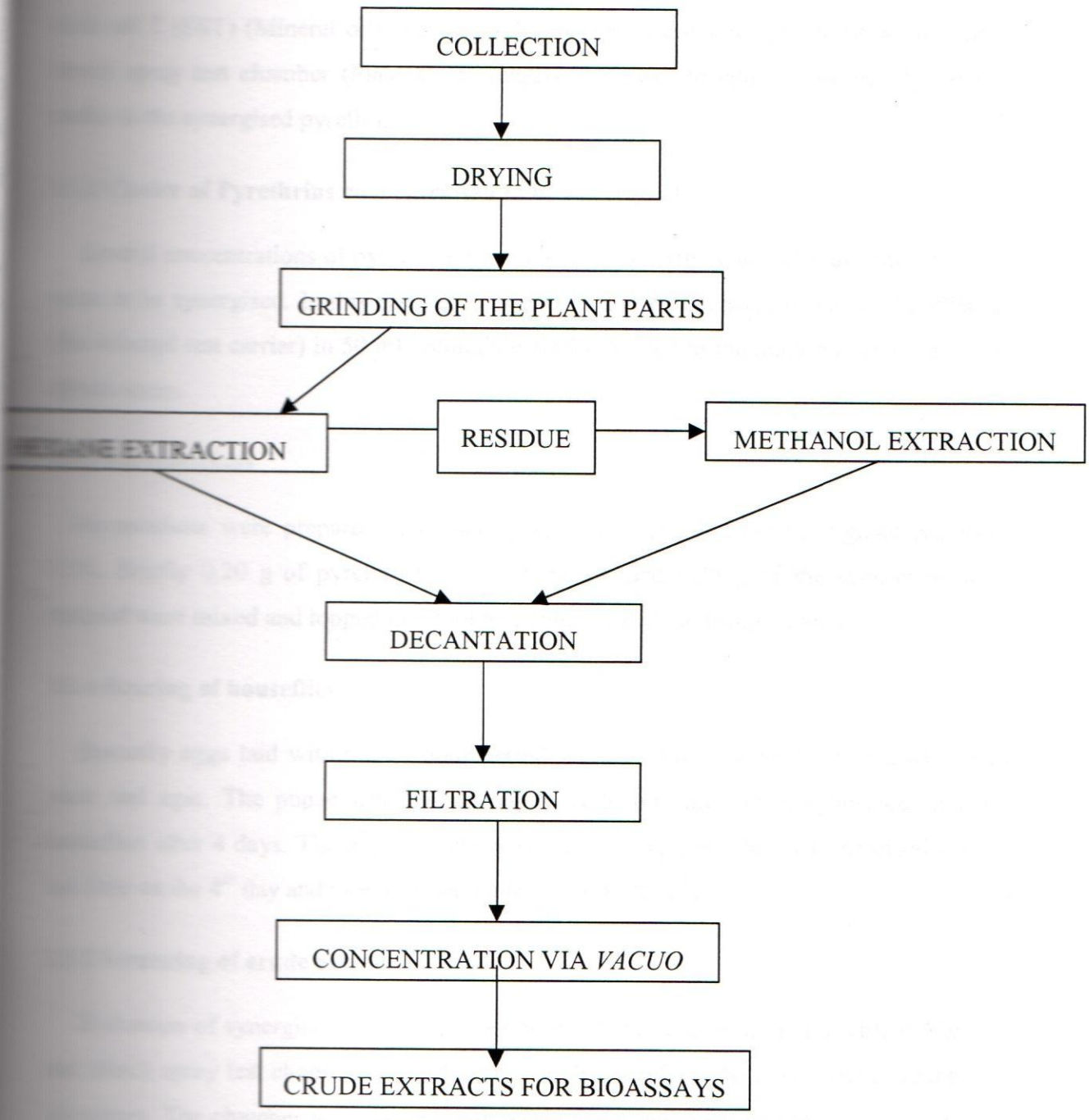
The dried plant parts were ground into fine powders at the Pyrethrum Board of Kenya's milling room to increase the surface area for maximum extraction of compounds. The grinding mills were cleaned thoroughly before the grinding process began to avoid mixing of the materials. Weights of the ground plant materials were recorded.

## 3.4 Extraction

Five ground plant materials were extracted, first with hexane at room temperature for 72 hours to allow for maximum extraction the extract decanted and filtered. The residue was again soaked in methanol and allowed to stand for another period of 72 hours at room temperature, the methanol extract decanted and filtered. Both hexane and methanol extracts were evaporated under reduced pressure to give crude extracts that were stored in well-labeled containers and preserved at 4 $^{o}$C. The root bark of *V. uguenensis* was

[illegible] with chloroform and methanol (9:1) whereas the seeds of *P. nigrum* were [illegible] with ethanol (Figure 1). Hexane was employed to extract non-polar compounds [illegible] methanol was used to extract the polar compounds.

Figure 1: Extraction scheme of the plant material



### 3.5 Screening bioassays

#### 3.5.1 Selection of test carrier media

To present synergised pyrethrins to any test insect requires a carrier medium, which is usually a solvent with no effect of their own on the houseflies. Ethanol, isopropyl alcohol, shell sol T (SST) (Mineral oil) and isopar M solvents were screened in the Kearns and Mutch spray test chamber (Plate 4) for relative toxicities to adult houseflies as carrier media to the synergised pyrethrins.

#### 3.5.2 Choice of Pyrethrins concentration to be synergised

Several concentrations of pyrethrins were tested to determine an optimal concentration value to be synergised. In this case, 0.05, 0.1, 0.15 and 0.20% were dissolved in ethanol (the selected test carrier) in 50 ml volumetric flasks, topped to the mark and evaluated for effectiveness.

#### 3.5.3 Formulations

Formulations were prepared to contain pyrethrins 0.1% and the synergistic material 0.5%. Briefly 0.20 g of pyrethrins 25% concentrate and 0.25 g of the synergistic test material were mixed and topped to 50 ml in a volumetric flask using ethanol.

#### 3.5.4 Rearing of houseflies

Housefly eggs laid within a 24-hour period were placed on a medium of milk powder, yeast and agar. The pupae weighed averagely 20.5 mg and metamorphosized into adult houseflies after 4 days. The adult insects were kept in cages and fed with fresh milk for the last time on the 4$^{th}$ day and used in insecticidal assays after 1 h.

#### 3.5.5 Screening of crude extracts

Evaluation of synergistic activity of the crude extracts was done in a modified Kearns and Mutch spray test chamber (Plate 5) using adult houseflies, *M. domestica* L. reared in laboratory. The chamber was washed with warm water and detergent before being dried. The spray jets were rinsed with isopropyl alcohol and acetone. The inner sides of the wooden doors of the chamber were lagged with clean white paper. Spray solution, (0.2 ml) to be tested was pipetted into the opposite spray jets and compressed air pump supplying

to the pressure gauge was connected using gate valves and pressure allowed to build up. Adult houseflies (60-90) were introduced and the chamber window closed. The timer was set before the spray gadgets were pressed to deliver the test sample twice in a quick succession. The number of houseflies knocked down were counted and recorded after every 1 min. for the next 10 min. At the end of each experiment the insects used were collected, put in clean and sterilized jars, supplied with fresh food and transferred to a temperature controlled post-test recovery room. The mortality (kill) for houseflies after twenty four hours were assessed and recorded.

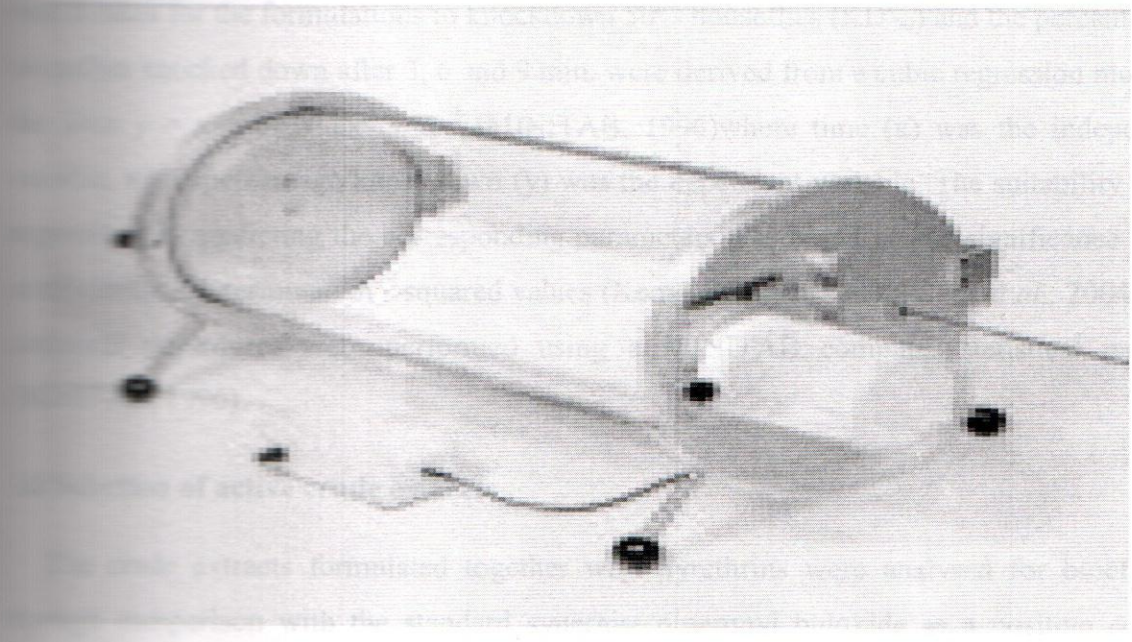


Plate 5: Kearns and Match spray test chamber

(Windows by picture and fax viewer)

The performance of the test sample in the 3rd, 6th and 9th min. the time it took to knockdown 50% of the houseflies ($KD^{t}_{50}$); the regressed performance and percent mortalities after 24 hours were employed in determining the bioefficacies of the different samples. The $KD^{t}_{50}$ and percent kill after 24 hours were compared with those specified by the Kenya Bureau of Standards for insecticides (Kenya Standard, 2000). The regressed percent knockdown after 3, 6 and 9 min. were used to categorize the samples as early, moderate, late and very poor knockdown formulations (Sum *et al.*, 2004). Early knockdown samples consisted of those formulations that scored over 50% knockdown within the 3rd minute. The moderate knockdown formulation were those that scored between 40-50% within the 3rd minute whereas late knockdown formulations were those that scored between 30-40% within

the 3rd minute while poor knockdown formulations were those that scored less than 30% knockdown.

**3.7 Data analysis**

The knocked down and killed insect percentages were transformed to normalize the variance and the means compared using a balanced analysis of variance procedure. Least significant difference (LSD) tests were used to make comparisons of knockdown based on the means of the 3rd, 6th and 9th at 95% confidence limits (Sum *et al.*, 2004). The expected time it takes for the formulations to knockdown 50% houseflies ($KD^{t}_{50}$) and the percentage of houseflies knocked down after 3, 6 and 9 min. were derived from a cubic regression model of the form $y = a + bx + cx^2 + dx^3$ (MINITAB, 1996)where time (x) was the independent variable, while percentage knockdown (y) was the dependent variable. The suitability of the regressions in predicting the corresponding parameters was based on the significance of the coefficients of determination r-squared values (Kenya Standard, 2000; Sum *et al.*, 2004). The statistical procedures were performed using a MINITAB computer statistical package (MINITAB, 1996).

**3.8 Selection of active crude extracts**

The crude extracts formulated together with pyrethrins were analysed for bioefficacy through comparison with the standard synergist piperonyl butoxide as a positive control, unsynergised pyrethrins 0.1% as the negative control and the specification for insecticides by the Kenya bureau of standards (Kenya Standard, 2000).

**3.9 Fractionation of active extracts**

The TLC analysis was used to select solvent systems for fractionation and column chromatographic analysis.

**3.10 Spectroscopic analysis**

The purified fractions were characterized at the School of Pure and Applied Sciences, University of KwaZulu Natal-Durban where the NMR spectroscopy was carried out using a Varian Gemini 300 MHz spectrometer with the chemical shifts recorded in ppm relative to TMS. The spectra were recorded at room temperature with deuterated chloroform [$CDCl_3$] The infrared spectra were recorded using a Nicolet impact 400D Fourier – Transform Infra – red (FT – IR) spectrometer. The solvents were dissolved in dichloromethane and analysed on

a NaCl window. The spectra were calibrated against an air background and the low-resolution mass spectra obtained for compounds recorded on an Agilent MS 5975 instrument connected to GC 6890. Melting points of the crystalline compound were determined on a Kolfer micro_hot stage melting point apparatus.

# CHAPTER FOUR

# EFFECTS OF SOLVENT AND PLANT EXTRACTS ON EFFICACY OF PYRETHRINS AGAINST THE HOUSEFLY, *MUSCA. DOMESTICA* L. (DIPTERA: MUSCIDAE)

## 4.1 Abstract

Formulations containing pyrethrins 0.1% (w/v) and 14 crude plant extracts as synergists (1:5) were prepared in ethanol and evaluated for bioefficacy against houseflies, *M. domestica* L. in the modified Kearns and Match spray test chamber. Ethanol was selected as the least toxic from solvents that were evaluated for effects against houseflies. The performances of the various plants extracts were evaluated through comparisons with standard synergist piperonyl butoxide as the positive control, unsynergised pyrethrins as the negative control and the specifications of insecticides by the Kenya Bureau of Standards. Pyrethrins mixtures at 0.05, 0.1, 0.15 and 0.2% w/v were screened for bioefficacy against the housefly to select a concentration to be used as the negative control. The knockdown time, $KD^{t}_{50}$ and mortality effects after 24 h of exposure that each concentration had on the housefly were analysed. Pyrethrins 0.1% w/v was selected for the study due to its moderate effectiveness both in knockdown and mortality effects. Formulations containing pyrethrins 0.1%, plant extracts as synergists and ethanol as the carrier medium were carefully evaluated and comparative analysis of the obtained data revealed that five plant crude extracts (D, F, I, P and U) exhibited synergism to the pyrethrins 0.1% w/v against the housefly and accordingly, P and U were superior as pyrethrins synergists.

## 4.2 Introduction

Solvents are usually used as carriers to present the synergised pyrethrins to the test insect. However, some solvents have contributed significantly to the overall toxicity effect observed for the drugs, pesticides and insecticides. Therefore, there is need to screen various solvents and select one that have minimal or no effect of its own to the test (Pyrethrum post, 1990). However, measure of this effect is tolerated in bioassays at < 5% kill after 24 h, where in cases the control mortality is between 5-20% the analysis has to be compensated through the use of the Abbot's formula as follows: -

$$K = \frac{P - C}{100 - C} * 100$$

Where: - P is the kill due to drug plus solvent (treatment effect), C is due to the solvent alone (control mortality) and, K is the adjusted mortality percent effect (Malaysian Standard, 1971).

Activities of synergised pyrethrins against several insect pests have been documented (Singh *et al.*, 1976; Maciver *et al.*, 1997). In the studies of the synergistic effects of various compounds to pyrethrins it has been found that there exists an optimum biological ratio for different pest species and probably for each individual synergist (Nash, 1954). Piperonyl butoxide is the main synergist employed to boost efficacy of low levels of pyrethrins by binding onto the cytochrome P-450 dependent microsomal oxidase, the defense mechanism employed by the insects in counteracting pyrethrins (Hamilton, 1995). However, there is need to replace piperonyl butoxide with a plant based synergist due to its increased toxicities, high market prices and shortage in its supply (Casida & Quistad, 1995). Several plants based synergists such as sesamin, sesamolin, haplophyllidine, safrole, dillapiole, karanjin, sesangolin and myristicin have been employed together with pyrethrins. However, none of these have the viability equivalent to that of piperonyl butoxide (Budowiski, 1964; Handa, 1975; Parmar, 1977; Santos, 1998). In this investigation, *B. napus, Z. chalybea, V. uguenensis, P. americana, S. indicum, A. vera* and *P. nigrum* from different plant families were selected for the study. Some of these plants have been cited to produce compounds with the 3, 4 – methylene dioxy phenyl group, a structural framework known to be responsible for the synergistic activity of piperonyl butoxide and other synergists (Casida & Quistad, 1995).

Unsynergised pyrethrins 0.3% (w/v) are known to produce 100% mortality against housefly in the modified Kearns and Match spray test chamber (Formulating Pyrethrum, 1996). In addition, a measure of the performance of any insecticidal formulation against household flying insects such as houseflies as per the specifications of the Kenya Standards should have a knockdown of < 50% in 5-9 min. and mortality of < 95% in 24 hours (Kenya standard, 2000).

With a low performance of unsynergised pyrethrins, the addition of the synergistic crude extracts that are non-insecticidal may increase bioefficacy. However, the ratio of formulations between the unsynergised pyrethrins and the synergistic materials is crucial just as the concentration of the unsynergised pyrethrins. Unsynergised formulations are rarely

applied for the control of insect pests, because it has been found that compounds that confer synergism enable the use of pyrethrins in very small quantities. Various ratios of synergists to pyrethrins exert different performances (Nash, 1954; Formulating Pyrethrum, 1987), the actual ratio for the synergistic samples needs to be evaluated. Evaluations of increasing the ratio of the synergistic test material to the unsynergised pyrethrins 0.1% to 8:1 as well as decreasing the ratio to 2:1 is intended to determine the linearity of the performance of the synergistic test material and whether lower levels of pyrethrins with the synergist would give the desired results.

The crude plant extracts were screened for insecticidal activity, because synergists being chemical compounds which when added to pyrethrins increase the biological activity of the mixture to a greater degree than the sum of the bioefficacy of the individual components used separately should have marginal or no insecticidal properties of its own (Kenya Standard, 2000; Hewlett, 1960). The intrinsic toxicities of the crude plant extracts to housefly are achieved by subjecting the crude plant extracts to similar tests as insecticides.

## 4.3 Materials and Methods

### 4.3.1 Effects of solvents to the houseflies

Samples of the four solvents namely EtOH, IPA, SST and isopar M were obtained from the formulations store of Pyrethrum Board of Kenya and screened to be used as carriers of synergised pyrethrins against the houseflies. $CO_2$ was analyzed for its effects on the houseflies to be used in the collection of the houseflies from the Kearns and Match test chamber.

### 4.3.2 Plant extracts

The plant extracts to be used as synergists to pyrethrins were extracted by the relevant solvents and given codes for easy identification (Table 1). Likewise, any formulation containing either of the plant extracts was given code similar to that code of the plant extract.

Table 1: Codes given to the different plant extracts

| Code | Extract | Code | Extract |
|---|---|---|---|
| **A** | MeOH extract of *S. indicum* | **I** | Hexane extract of *B. napus* |
| **B** | Hexane extract of *B. napus* | **VC** | $CHCl_3$/MeOH extract of *A. vera* |
| **C** | MeOH extract of *P. americana* | **VH** | Hexane extract of *A. vera* |
| **D** | Hexane extract of *S. indicum* | **VM** | MeOH extract of *A. vera* |
| **E** | MeOH extract of *Z. chalybea* | **P** | Ethanol extract of *P. nigrum* |
| **F** | Hexane extract of *P. americana* | **U** | $CHCl_3$/MeOH extract of *V. uguenensis* |
| **G** | MeOH extract of *P. longum* | **STD** | Piperonyl butoxide |
| **H** | Hexane extract of *Z. chalybea* | | |

### 4.3.3 Factor of synergism

**a) The knockdown factor of synergism (*S*)**

$$S = \frac{KD^t_{50} \text{ of unsynergised pyrethrins}}{KD^t_{50} \text{ of synergised pyrethrins}}$$

It was assumed that unsynergised pyrethrins 0.1% have an *S* factor of 1, then the higher the figure the greater the synergism.

**b) The mortality factor of synergism (*M*)**

$$M = \frac{\text{Per cent kill due to synergised Pyrethrins 0.1\%}}{\text{Per cent kill due to unsynergised Pyrethrins 0.1\%}}$$

It was assumed that unsynergised pyrethrins 0.1% has *M* factor of 1, then the higher the figure the greater the synergism.

### 4.4 Results

### 4.4.1 $KD^t_{50}$ and percent knockdown of the solvents exposed to houseflies, *M. domestica* L.

Irrespective of the time of exposure, ethanol recorded no knockdown effect to the houseflies. IPA and isopar M had no knockdown effect in the 3rd min. but recorded increased knockdown effects in the 6th and 9th min. SST was the most toxic solvent recording high

levels of knockdown at all the three levels compared to ethanol, IPA and isopar M (Table 2). The $KD^{t}_{50}$ of SST was higher than those of other solvents (Figure 2). The $KD^{t}_{50}$ of SST is 8.32 min. whereas it would take an infinite time for ethanol, IPA and isopar M to achieve a knockdown effect of 50% houseflies. The effect of ethanol with time to the houseflies was constant whereas the effects of the IPA, SST and isopar M solvents increased with time.

### 4.4.2 Mean percent mortalities of the solvents against houseflies, *M. domestica* L.

Isopar M was the superior killing solvent with mean percent mortality of 17.40 compared to all others tested. This was followed by SST (3.70), IPA (1.17) and ethanol, which showed the least mortality (0.95). Carbon dioxide ($CO_2$) gas had very low kill effects at 0.51% (Figure 3). There were significant difference between isopar M on one hand and the rest of the solvents on the other. Though there were variations in the level of kill between SST, IPA and ethanol, their performances were not significantly different from each other.

Table 2: $KD^{t}_{50}$ and percent knockdown performances of the solvents exposed to houseflies, *M. domestica* L.

| | | | | % KD | | |
|---|---|---|---|---|---|---|
| **Solvents** | **Polynomial equation** | **$r^2$** | **$KD^{t}_{50}$** | **3 Min** | **6 Min** | **9 Min** |
| **Ethanol** | $Y = 0.00 - 0.00X + 0.00X^2 - 0.00X^3$ | 0.999 | Infinity | 0.00 (0.00$^{b}$) | 0.00 (0.00$^{c}$) | 0.00 (0.00$^{c}$) |
| **IPA** | $Y = 1.47 - 1.58X + 0.39X^2 - 2.20E\text{-}02X^3$ | 0.324 | Infinity | 0.34 (0.00$^{b}$) | 1.32 (0.90$^{c}$) | 2.88 (2.70$^{c}$) |
| **Isopar M** | $Y = 0.40 - 1.65X + 0.76X^2 - 3.14E\text{-}02X^3$ | 0.8520 | Infinity | 1.44 (0.00$^{b}$) | 11.05 (13.20$^{b}$) | 24.13 (23.15$^{b}$) |
| **SST** | $Y = 1.86 - 4.09X + 2.27X^2 - 0.13X^3$ | 0.973 | 8.32 | 6.49 (4.65$^{a}$) | 30.62 (27.35$^{a}$) | 52.82 (54.20$^{a}$) |
| **$CO_2$** | $Y = 0.00 - 0.00X + 0.00X^2 - 0.00X^3$ | 0.999 | Infinity | 0.00 (0.00$^{b}$) | 0.00 (0.00$^{b}$) | 0.00 (0.00$^{b}$) |

Figures in parenthesis represent the actual figures obtained whereas the others represent the fitted values as per the equation of regression. Any two means in the same column sharing a common letter are not significantly different at 5% level (LSD test).

Figure 2: $KD^{t}_{50}$ and percent knockdown performance of solvents against houseflies, *M. domestica* L.

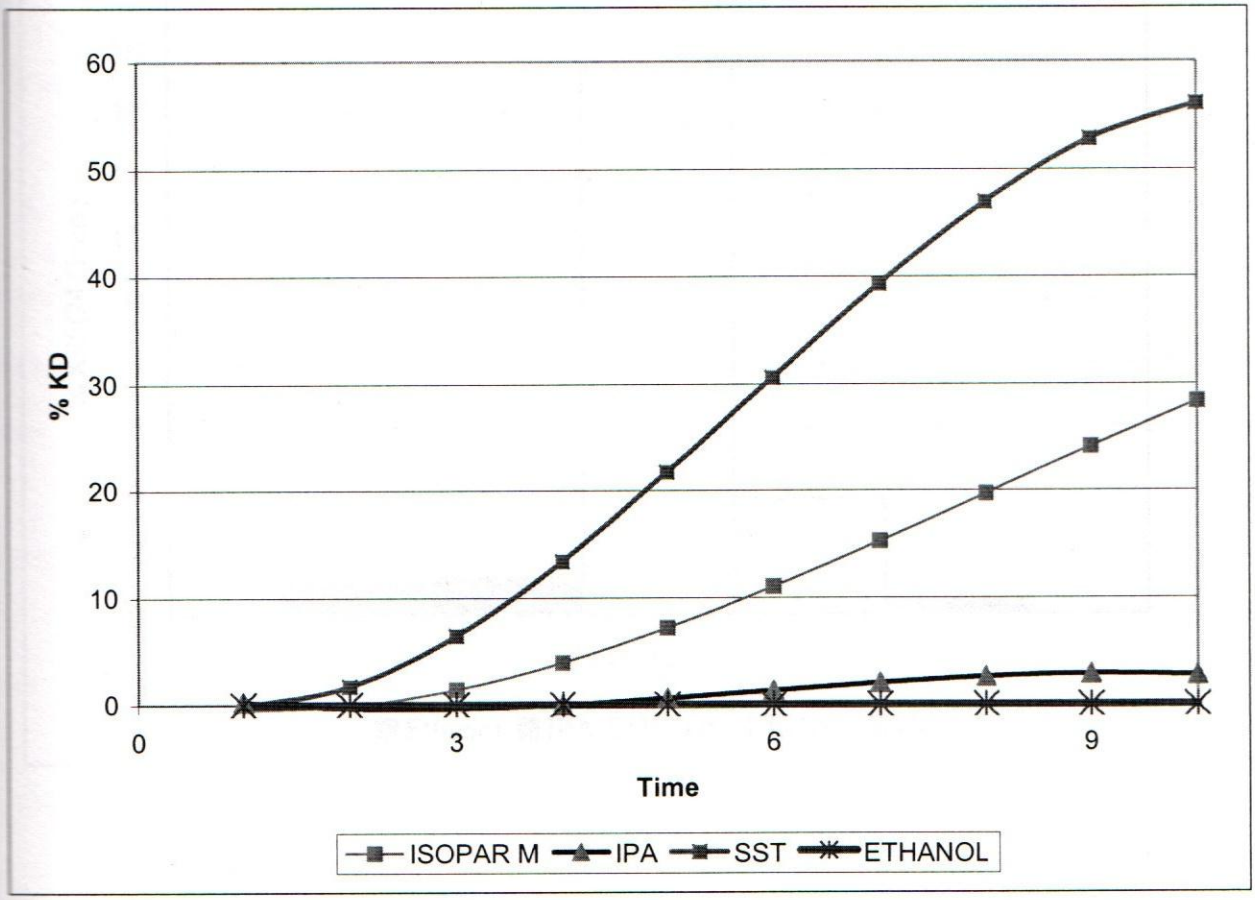
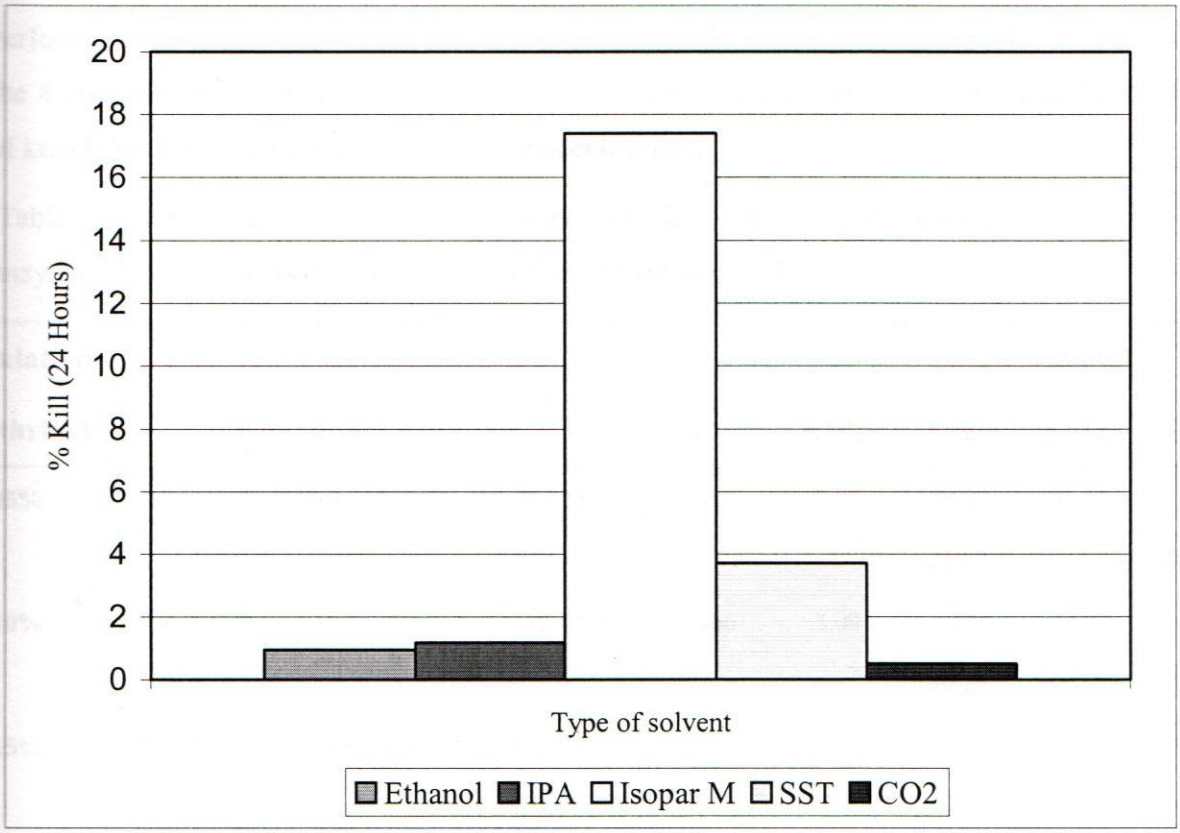


Figure 3: Percent Mortalities of houseflies, *M. domestica* L. exposed to different types of solvents




### 4.4.3 $KD^{t}_{50}$ and percent knockdown of unsynergised pyrethrins against houseflies

The study revealed that unsynergised pyrethrins 0.05% formulation is the least potent knockdown agent compared to the other three levels of concentrations of the unsynergised pyrethrins. In the 3rd min. pyrethrins 0.05% recorded a mean performance of 23.13% which was exactly half the performance of pyrethrins 0.1% with a mean performance of 48.80%. It is interesting to note that the performance of pyrethrins 0.1% was superior to pyrethrins 0.15% at 48.26%. Pyrethrins 0.20% was the most potent knockdown agent compared to the other three concentrations at 66.67%. The knockdown performance of the unsynergised pyrethrins was linear in both the 6th and 9th min. with pyrethrins 0.05% the least potent knockdown agent and pyrethrins 0.2% the most superior knockdown agent (Table 3).

The $KD^{t}_{50}$ for pyrethrins 0.05% was the longest (5.89 min.) followed by 0.1% and 0.15% with $KD^{t}_{50}$ of 3.09 and 3.08 min. respectively whereas pyrethrins 0.2% was a very good knockdown agent with the shortest $KD^{t}_{50}$ of 2.19 min.(Figure 4), classified as an early knockdown formulation that recorded 66.67% knockdown activity after 3 min. of exposure.

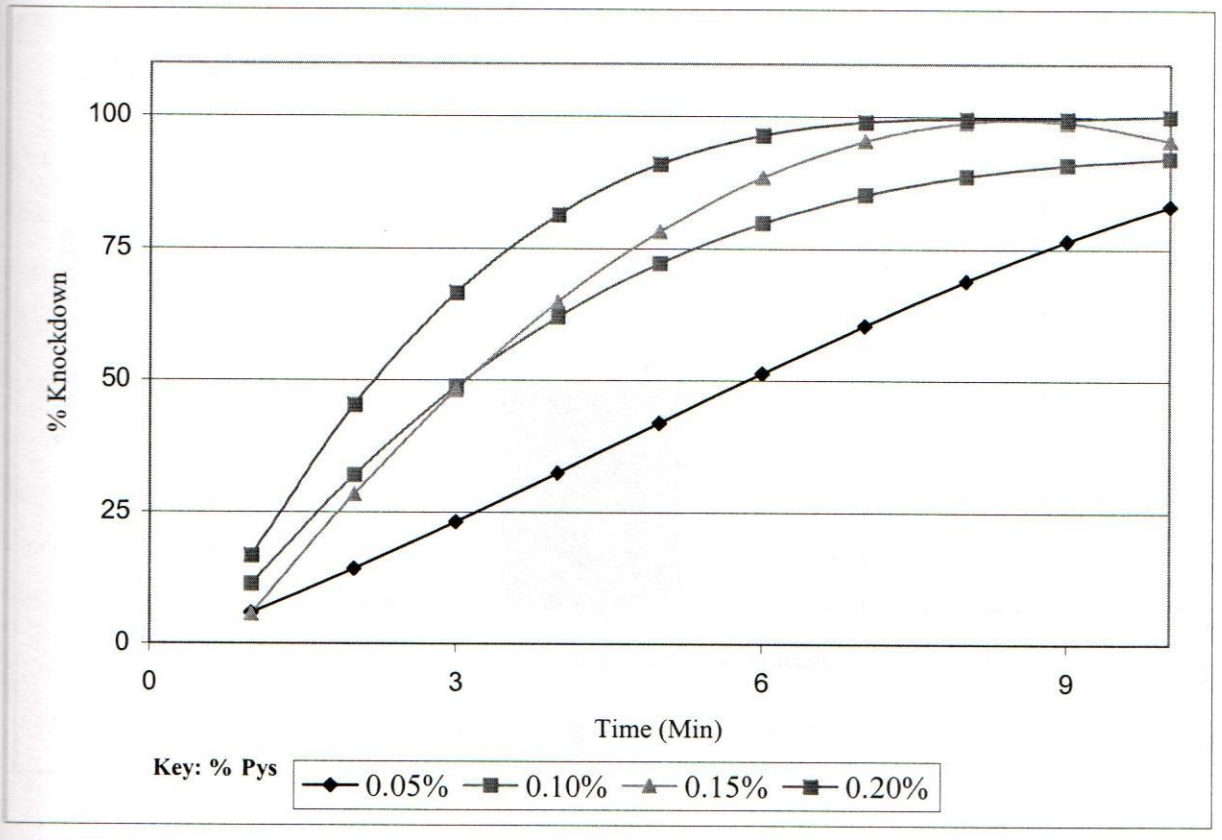
At 0.15 and 0.1% pyrethrins had moderate knockdown effect with a performance of 48.80 and 48.26%, respectively. At 0.05% they had very poor knockdown effect with a performance of 23.13% (Table 3). The correlation coefficients, $r^2$ were relatively large for all the 4 concentrations, an indication that the regressions were suitable in indicating the effect of knockdown with time for the specific concentrations.

Table 3: $KD^t_{50}$ and percent knockdown of the various concentrations of unsynergised pyrethrins exposed to houseflies, *M. domestica* L

| Formulation (Pyrethrins) | Polynomial equation | $r^2$ | $KD^t_{50}$ | % KD 3 Min | % KD 6 Min | % KD 9 Min |
|---|---|---|---|---|---|---|
| 0.05% | $Y = -1.50 + 6.86X + 0.57X^2 - 4.14E\text{-}02X^3$ | 0.988 | 5.89 | 23.13 (24.07$^d$) | 51.41 (49.45$^c$) | 76.62 (76.70$^d$) |
| 0.10% | $Y = -13.72 + 27.35X - 2.38X^2 + 7.05E\text{-}02X^3$ | 0.966 | 3.09 | 48.80 (48.20$^b$) | 79.87 (81.10$^b$) | 90.91 (90.48$^c$) |
| 0.15% | $Y = -20.37 + 27.43X - 1.49X^2 - 9.32E\text{-}03X^3$ | 0.976 | 3.08 | 48.26 (44.72$^c$) | 88.54 (91.07$^b$) | 98.97 (97.24$^b$) |
| 0.20% | $Y = -20.65 + 42.11X - 4.90X^2 + 0.19X^3$ | 0.971 | 2.19 | 66.67 (63.61$^a$) | 96.53 (93.35$^a$) | 99.72 (100$^a$) |

Figures in parenthesis represent the actual figures obtained whereas the others represent the fitted values as per the equation of regression. Any two means in the same column sharing a common letter are not significantly different at 5% level (LSD test).

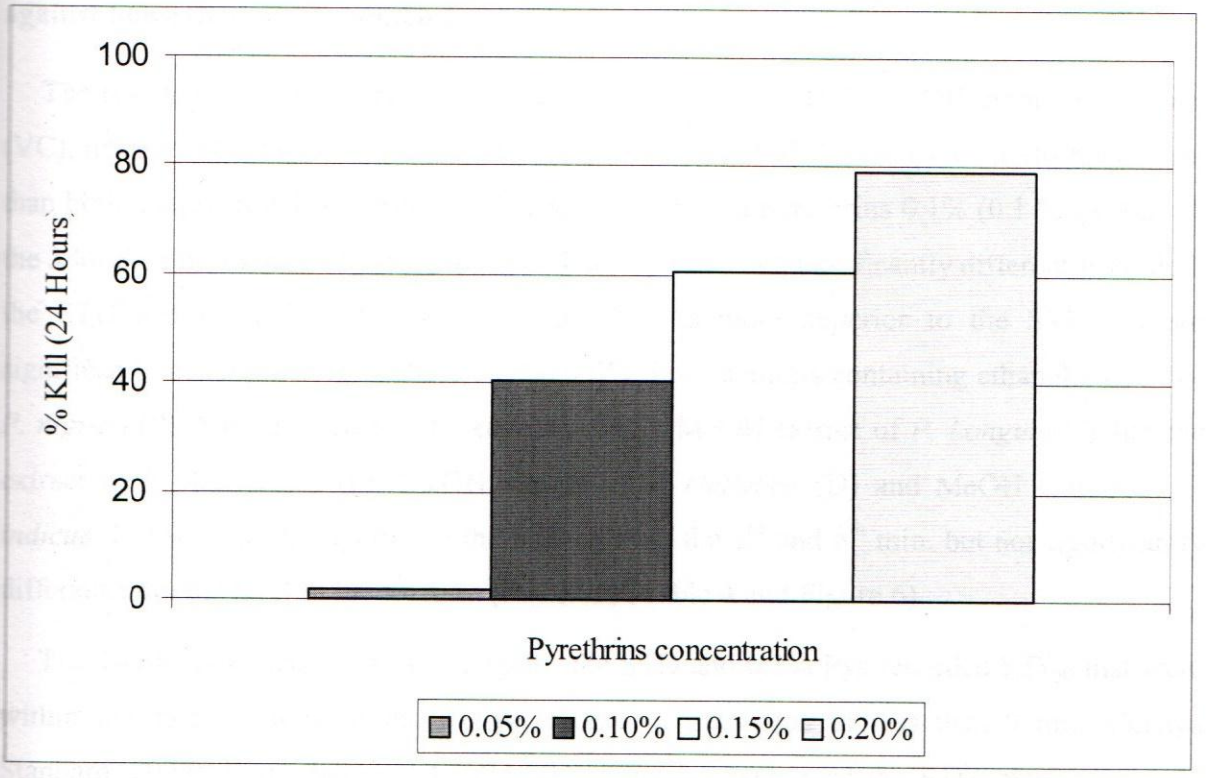
Figure 4: $KD^{t}_{50}$ and percent knockdown of the various concentrations of unsynergised pyrethrins exposed to houseflies, *M. domestica* L




### 4.4.4 Percent mortalities of houseflies exposed to various concentrations of unsynergised pyrethrins

The percentage mortality of the 4 concentrations of unsynergised pyrethrins were significantly different ($P < 0.05$) from each other. The performance significantly increased with increasing dose. Pyrethrins 0.05% had the lowest percent kill (1.76%) while at 0.2% they had the highest percent kill (79.12%) (Figure 5).

Figure 5: Percent mortalities of houseflies exposed to different concentrations of unsynergised pyrethrins



### 4.4.5 $KD^{t}_{50}$ and percent knockdown of the crude plant extracts synergised pyrethrins against houseflies, *M. domestica* L.

The results revealed that hexane extract of *B. napus* (I), $CHCl_3$/MeOH extract of *A. vera* (VC), hexane extract of *A. vera* (VH) and methanol extract of *A. vera* (VM) performed lower than both the piperonyl butoxide (STD) and unsynergised pyrethrins 0.1% (0.1 % Pys) in all the 3 levels. Formulations incorporating U, P and C were not significantly different from both the STD and 0.1% Pys. However, sample B was more superior to the STD but not significantly different from 0.1% Pys ($P < 0.05$). Formulations containing ethanol extract of *P. nigrum* (P), MeOH extract of *P. Americana* (C), MeOH extract of *P. Longum*(G), hexane extract of *P. Americana* (F), MeOH extract of *Z. chalybea* (D) and MeOH extract of *S. indicum* (A) were more superior to the STD in both the 3rd and 6th min. but not significantly different from the STD in the 9th min. ($P < 0.05$) (Table 4 and Figure 6).

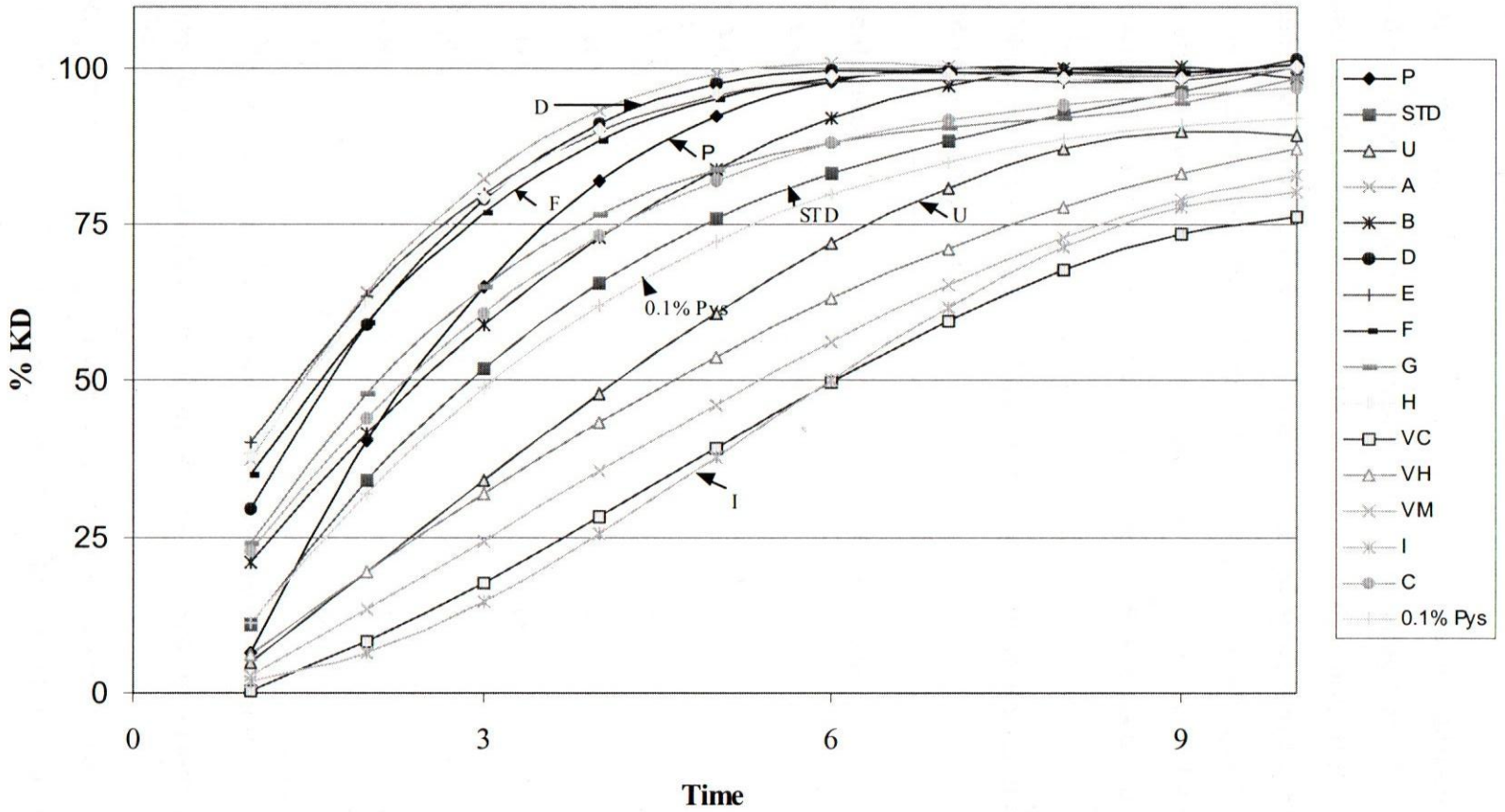
The 14 plant extracts used as synergists, the STD and 0.1% Pys recorded $KD^{t}_{50}$ that were within the specifications of the Kenya Bureau of Standards of less than 9 min. (Kenya Standard, 2000). Formulations VC VM VH U and I antagonized the $KD^{t}_{50}$ performance of 0.1% Pys (3.09 min.). Formulations G, B, P, C, F, H, E, D and A recorded a more superior performance of a shorter $KD^{t}_{50}$ to both the 0.1% Pys and the STD. Therefore, formulations G, B, P, C, F, H, E, D and STD were early knockdown agents, with a performance of over 50% within 3 min. Unsynergised pyrethrins 0.1% was classified as moderate knockdown agent. While formulations incorporating VH, U and A as late knockdown agents and those incorporating I, VC and VM as very poor knockdown agents (Table 4 and Figure 6). The performance of all these crude plant extracts were compared further by use of the knockdown factor of synergism and ranked accordingly (Appendix I).

Table 4: $KD^{t}_{50}$ and percent knockdown of the plant crude extracts synergised pyrethrins against houseflies, *M. domestica*

| Formulations | Polynomial equation | $r^2$ | $KD^{t}_{50}$ | % KD | | |
|---|---|---|---|---|---|---|
| | | | | 3 Min | 6 Min | 9 Min |
| **I** | $Y = 2.19 - 3.01X + 2.95X^2 - 0.19X^3$ | 0.841 | 6.06 | 14.67(16.33$^{k}$) | 49.99(51.03$^{e}$) | 77.91(77.03$^{de}$) |
| **VC** | $Y = -5.42 + 4.19X + 1.50X^2 - 0.11X^3$ | 0.995 | 6.03 | 17.66(17.20$^{k}$) | 49.89(51.60$^{e}$) | 73.43(72.60$^{e}$) |
| **VM** | $Y = -7.24 + 9.52X + 0.52X^2 - 5.70E\text{-}02X^3$ | 0.925 | 5.37 | 24.44(23.60$^{kj}$) | 56.22(58.30$^{e}$) | 78.89(79.10$^{cde}$) |
| **VH** | $Y = -8.21 + 14.62X - 0.38X^2 - 1.30E\text{-}02X^3$ | 0.982 | 4.63 | 31.90(32.60$^{j}$) | 63.09(62.03$^{ed}$) | 83.25(84.40$^{c}$) |
| **U** | $Y = -9.14 + 13.69X + 0.50X^2 - 8.89E\text{-}02X^3$ | 0.779 | 4.17 | 34.07(32.89$^{jg}$) | 71.94(75.24$^{dc}$) | 90.06(88.27$^{cde}$) |
| **STD** | $Y = -18.25 + 32.34X - 3.36X^2 + 0.130X^3$ | 0.974 | 2.89 | 52.07(47.34$^{hg}$) | 83.14(81.27$^{cb}$) | 96.21(96.64$^{ba}$) |
| **0.1% Pys** | $Y = -13.72 + 27.35X - 2.38X^2 + 7.05E\text{-}02X^3$ | 0.966 | 3.09 | 48.80(48.20$^{he}$) | 79.87(81.10$^{cb}$) | 90.91(90.48$^{cba}$) |
| **B** | $Y = -3.32 + 26.07X - 1.86X^2 - 2.74E\text{-}02X^3$ | 0.948 | 2.48 | 58.87(62.07$^{ge}$) | 91.97(92.70$^{ha}$) | 100(99.37$^{ba}$) |
| **P** | $Y = -38.58 + 50.78X - 6.14X^2 - 0.25X^3$ | 0.949 | 2.35 | 65.08(63.23$^{fed}$) | 97.85(96.34$^{a}$) | 99.46(100$^{a}$) |
| **C** | $Y = -4.18 + 29.94X - 3.09X^2 + 0.11X^3$ | 0.978 | 2.33 | 60.8(63.31$^{fedc}$) | 88.05(87.91$^{a}$) | 95.60(95.96$^{ba}$) |
| **G** | $Y = -7.75 + 36.15X - 4.57X^2 + 0.20X^3$ | 0.917 | 2.11 | 65.01(65.57$^{ecb}$) | 88.17(88.13$^{a}$) | 94.39(95.10$^{ba}$) |
| **F** | $Y = 1.82 + 37.56X - 4.79X^2 + 0.20X^3$ | 0975 | 1.59 | 76.9(77.4$^{edcba}$) | 98.41(98.07$^{a}$) | 99.26(100$^{a}$) |
| **H** | $Y = 4.61 + 38.18X - 5.09X^2 + 0.22X^3$ | 0.953 | 1.46 | 79.37(78.2$^{dcba}$) | 98.52(97.40$^{a}$) | 98.64(99.27$^{ba}$) |
| **E** | $Y = 7.84 + 36.98X - 4.99X^2 - 0.22X^{3n}$ | 0.942 | 1.39 | 79.83(80.93$^{a}$) | 97.92(96.57$^{a}$) | 98.10(99.10$^{ba}$) |
| **D** | $Y = -11.18 + 46.92X - 6.50X^2 + 0.29X^3$ | 0.942 | 1.66 | 79.00(81.57$^{a}$) | 98.98(98.83$^{a}$) | 99.72(100$^{a}$) |
| **A** | $Y = 0.62 + 42.38X - 5.84X^2 + 0.26X^3$ | 0.932 | 1.44 | 84.00(84.00$^{a}$) | 99.30(99.30$^{a}$) | 100(100$^{a}$) |

Figures in parenthesis represent the actual values obtained whereas the others represent the fitted values as per the equation of regression. Any two means in the same column sharing a common letter are not significantly different at 5% level (LSD test).

Figure 6: $KD^{I}_{50}$ and percent knockdown of the various formulations of synergised pyrethrins exposed to houseflies, *M. domestica* L



### 4.4.6 Mean percent mortality of houseflies exposed to crude plant extracts synergised pyrethrins

The mean mortality of houseflies exposed to various formulations of plant extracts synergised pyrethrins revealed that formulations incorporating VM, VC, VH, H, G, B, A, E and C antagonized the performance of the unsynergised pyrethrins 0.1%. On the other hand, formulations incorporating F, U, D, P and I exhibited synergism by boosting the percent mortality of treated houseflies. Formulation incorporating P significantly matched the mortality effect of the STD ($P < 0.05$) (Table 5). The mortality performance of the crude plant extracts were compared using the mortality factor of synergism and ranked accordingly (appendix I).

Table 5: Mean percent mortality of houseflies exposed to various formulations of plant extract synergised pyrethrins

| Formulations | % Kill | Formulations | % Kill |
|---|---|---|---|
| **VM** | $15.43^{h}$ | **C** | $32.46^{fd}$ |
| **VC** | $18.58^{h}$ | **0.1% Pys** | $40.46^{de}$ |
| **VH** | $21.00^{hf}$ | **F** | $44.40^{d}$ |
| **H** | $21.23^{hf}$ | **U** | $58.96^{c}$ |
| **G** | $24.4^{hf}$ | **D** | $60.23^{c}$ |
| **B** | $29.43^{ge}$ | **I** | $73.60^{b}$ |
| **A** | $32.10f^{d}$ | **STD** | $100.00^{a}$ |
| **E** | $32.17^{fd}$ | **P** | $100.00^{a}$ |

*Any two means sharing a common letter are not significantly different at 5% level (LSD test).

### 4.4.7 Mean percent knockdown of houseflies exposed to crude plant extracts as insecticides

All the five plant extracts used as insecticides recorded insignificant knockdown effects in the $3^{rd}$, $6^{th}$ and $9^{th}$ min. The STD was more toxic to the housefly in all the three levels compared to the plant extracts synergised pyrethrins (Table 6).

### 4.4.8 Plant extracts synergised pyrethrins 0.1% in the ratio 1:2 and 1:8 against houseflies

In the 3rd min. formulations P (65.04 %), I (67.70%) and the STD (38.30%) recorded a higher knockdown compared to the other extracts D, (26.16%), F, (19.60%) and U, (17.93%)). In the 6th minute, the trend was the same as in the 3rd min. However, formulation D (79.56%) showed a remarkable improvement than formulation F and U. Formulations D, P, I and the STD were not significantly indifferent and recorded knockdown performance higher than samples F and U in the 9th minute. When the concentration of the plant extract was increased, the knockdown performance of the formulation D, F, P and the STD increased, unlike the performance witnessed in formulations U and I in all the three levels (Table 6).

### 4.4.9 $KD^{t}_{50}$ and knockdown factor of synergism of the crude plant extract formulations on houseflies

#### 4.4.9.1 Formulations containing plant extracts as insecticides

Taking the $KD^{t}_{50}$ as a parameter, it was found that it took all the five formulations, an infinite period of time to achieve a 50% knockdown of test flies while the STD took 3.80 min. to achieve the same performance. The knockdown factors of synergism of all the selected plant extracts were very poor, indicating that none of the extracts could be employed as knockdown agents. The knockdown factor of synergism of the STD was higher than those of the plant extracts but below one (Table 7).

#### 4.4.9.2 Plant extracts synergised pyrethrins in the ratio 1:2 and 1:8 against houseflies

At ratio 1:2, the formulations D, I, P and the STD had very short $KD^{t}_{50}$ which translated to higher factor synergism, whereas formulations F and U recorded longer $KD^{t}_{50}$ translating to smaller factor of synergism. Upon ranking the extracts, in the order of boosting the knockdown effects of the unsynergised pyrethrins, formulations D, P and I were effective, surpassing the synergism imparted by the standard (Table 7). On the other hand, at ratio 1:8 formulations D, F, P and the STD recorded very short $KD^{t}_{50}$ compared to formulations I and U. The factors of synergism follow a similar trend as when the ratio is 1:2. However, upon comparing the two ratios per each sample it is seen that formulation D maintained its performance in both the employed ratios. However, formulations P and I antagonized the insecticidal activity upon increment of the extract since, the smaller the quantities of the

extracts the better the knockdown. For formulations F, U and the STD, the higher the quantity of the extract the better the synergism (Table 7).

### 4.4.10 Mortality factors of synergism of the formulations against houseflies

As shown by the mortality factor of synergism, the extracts cannot be employed as insecticides. However, when the plant extracts are used as synergists, they boost the mortality of pyrethrins. Formulation P matched the performance of the STD with an *M* factor of 2.47 in ratio 1:8. At a ratio of 1:2 the *M* factor of P is high but does not match that of the standard. Sample U has an *M* factor of 2.05, when used in ratio 1:8 (Table 8).

Table 6: Mean percent knockdown of houseflies exposed to the various formulations of synergised pyrethrins

| Extracts | As insecticides, 0.1% | | | Ratio 1:2 | | | Ratio 1:8 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 3 Min | 6 Min | 9 Min | 3 Min | 6 Min | 9 Min | 3 Min | 6 Min | 9 Min |
| **D** | $0.00^{b}$ | $0.00^{b}$ | $0.00^{c}$ | $26.16^{b}$ | $79.56^{b}$ | $94.62^{a}$ | $68.33^{a}$ | $94.19^{a}$ | $99.16^{a}$ |
| **F** | $0.00^{b}$ | $0.00^{b}$ | $0.00^{c}$ | $19.60^{b}$ | $59.92^{c}$ | $86.25^{b}$ | $66.55^{a}$ | $90.88^{a}$ | $98.87^{a}$ |
| **I** | $0.5^{b}$ | $0.00^{b}$ | $0.00^{c}$ | $67.70^{a}$ | $96.20^{a}$ | $100^{a}$ | $16.63^{b}$ | $62.53^{b}$ | $86.50^{b}$ |
| **P** | $0.54^{b}$ | $0.50^{b}$ | $2.56^{b}$ | $65.04^{0}$ | $97.73^{a}$ | $100^{a}$ | $59.08^{b}$ | $96.62^{a}$ | $100^{a}$ |
| **U** | $0.00^{b}$ | $0.46^{b}$ | $1.78b^{c}$ | $17.93^{b}$ | $54.85^{c}$ | $82.56^{b}$ | $26.83^{a}$ | $69.77^{b}$ | $86.67^{b}$ |
| **STD** | $37.47^{a}$ | $77.47^{a}$ | $89.00^{a}$ | $38.30^{a}$ | $92.02^{a}$ | $100^{a}$ | $59.8^{b}$ | $95.12^{a}$ | $100^{a}$ |

Any two means in the same column sharing a common letter are not significantly different ($P < 0.05$).

Table 7: $KD^{t}_{50}$ and knockdown factor of synergism (*S*) of the crude plant extracts as insecticides, in the ratios 1:2 and 1:8 against houseflies, *M. domestica* L.

| **Extracts** | **As insecticides** | | | **Ratio 1:2** | | | **Ratio 1:8** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time (Min)** | | | | | | | | | |
| | **$KD^{t}_{50}$** | ***S*** | **Rank** | **$KD^{t}_{50}$** | ***S*** | **Rank** | **$KD^{t}_{50}$** | ***S*** | **Rank** |
| **D** | Inf. | Misc. | 2 | 2.02 | 1.52 | 1 | 2.02 | 1.52 | 1 |
| **F** | Inf. | Misc. | 2 | 5.20 | 0.59 | 5 | 2.16 | 1.43 | 2 |
| **I** | Inf. | Misc. | 2 | 2.25 | 1.37 | 3 | 5.19 | 0.60 | 6 |
| **P** | Inf. | Misc. | 2 | 2.11 | 1.46 | 2 | 2.46 | 1.26 | 3 |
| **U** | Inf. | Misc. | 2 | 5.68 | 0.54 | 6 | 4.50 | 0.69 | 5 |
| **STD** | 3.80 | 0.81 | 1 | 3.41 | 0.91 | 4 | 2.59 | 1.19 | 4 |

Key: Inf. refers to infinity; Misc. refers to Miscellaneous

Table 8: Mortality factor of synergism (*M*) of the crude extracts as insecticides, in the ratios 1:2 and 1:8 against houseflies

| Extracts | As insecticides | | | Ratio 1:2 | | | Ratio 1:8 | | |
|---|---|---|---|---|---|---|---|---|---|
| | % Kill | *M* | Rank | % Kill | *M* | Rank | % Kill | *M* | Rank |
| **D** | $1.97^{b}$ | 0.05 | 5 | $53.74^{c}$ | 1.33 | 4 | $64.22^{c}$ | 1.59 | 4 |
| **F** | $4.13^{b}$ | 0.10 | 3 | $34.80^{d}$ | 0.86 | 6 | $57.70^{c}$ | 1.43 | 6 |
| **P** | $2.03^{b}$ | 0.05 | 5 | $93.56^{a}$ | 2.31 | 2 | $100^{a}$ | 2.47 | 1 |
| **I** | $2.84^{b}$ | 0.07 | 4 | $67.52^{b}$ | 1.67 | 3 | $63.97^{c}$ | 1.58 | 5 |
| **U** | $4.49^{b}$ | 0.11 | 2 | $49.76^{c}$ | 1.23 | 5 | $83.20^{b}$ | 2.05 | 3 |
| **STD** | $51.45^{a}$ | 1.27 | 1 | $98.72^{a}$ | 2.44 | 1 | $100^{a}$ | 2.47 | 1 |

Any two means in the same column sharing a common letter are not significantly different ($P < 0.05$).

## 4.5 Discussion

There are various solvents in use with pyrethrum such as ethanol, hexane, isopropyl alcohol, acetone, distilled water and SST (Fiero, 1964). Investigations on toxicity of solvents to insect pests are less cited therefore, solvents should be classified in terms of their intrinsic toxicities to various test insects. Other solvents have been screened as documented in Pyrethrum Post (1990) edition against three strains of the housefly, the standard laboratory II (WHO susceptible), the F58-WT, the DDT- and the permethrin-resistant strains.

From this study it can be seen that ethanol and IPA, which were the less toxic solvents should be used for bioassay evaluation as carriers of synergised pyrethrins while the more toxic solvents SST and isopar M should be used for formulations of insecticides or perhaps the standardization of the pyrethrins for sale. Ethanol was selected to be the test carrier medium for further experiments in the study due to its less toxicity and its ability to dissolve pyrethrins and the plant extracts (synergists). From the study it can also be seen that $CO_2$ have no effect on the houseflies, therefore can be used to knockdown the houseflies that were not initially knocked by the formulations for collection at the end of the exposure periods in the Kearns and Match spray test chamber.

The 4 concentrations of the unsynergised pyrethrins, (0.05, 0.1, 0.15 and 0.2%) matched the knockdown specifications of the Kenya Standard. However, none of these concentrations achieved the required killing effect of an insecticide (Kenya Standard, 2000). Pyrethrins 0.1%, which recorded 40.46% mortality, was the best concentration to be synergised because it had the lowest killing potency, while at 0.05 it showed an insignificant killing effect that was not economical to be synergised. At 0.15 and 0.20% had moderate killing potency and hence unsuitable for synergistic studies

The percent mortality of the selected unsynergised pyrethrins 0.1% was lower than those required standards set by the Kenya Bureau of Standards. This can be enhanced either by increasing the pyrethrins content or formulating the same concentration of pyrethrins together with a synergist. However, increasing the concentration of pyrethrins is prohibitive since the cost of production is very high and would increase the cost. The use of a synergist that is cheaper than the STD and much safer in boosting the activity of the otherwise low concentration of pyrethrins would be appropriate.

In this study, low concentration of pyrethrins was selected in order to lower the cost of formulations yet endeavor to boost their effectiveness using crude plant extracts as synergists. The effectiveness of plant crude extracts as synergists were compared via two parameters, the knockdown activity and the mortality effects exerted by the plant crude extracts against those of the unsynergised pyrethrins 0.1%, the STD (piperonyl butoxide) synergised pyrethrins and the specifications of insecticides by the Kenya Bureau of Standards. Crude plant extracts were used as synergists to pyrethrins so that a clear line could be drawn between the formulated synthetic insecticides and naturally occurring insecticides. The study clearly revealed that all the 14 crude plant extracts were potent knockdown agents because they all recorded $KD^{t}_{50}$ that were lower than 9 min. (Kenya Standard, 2000). On considering the time of the occurrence of the knockdown, the plant extracts showed differences on how the houseflies responded. The early knockdown agents which included A, B, C, D, E, F, G, H and P were the best formulations whereas VC, VH, VM, I and U were late or poor knockdown agents.

The use of knockdown effects in selecting the best synergist could not be achieved without considering the overall killing effect of the formulations. A factor that was clearly shown by the mortality effect that increased from 40.46% due to the unsynergised pyrethrins 0.1% to either 95% that is specified by the Kenya Bureau of Standards or 100% that was achieved by the STD. The results indicated that only one crude plant extract, formulation having ethanol extract of *P. nigrum* (P) met these requirements by matching the performance of the piperonyl butoxide (STD) and exceeding the performance stipulated by the Kenya Standards. However, formulations F, U, D, and I recorded an improved mortality effect at 44.40 58.96, 60.23 and 73.60% respectively and it is clear that formulation P is a potent synergist hence the structure of the compound responsible in synergising pyrethrins 0.1% should be elucidated and compared to that of the standard. However, further investigations on their insecticidal activities need to be described.

Formulations P, F, U, D and I were selected for further investigations whereby the ratio of the formulation should be increased to 8:1 and also decreased to 2:1 to evaluate the linearity imparted by the concentration. Formulations A, B, D, E, F, G and H that were categorized as early knockdown formulations showed very poor killing potency which antagonized the activity recorded by the unsynergised pyrethrins 0.1%. These plant extracts were recommended for further isolation so as to elucidate compounds that are responsible for the knockdown potency and those for antagonism of the kill effects of the unsynergised

pyrethrins 0.1%. The formulations VH, VC, C, and VM that are categorized as late knockdown formulations recorded very poor killing effects thus were not followed.

In summary, the study has shown that local botanicals exhibit both pyrethrins antagonistic and synergistic activity and that structural elucidation of the compounds involved will go a long way in lowering the cost of production of pyrethrum-based insecticides resulting in a cheaper and environmentally friendly pest and vector management in the field or at household level.

The Kenya standard specifies that an insecticide qualifies to be good if it attains a $KD^{t}_{50}$ of 5-9 minutes and 95% kill after 24 hours (Kenya standard, 2000). Therefore, a plant material that boosts a lower concentration of pyrethrins with insignificant insecticidal activities to performances similar or higher than the performances specified by the Kenya standard was regarded as a potential synergist. Under these specifications, it is a requirement that the plant material do not exert any insecticidal activity on their own (Hewlett, 1960).

The ethanol extract of *P. nigrum* (P) is a good synergist in the two levels measured in this study where $KD^{t}_{50}$ was better than the specifications of the Kenya Bureau of Standards and the performance of the standard, piperonyl butoxide. The extract was very poor as an insecticidal agent. The percent mortality of this plant extract was very good specifically in the higher ratio (Hewlett, 1960). It is noted that the plant extract has not been cited before for any compounds that were synergistic to pyrethrins, though the plant has been mentioned as yielding compounds possessing the 3, 4-methylenedioxy phenyl groups (Parmar *et al.*, 1998; Lee *et al.*, 2000; Siddiqui *et al.*, 2003). The compound that exerts the observed synergism to pyrethrins needs to be characterized.

The $CHCl_3$/MeOH extract (U) exhibited consistencies upon comparing the percent kill after 24 hours of the two levels yet is a poor insecticidal agent by itself. The crude plant extract is a potent synergist although its knockdown effects were below expectation. The extract has not been cited before for any chemical or biological activity, thus the need to elucidate the structures present in the extract and relate them to the known synergists.

The hexane extract of *S. indicum* (D) showed consistent biological efficacies both in $KD^{t}_{50}$ and mean percent kill after 24 hours as has been known to exert synergism to pyrethrins due to sesamin and sesamolin (Budowiski, 1964). However, since the hexane extracts of *S. indicum, P americana* and *B. napus* exhibited synergism to unsynergised pyrethrins 0.1% to a smaller extent, they were not considered for further investigation.

## CHAPTER FIVE

# PIPERINE, A PIPERIDINE ALKALOID FROM *P. NIGRUM* AS A SYNERGIST TO PYRETHRINS FOR THE CONTROL OF THE HOUSEFLY, *MUSCA. DOMESTICA* L.

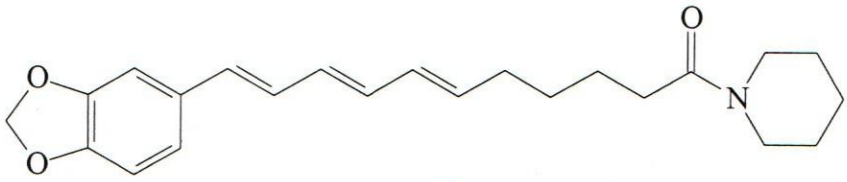

### 5.1 Abstract

The possibility of obtaining a naturally occurring synergist was evaluated through screening ethanol extract of *P. nigrum* L. (Piperaceae) against the housefly in the modified Kearns and Match spray test chamber. The crude ethanol extract of *P. nigrum* showed synergism to pyrethrins with 100% mortality after 24 h and $KD^{t}_{50}$ of 2.85 min. a performance that warranted an investigation on the compound that exhibited this synergism. Column chromatography of the ethanol extract yielded two semi-pure fractions ($P_1$ and $P_2$). Bioassays revealed that $P_1$ was the most superior fraction as a potent synergist. $^{1}$H-NMR, $^{13}$C-NMR, IR, GC-MS and DEPT spectra as well as melting point confirmed that piperine was the compound responsible for the synergistic activity of the ethanol extract of the *P. nigrum* to the unsynergised pyrethrins 0.1%.


### 5.2 Introduction

*P. nigrum,* a member of Piperaceae family is known to yield compounds with 3, 4-methylenedioxy phenyl groups (Xavier *et al.,* 1997; Parmar *et al.,* 1998; Lee *et al.*, 2000; Siddiqui *et al.,* 2003; Martins *et al.,* 2003). The presence of 3, 4-methylenedioxy phenyl groups in compounds used as pyrethrins synergists have long been known (Hopkins and Maciver, 1965). The observation that sesame oil synergised pyrethrins (Eaglestone, 1942) and followed by identification of sesamin and sesamolin, both compounds with the 3, 4-methylenedioxy phenyl moiety (Budowiski 1964) cemented this observation.

Phytochemical investigation of most of *Piper* plants has yielded many polyphenols and alkaloids (Parmar *et al.*, 1998; Lee, *et al.*, 2000 and Siddiqui *et al.*, 2003). Over 38 compounds have been isolated and reported from the genus *Piper*. The most recently reported compounds include piptigrine (**51**), furacridone (**52**) and B-sitosterylpalmitate (**53**) (Parmar *et al.*, 1998; Siddiqui *et al.,* 2003). Piptigrine was tested for insecticidal activity against fourth instar larvae of *Aedes aegypti* (Siddiqui *et al.*, 2003).



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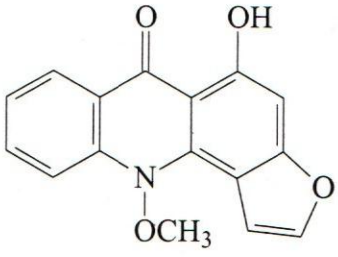
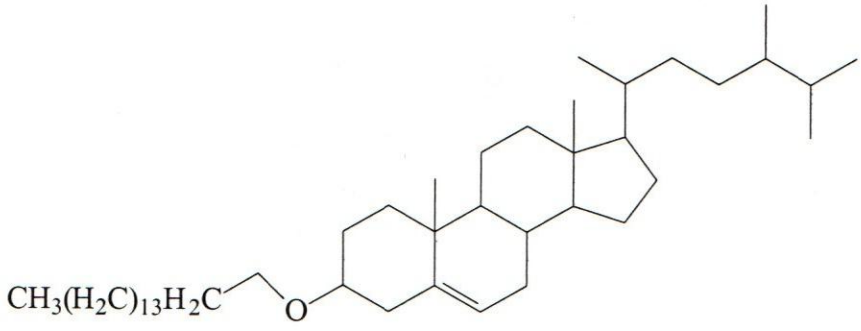


**52**



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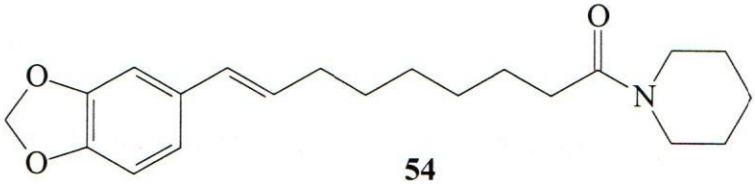
Two new tetrahydrofuran lignans have also been reported from *P. solmsianun*. They have been evaluated against trypanomastigote form of *Trypanossoma cruzi*. A piperidine alkaloid, pipernonaline (**54**) has been isolated from *P. longum* fruits and tested against phytopathogenic fungi *in vivo* (Martins *et al.,* 2003). Piperdardine (7) have been isolated from *P. tuberculatum* (Xavier *et al.,* 1997)



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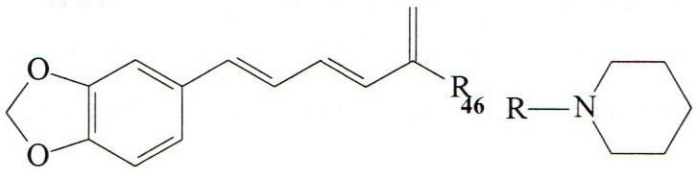
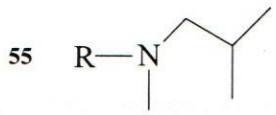
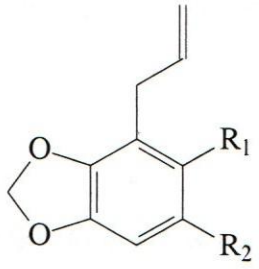
Other polyphenols and alkaloids isolated from *Piper* species (Parmar, *et al.*, 1998) include piperine (**46**), piperlonguminine (**55**), parsley apiole (**56**), safrole (**31**), cepharadione (**57**) and myristicin (**39**).



46 R—N



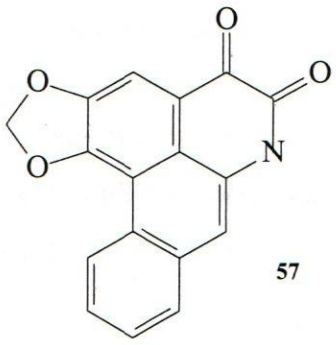
55 R—N



56 $R_1=R_2=OCH_3$

39 $R_1=H$ $R_2=OCH_3$

31 $R_1=H$ $R_2=H$



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Structural analysis revealed that most of these compounds extracted form *Piper* species clearly furnishes presence of a 3, 4 - methylenedioxy phenyl group. In this study, synergistic activities of the ethanol extract of *P. nigrum* to unsynergised pyrethrins 0.1% were screened against housefly, *M. domestica* L. with the aim of producing cheap and safe pyrethrins synergist, alternative to the synthetic piperonyl butoxide.

## 5.3 Materials and Method

### 5.3.1 Fractionating ethanol extract of the seeds of *P. nigrum*

Several solvent systems were first compared to determine the best combination for TLC resolution. Hexane: ethyl acetate mixture (3:1) gave a good separation. The plates were analyzed with anisaldehyde spraying reagent (anisaldehyde: concentrated sulphuric acid: methanol in a ratio of 1:2:97). This solvent system was then chosen for column

chromatography. Silica gel was packed in a column and eluted with hexane: ethyl acetate (3:1) and TLC analysis used to determine the purity of the eluent fractions. A formulation of pyrethrins and fractions obtained ($P_1$ and $P_2$) was made (1:5) and bioassayed. Results on knockdown and percent mortality were observed, recorded and interpreted.

### 5.3.2 Effects of fractionated portions of the ethanol extract of *P. nigrum* synergised pyrethrins against houseflies

The two fractions $P_1$ and $P_2$ synergised pyrethrins were subjected to bioassay (Section 3.5.5).

## 5.4 Results

### 5.4.1 $KD^t_{50}$ and percent knockdown of $P_1$ and $P_2$ synergised pyrethrins against houseflies

Fraction $P_1$ recorded an excellent performance compared to fraction $P_2$ in all three levels namely the 3rd, 6th and 9th min. after exposure (Table 9). Fraction $P_2$ did not exhibit any synergism to pyrethrins at any concentration whereas fraction $P_1$ performed better than the unsynergised pyrethrins and the STD at all the levels. However, the performance shown by the crude extract was significantly higher than fraction $P_1$ in the 3rd and 6th min. but lower in the 9th min.

Fraction $P_1$ recorded the best $KD^t_{50}$ at 2.85 min. a performance better than those recorded by the STD synergised pyrethrins and the unsynergised pyrethrins but lower than that recorded by the crude extract. Fractions $P_1$ and $P_2$ performed lower than the specifications of the Kenya standard (Kenya Standard, 2000). Fraction $P_1$ maintained the early knockdown activity witnessed in the crude extract whereas fraction $P_2$ was a very poor knockdown agent (Table 10).

### 5.4.2 Mean percent mortalities of houseflies exposed to $P_1$ and $P_2$ synergised pyrethrins

Fractions $P_1$ and $P_2$ separately enhanced the mortality performance of the unsynergised pyrethrins 0.1% from 40.46 to 100 and 56.23%, respectively. The mean mortality confirms that fraction $P_1$ is a synergist to pyrethrins (Table 10). $P_2$ was excluded for further investigation due to its low bioefficacy.

Table 9: $KD^{t}_{50}$ and percent knockdown of $P_1$ and $P_2$ synergised pyrethrins exposed to houseflies, *M. domestica* L

| Fractions | Linear equation | $r^2$ | $KD^{t}_{50}$ | % KD | | |
|---|---|---|---|---|---|---|
| | | | | 3 Min | 6 Min | 9 Min |
| **0.1% PYS** | $Y = -13.72 + 27.35X + 0.57X^2 - 4.14E\text{-}02X^3$ | 0.966 | 3.09 | 48.80 | 79.87 | 90.91 |
| | | | | (48.20$^b$) | (81.10$^b$) | (90.48$^b$) |
| **$P_1$** | $Y = -40.79 + 42.5X + 4.06X^2 - 0.12X^3$ | 0.965 | 2.85 | 53.4 | 94.10 | 100 |
| | | | | (52.00$^b$) | (94.70$^a$) | (100$^a$) |
| **$P_2$** | $Y = -6.24 + 4.86X + 0.60X^2 + 0.12X^3$ | 0.779 | 6.45 | 13.73 | 44.46 | 85.95 |
| | | | | (7.29$^c$) | (30.42$^c$) | (63.44$^b$) |
| **P** | $Y = -38.58 + 50.78X - 6.14X^2 - 0.25X^3$ | 0.949 | 2.35 | 65.08 | 97.85 | 99.46 |
| | | | | (63.23$^a$) | (96.34$^a$) | (100$^a$) |
| **STD** | $Y = -18.25 + 32.34X - 3.36X^2 + 0.13X^3$ | 0.974 | 2.89 | 52.07 | 83.14 | 96.21 |
| | | | | (47.34$^b$) | (81.27$^b$) | (96.64$^b$) |

Figures in parenthesis represent the actual values obtained whereas the others represent the regressed values as per the equation of regression. Any two means in the same column sharing a common letter are not significantly different ($P < 0.05$).

Table 10: Mean percent mortalities of $P_1$ and $P_2$ synergised pyrethrins against houseflies

| Formulations | % Mortality (24 hours) |
|---|---|
| **0.1 % PYS** | $40.46^{c}$ |
| **$P_1$** | $100^{a}$ |
| **$P_2$** | $56.23^{b}$ |
| **P** | $100^{a}$ |
| **STD** | $100^{a}$ |

Any two means in the same column sharing a common letter are not significantly different ($P < 0.05$).

### 5.4.3 Structural elucidation of piperine

The purity of the compound was determined using the TLC analysis whereby the calculated retention factor was found to be 0.25 (Ikan, 1991). The melting point recorded at 127 – 128 °C (129 °C, Aldrich, 1985) was also used in verifying the purity. Piperine was identified by comparison of $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data with those in literature (Table 11) (Xavier *et al.*, 1997).

The IR spectrum showed absorptions at 1656.33 $cm^{-1}$ for the carbonyl group and four absorptions between 1582.86 and 1252.24 $cm^{-1}$ for aromatic and aliphatic C = C. Absorptions at 2942.04 $cm^{-1}$ is for the carbon due to – $CH_2$ – group of the methylenedioxy phenyl moiety (Figure 7.1). The GC-MS spectrum showed the molecular ion peak at m/z 285 accounting for the elemental composition of $C_{17}H_{19}NO_3$. The peak at m/z 84 ($C_5H_{10}N^+$) indicated the presence of a piperidine ring (Figure 7). The piperidine ring was confirmed by the $^{1}$H-NMR spectrum, which had broad singlet at δ 3.49 (2H, H - 2) and 3.49 (2H – 6''), multiplet at δ 1.49 (2H, H - 3) and 1.47 (2H, H - 5) and a multiplet at δ 1.50 (2H, H - 4). The $^{1}$H-NMR spectrum (Table 11) further showed signals for three aromatic protons at δ 6.74 (m, H – 6''), 6.82 (d, H – 2'') and 6.60 (d, H – 7'') assigned to the 1, 3, 4 – trisubstituted benzene ring. A two-proton singlet at δ 5.81 (s, H – 2a'') was indicative of a –$OCH_2O$-. The respective carbons for these functionalities were observed at δ 128.4 (C – 6''), 105.5 (C – 2'') and 108.4 (C – 7'') and the δ 100.9 for the methylenedioxy carbon in the $^{13}$C-NMR spectrum. The signals at δ 165.3 (C – 1') for carbonyl carbon, and at δ 148.0, 148.0 and 130.9 for three aromatic quaternary carbons (C – 3'', C – 4'' and C – 5'' respectively) were also observed in the $^{13}$C-NMR spectrum. The DEPT spectrum indicated 6 – $CH_2$ groups. These observations led to the confirmation of structure of piperine as 1' – [5' – (3'', 4'' – methylenedioxy phenyl) – 2', 4' – Pentadienyol] Piperidine (Appendices II, III and IV).

Table 11: NMR data for piperine

| Position | $^{1}H$ | $^{1}H$ lit (Xavier *et al.*, 1997) | $^{13}C$ | $^{13}C$ lit(Xavier *et al.*, 1997) |
|---|---|---|---|---|
| 2 | 3.49 br s | 3.48 br s | 43.2 | 43.0 |
| 3 | 1.49 m | 1.49 m | 25.6 | 25.7 |
| 4 | 1.50 m | 1.56 m | 24.6 | 24.5 |
| 5 | 1.49 m | 1.49 m | 26.7 | 26.9 |
| 6 | 3.49 br s | 3.48 br s | 46.8 | 47.1 |
| 1' | — | — | 165.3 | 165.2 |
| 2' | 6.59 m | 6.63 d (*J*=1.46 Hz) | 120.0 | 120.0 |
| 3' | 7.30 m | 7.31 m | 142.4 | 142.3 |
| 4' | 6.63 m | 6.64 m | 125.3 | 125.3 |
| 5' | 6.62 m | 6.65 m | 138.1 | 138.0 |
| 2a" | 5.81 s | 5.86 s | 101.2 | 101.2 |
| 3" | — | — | 148.0 | 148.1 |
| 2" | 6.82 d (*J*=1.6 Hz) | 6.88 1H d (*J*=1.6 Hz) | 105.5 | 105.5 |
| 5" | — | — | 130.9 | 130.9 |
| 6" | 6.74 m | 6.79 dd (*J*=1.6, 8.0 Hz) | 122.4 | 122.3 |
| 7" | 6.60 d (*J*=8.0 Hz) | 6.67 d (J=8.0 Hz) | 108.4 | 108.3 |
| 4" | — | — | 148.0 | 148.0 |

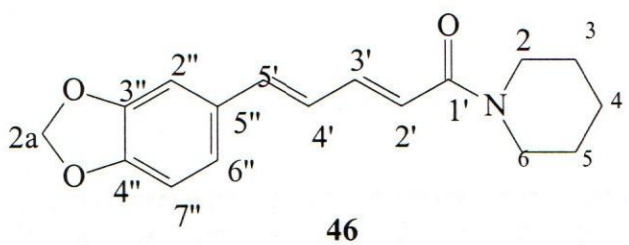


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Figure 7: The Infra red spectrum of piperine

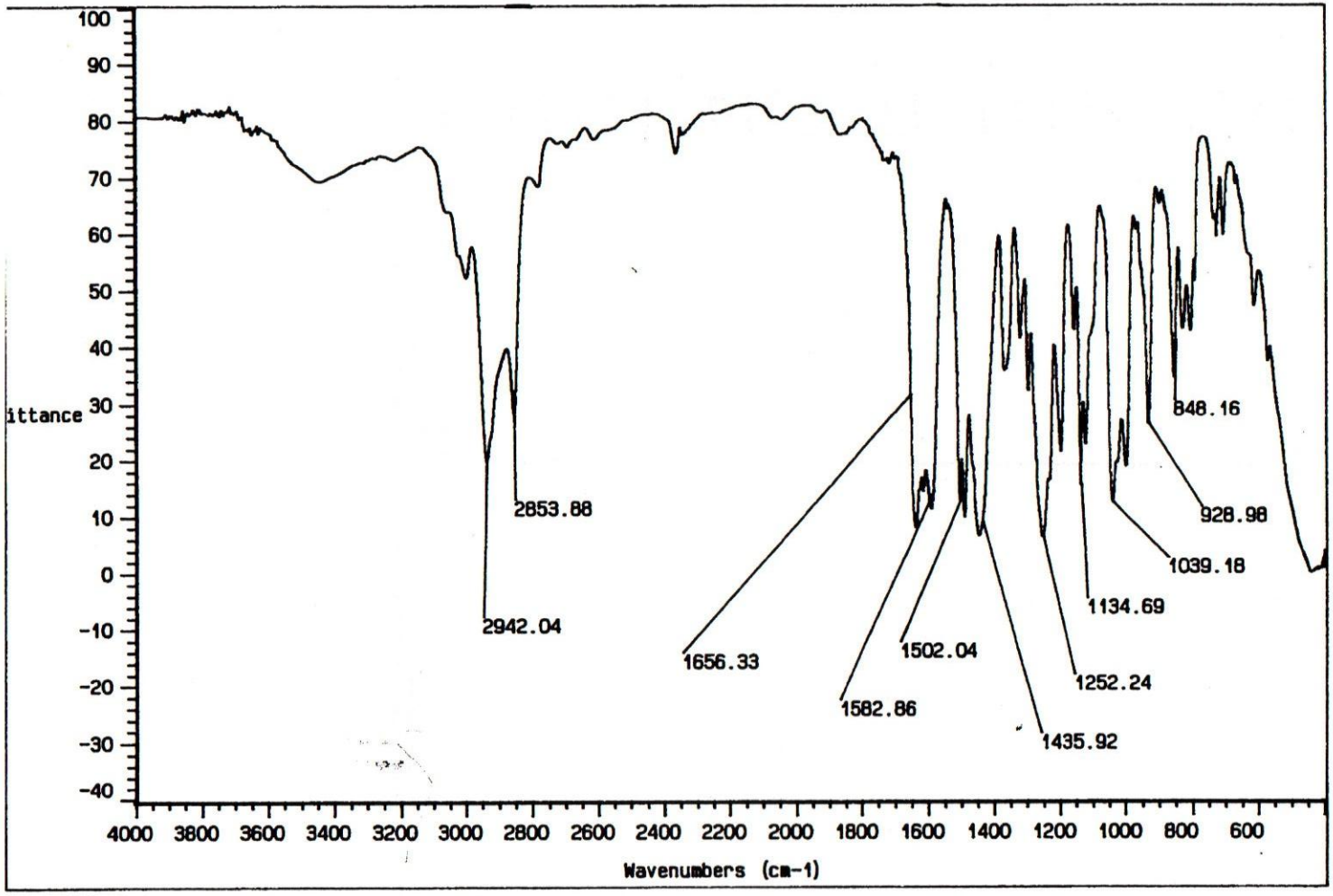


Figure 8: The GC profile of piperine

```
File       : H:\PETER\PEP1.D
Operator   : Peter
Acquired   : 28 Jan 2005    8:06      using AcqMethod NEW
Instrument :   Instrumen
Sample Name: PEP1
Misc Info  : 1µl inject, splitless, methylene chloride
Vial Number: 1
```

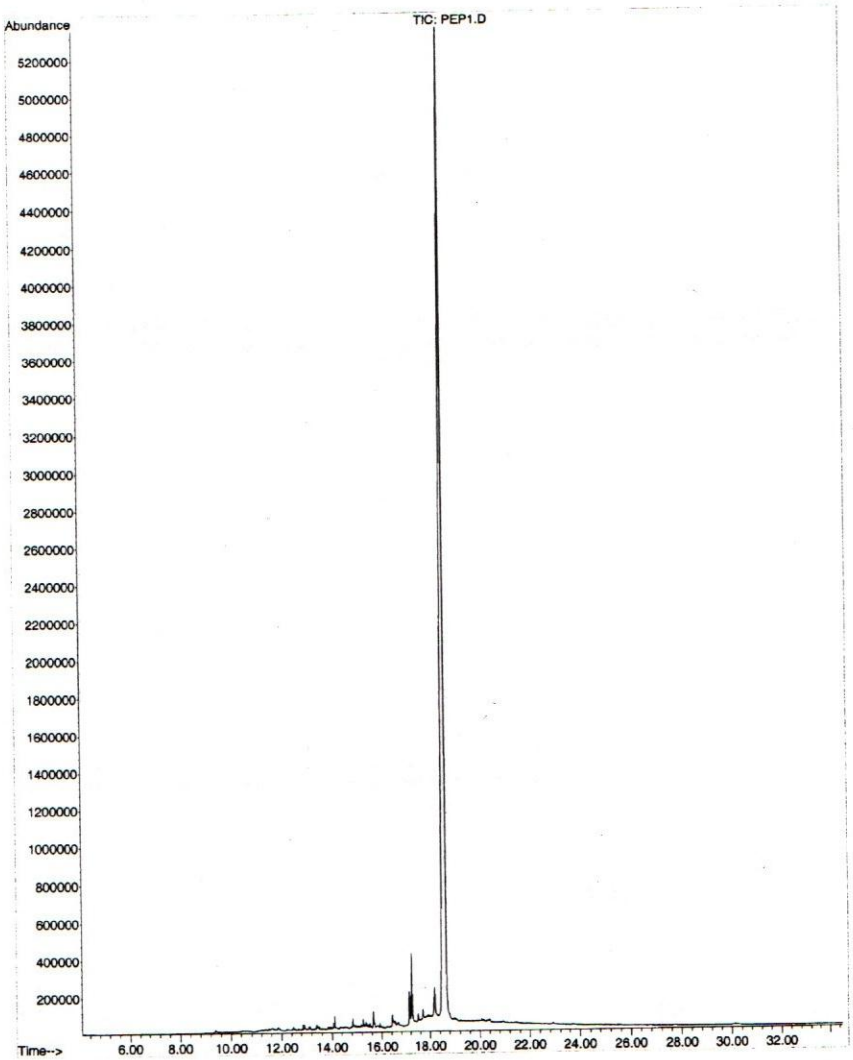
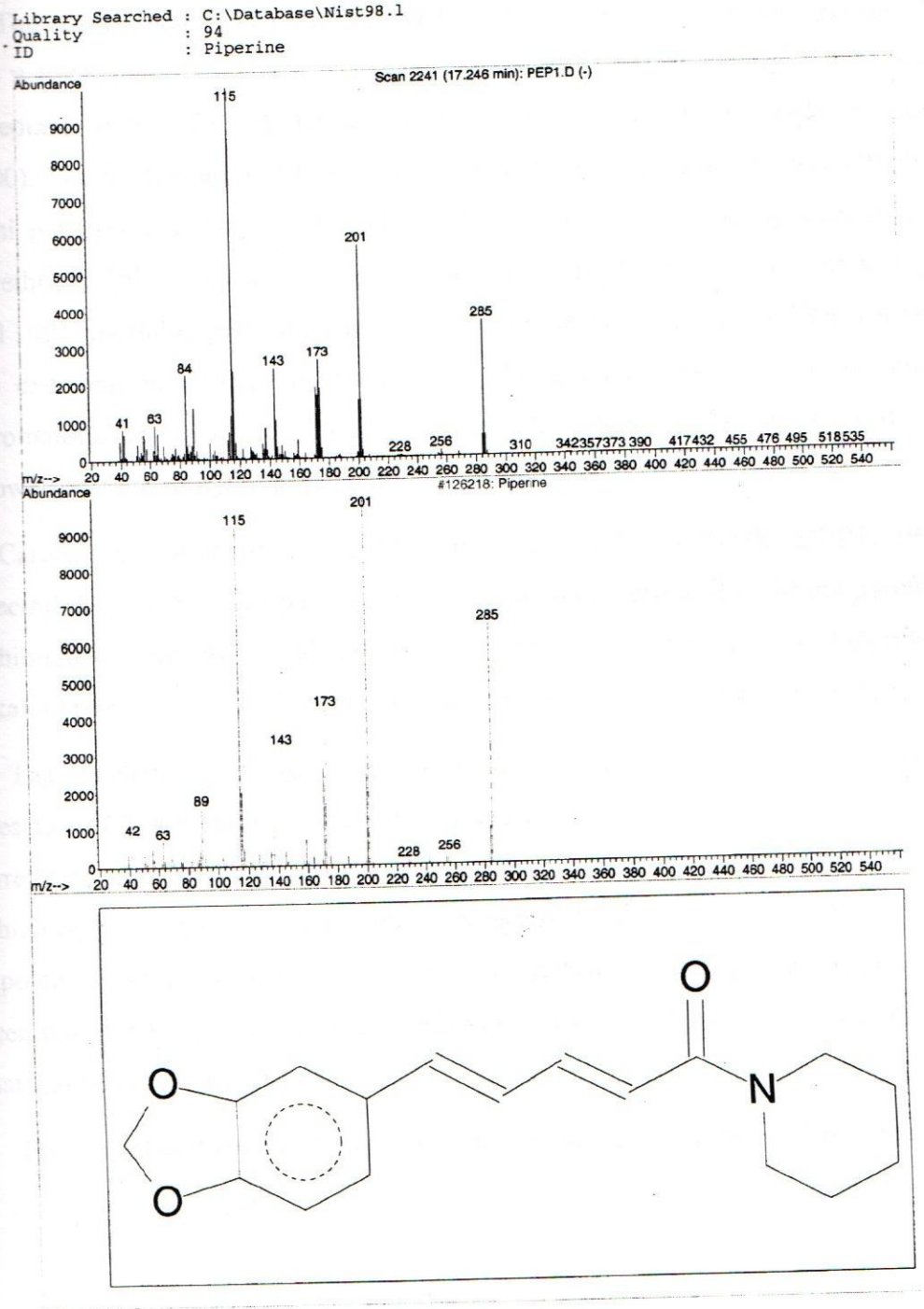


Figure 9: The MS spectrum of piperine




The comparisons of the MS spectrum of the extracted compound to the one obtained from the Aldrich Library confirmed the structure of piperine (Aldrich, 1985).

## 5.5 Discussion

The crude ethanol extract of the seeds of *P. nigrum* L. is a potent synergist to pyrethrins, this was evidenced by the $KD^{t}_{50}$ of 2.35 min. and 100% mortality, which were within the acceptable limits of the specifications by the Kenya Bureau of Standards (Kenya standard, 2000). The fractionation of this active crude extract via column chromatography yielded two semi pure portions labeled $P_1$ and $P_2$. Fraction $P_1$, upon bioassay exhibited synergism to pyrethrins. This yellow crystalline compound recorded a performance of $KD^{t}_{50}$ of 2.85 min. and 100% mortality performances that meets the specification of the Kenya Standards. Due to the excellent bioefficacy of fraction $P_1$, the fraction was purified by repeated column chromatography on silica gel using hexane: ethyl acetate (1:3) until when the TLC analysis showed that the compound was pure.

Careful evaluation of the melting point, $^{1}$H-NMR, $^{13}$C-NMR, DEPT, IR and GC-MS spectral data showed that piperine was the compound responsible for the pyrethrin synergism exhibited by fraction $P_1$ and the crude ethanol extract of *P. nigrum*. Piperine, a piperidine alkaloid possesses the 3, 4-methylenedioxy phenyl moiety (Hopkins and Maciver, 1965).

The excellent bioefficacy of the formulation containing piperine as a synergist and the presence of 3, 4-methylenedioxy phenyl moiety showed that piperine is a potent synergist to pyrethrins. Piperine, can serve as a cheaper and safe alternative to the piperonyl butoxide which is currently used as a pyrethrins synergist. However, inspite of the fact that piperine is a potent synergist there is need to check its stability to sunlight and heat. Arguments have been posed that piperonyl butoxide behaves as a synergist and as a mild stabilizer, a property that can be checked for piperine.

The study has shown that plants have the potential to be exploited as pyrethrum synergists.

# CHAPTER SIX

# FLINDERSIAMINE, A FUROQUINOLINE ALKALOID FROM *V. UGUENENSIS* AS A SYNERGIST TO PYRETHRINS FOR THE CONTROL OF THE HOUSEFLY, *M. DOMESTICA* L. (DIPTERA: MUSCIDAE)

## 6.1 Abstract

Bioassay guided fractionation of a $CHCl_3$/MeOH extract of the root barks of *V. uguenensis* resulted in the isolation of a known compound flindersiamine, a furoquinoline alkaloid. Column chromatography of the $CHCl_3$/MeOH extract of the root barks of *V. uguenensis* yielded six semi-pure fractions that were labeled $V_1$, $V_2$, $V_3$, $V_4$, $V_5$ and $V_6$. Fraction $V_1$, $V_2$ and $V_3$ exhibited synergism to the unsynergised pyrethrins 0.1% against the housefly, *M. domestica* L. in the modified Kearns and Match spray test chamber. Extensive column chromatography of fraction $V_1$, the most superior fraction in synergising pyrethrins furnished flindersiamine a known furoquinoline alkaloid whose structure was established by comparing its $^1$H-NMR and $^{13}$C-NMR with those in the chemical literature and the IR spectra.

## 6.2 Introduction

The genus *Vepris* (Rutaceae) is known to comprise of over 80 species that are distributed in tropical Africa as well as other parts of the world (Chaturverdula *et al.*, 2003). *V. uguenensis* is a shrub growing to 3-4 m tall. The extracts were selected for bioassay-guided fractionation based on lack of reports on its synergistic or cytotoxic effects. However, the genus *Vepris* is cited to be a rich source of alkaloids and limonoids most of which possess the 3, 4-methylenedioxy phenyl moieties (Chaturverdula *et al.*, 2003). The wood of *V. punctata* is known to yield compounds possessing the 3,4-methylenedioxy phenyl groups (Chaturverdula *et al.*, 2003). 5-Methoxymaculine, 5,8-dimethoxymaculine, maculine and flindersiamine are compounds with the 3,4-methylenedioxy phenyl groups that have been isolated from *Vepris* species. The root bark of *Araliopsis soyauxii* (Rutaceae) yields maculine and flindersiamine (Vaquette *et al.*, 1976).

With the assumption that most plants of the genus *Vepris* yield compounds possessing the 3,4-methylenedioxy phenyl group and the fact that compounds possessing the 3,4-methylenedioxy phenyl groups have synergistic properties (Beroza and Barthel, 1952). We embarked on investigations on dynergistic activities of *V. uguenensis*.

## 6.3 Materials and Methods

### 6.3.1 Isolation and Fractionation of the $CHCl_3$/MeOH extract of the root barks of *V. uguenensis*

The TLC analysis was done using several solvent systems and the system chosen for elution was 100% hexane, followed by gradient elution that proceeded by addition of a small proportion of 5% chloroform introduced at a time, up to when 100% chloroform had been used. TLC analysis was used to determine the purity of the eluent fractionates. The isolated fractions ($V_1$, $V_2$, $V_3$, $V_4$, $V_5$ and $V_6$) were evaluated for synergism to pyrethrins against the housefly, *M. domestica* L. in the Modified Kearns and Match spray test chamber.

### 6.3.2 Effect of the $CHCl_3$/MeOH *V. uguenensis* extracts synergised pyrethrins on houseflies, *M. domestica* L.

The synergistic activities of the six fractions $V_1$, $V_2$, $V_3$, $V_4$, $V_5$ and $V_6$ were evaluated by bioassays (Section 3.5.5)

## 6.4 Results

### 6.4.1 $KD^t_{50}$ and percent knockdown of houseflies exposed to $CHCl_3$/MeOH fractions of *V. uguenensis* synergised pyrethrins

Fraction $V_1$ recorded a higher performance compared to all the others obtained from the $CHCl_3$/MeOH extracts of *V. uguenensis* in paralyzing the houseflies in the 3rd min. of exposure (Table 12). However, the performance of the fraction was not significantly ($P < 0.05$) different from that of fractions $V_2$, $V_5$ and $V_6$. Fractions $V_3$ and $V_4$ which were not significantly different from each other ($P < 0.05$) recorded poor performances compared to the other fractions. In the 6th and 9th min. the performances of the 6 fractions were varied but not significantly different from each other (Table 12). Upon comparison of the knockdown performances of the crude extract to the fractions of the extract, fraction $V_1$ was superior than other fractions and to the $CHCl_3$/MeOH extract of *V. uguenensis*.

Fraction $V_1$ recorded the best $KD^t_{50}$ at 3.98 min. followed by $V_2$ at 4.88 min. and $V_6$ at 4.93 min (Figure 10). All the six fractions performed below the specifications of the Kenya Bureau of Standards (Kenya Standard, 2000). The $KD^t_{50}$ of fraction $V_1$ was better than that of the crude extract, 4.17 min. However, fraction $V_1$ was a late knockdown agent with a

performance of 38.70% after 3 min. similar to that of the crude extract. Fractions $V_2$, $V_3$, $V_4$, $V_5$ and $V_6$ were very poor knockdown agents.

### 6.4.2 Mean percent mortalities of the houseflies exposed to $CHCl_3$/MeOH extract of *V. uguenensis* synergised pyrethrins

Fractions $V_1$, $V_2$, $V_3$ and $V_5$ exhibited synergism by boosting the mortality effects of the unsynergised pyrethrins 0.1%. $V_1$ and $V_2$ were not significantly different from each other and were the best killing agents, enhancing the mortality effects to 77.65 and 77.98%, respectively (Figure 11). However, all the six fractions did not match the performance of the STD nor did they meet the specifications of the Kenya Bureau of Standards at 95% kill after 24 hours (Kenya Standard, 2000).

Table 12: $KD^{t}_{50}$ and percent knockdown of houseflies exposed to $CHCl_3$/MeOH fractions of *V. uguenensis* synergised pyrethrins

| Sample. | Polynomial equation | $r^2$ | Time $KD^{t}_{50}$ | % KD at 3 Min | 6 Min | 9 Min |
|---|---|---|---|---|---|---|
| $V_1$ | $Y = -11.87 + 21.59X - 1.77X^2 - 6.14E\text{-}02X^3$ | 0.791 | 3.98 | 38.7 | 67.4 | 84.2 |
| | | | | (38.22[a]) | (67.00[a]) | (84.80[a]) |
| V+ | $Y = -5.77 + 8.12X + 1.25X^2 - 0.12X^3$ | 0.827 | 4.88 | 26.7 | 62.7 | 83.4 |
| | | | | (24.81[ab]) | (63.14[a]) | (82.14[a]) |
| $V_3$ | $Y = 0.64 - 0.64X + 1.85X^2 - 0.11X^3$ | 0.905 | 7.09 | 12.4 | 39.7 | 64.8 |
| | | | | (11.10[b]) | (39.99[a]) | (64.07[a]) |
| $V_4$ | $Y = -2.92 + 3.51X + 1.05X^2 - 7.30E\text{-}02X^3$ | 0.786 | 7.26 | 15.1 | 40.2 | 60.5 |
| | | | | (13.76[b]) | (40.14[a]) | (60.34[a]) |
| $V_5$ | $Y = -5.2`9 + 6.24X + 0.55X^2 - 4.06E\text{-}02X^3$ | 0.778 | 6.82 | 17.3 | 43.2 | 65.8 |
| | | | | (18.88[ab]) | (43.65[a]) | (64.52[a]) |
| $V_6$ | $Y = -4.99 + 7.03X + 1.43X^2 - 0.12X^3$ | 0.961 | 4.93 | 25.7 | 62.6 | 86.0 |
| | | | | (22.12[ab]) | (66.04[a]) | (86.53[a]) |

Figures in parenthesis represent the actual values obtained whereas the others represent the regressed values as per the equation of regression. Any two means in the same column sharing a common letter are not significantly different ($P < 0.05$).

Figure 10: $KD^{t}50$ and mean percent knockdown of the houseflies exposed to $CHCl_3$/MeOH extract of *V. uguenensis* synergised pyrethrins

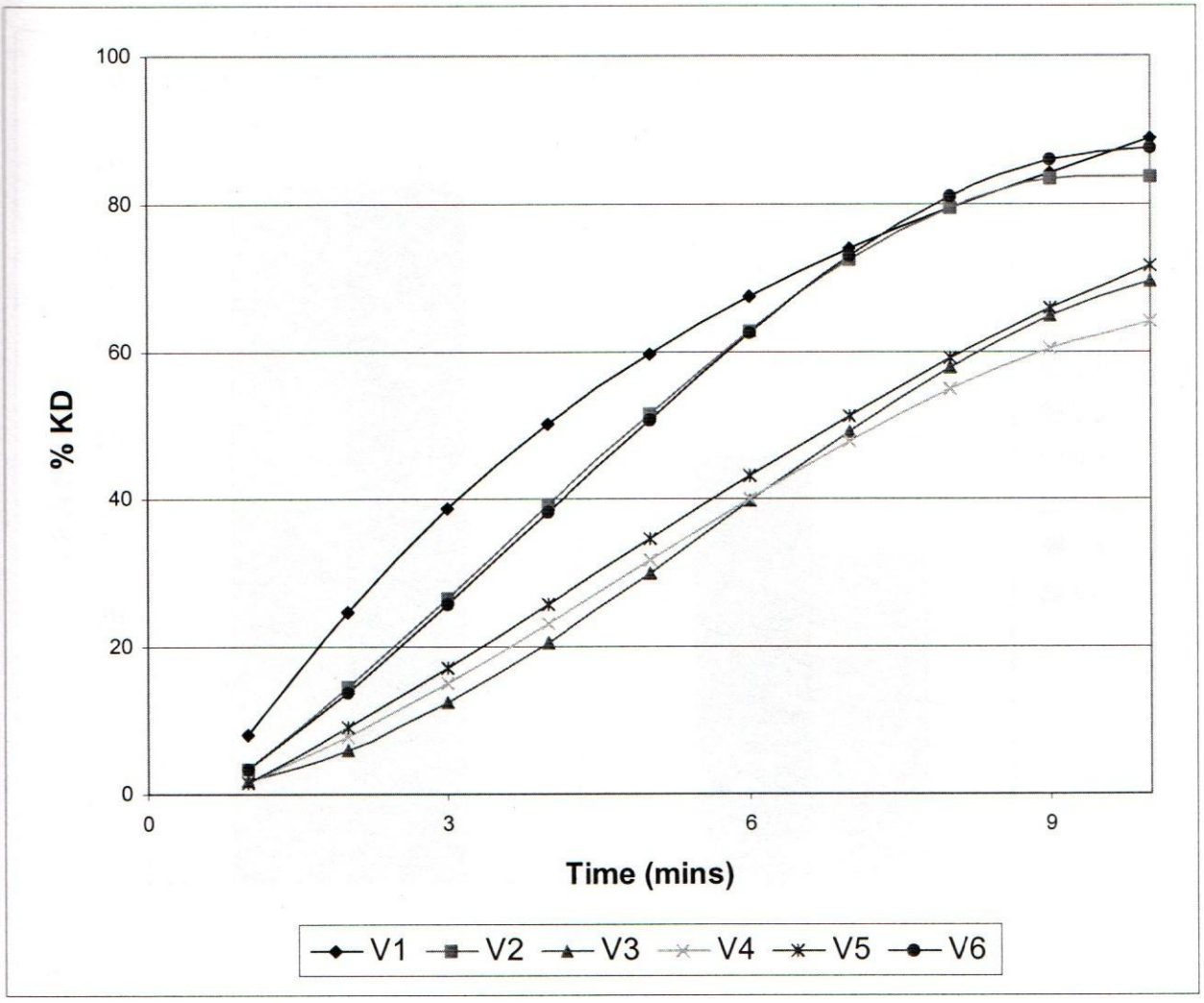
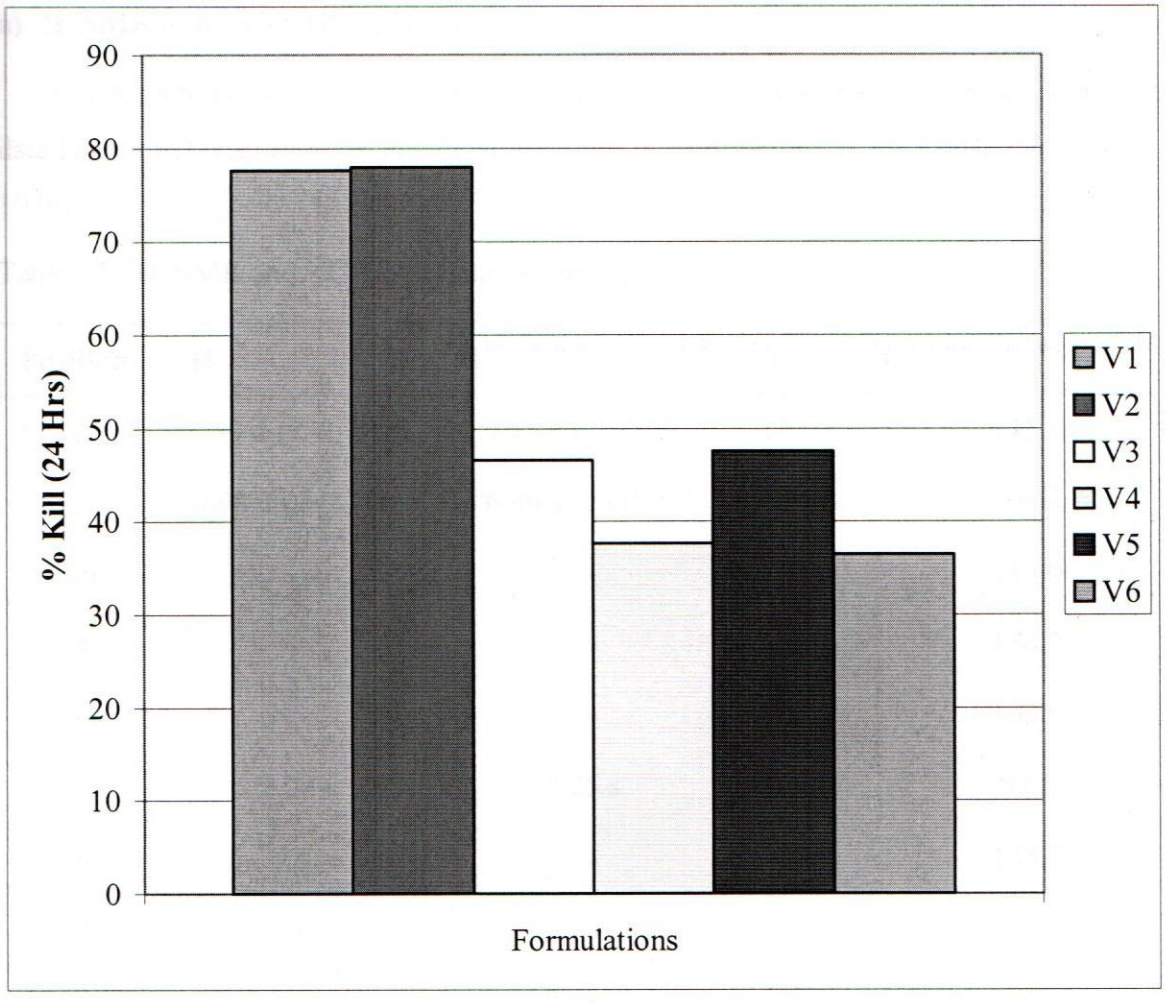


Figure 11: Mean percent mortalities of the houseflies exposed to $CHCl_3$/MeOH extract of *V. uguenensis* synergised pyrethrins



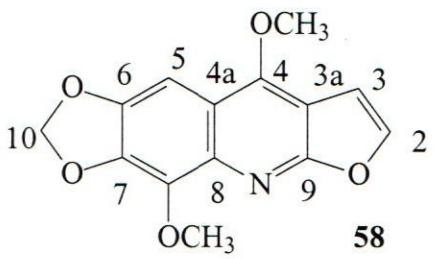
### 6.4.3 Structural elucidation of flindersiamine

#### a) $^{1}$H-NMR and $^{13}$C-NMR spectra

Flindersiamine was identified by comparison of $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data (Table 13) with those in chemical literature (Chaturverdula *et al.*, 2003; Vaquette *et al.*, 1976).

Table 13: $^{1}$H-NMR and $^{13}$C-NMR data for flindersiamine

| Position | $^{1}$H | $^{1}$H lit (Vaquette *et al.*, 1976) | $^{13}$C | $^{3}$C lit(Chaturverdula *et al.*, 2003) |
|---|---|---|---|---|
| 2 | 7.55 d (*J*=2.7Hz) | 7.55 d (*J*=3Hz) | 143.0 | 143.1 |
| 3 | 6.98 d (*J*=2.7Hz | 6.96 d (*J*=3Hz) | 104.4 | 104.4 |
| 3a | — | — | 114.9 | 115.0 |
| 4 | — | — | 156.1 | 156.2 |
| 4a | — | — | 102.9 | 103.0 |
| 5 | 7.24 s | 7.25 s | 92.4 | 92.5 |
| 6 | — | — | 138.0 | 138.2 |
| 7 | — | — | 137.7 | 137.9 |
| 8 | — | — | 135.9 | 136 |
| 8a | — | — | 146.7 | 146.8 |
| 9 | — | — | 162.6 | 162.8 |
| $OCH_3$-4 | 4.38 s | 4.36 s | 60.6 | 60.7 |
| $OCH_3$-8 | 4.21 s | 4.25 s | 58.9 | 59.0 |
| 10 | 6.01 s | 6.05 s | 101.5 | 101.6 |

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The melting point of flindersiamine was determined to be 204-206 $^{o}$C compared to that of the literature value of 206 - 207 $^{o}$C (Vaquette *et al.*, 1976). The IR spectrum was used to confirm the main structural features of flindersiamine. Absorption at 2922.45 $cm^{-1}$ was assigned to carbon due to $-CH_2$ group of the methylenedioxy, 2850.61 $cm^{-1}$ to the tetrahedral carbon due to $-CH_3$ group of the methoxy groups (Figure 12).

Figure 12: The Infra red spectrum of flindersiamine

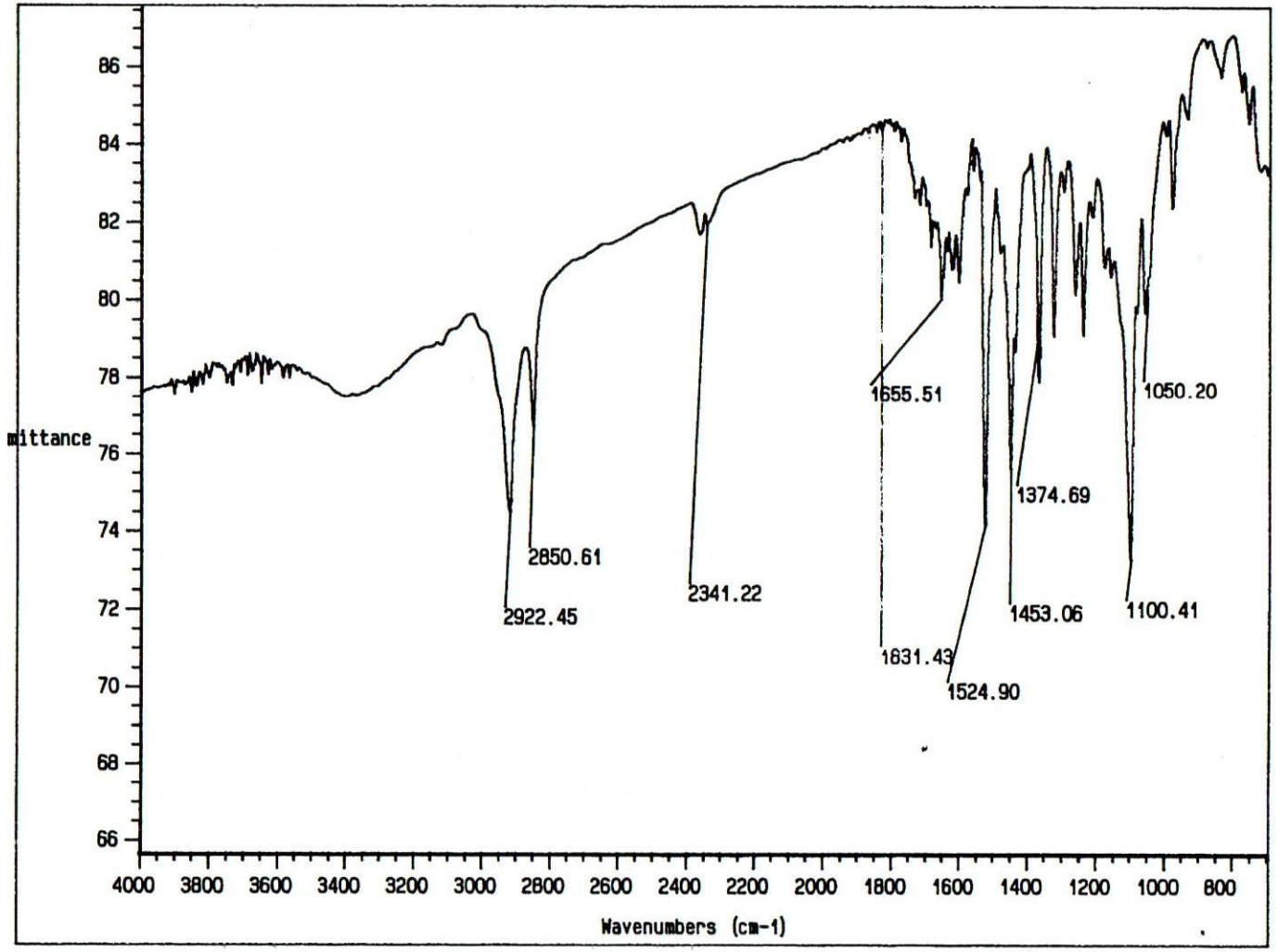

Figure 13: The GC profile of flindersiamine

```
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Acquired   :  3 Mar 2005  14:55     using AcqMethod NEW
Instrument :   Instrumen
Sample Name: V8-40-57.12-15
Misc Info  : 1µl inject, splitless, methylene chloride
Vial Number: 10
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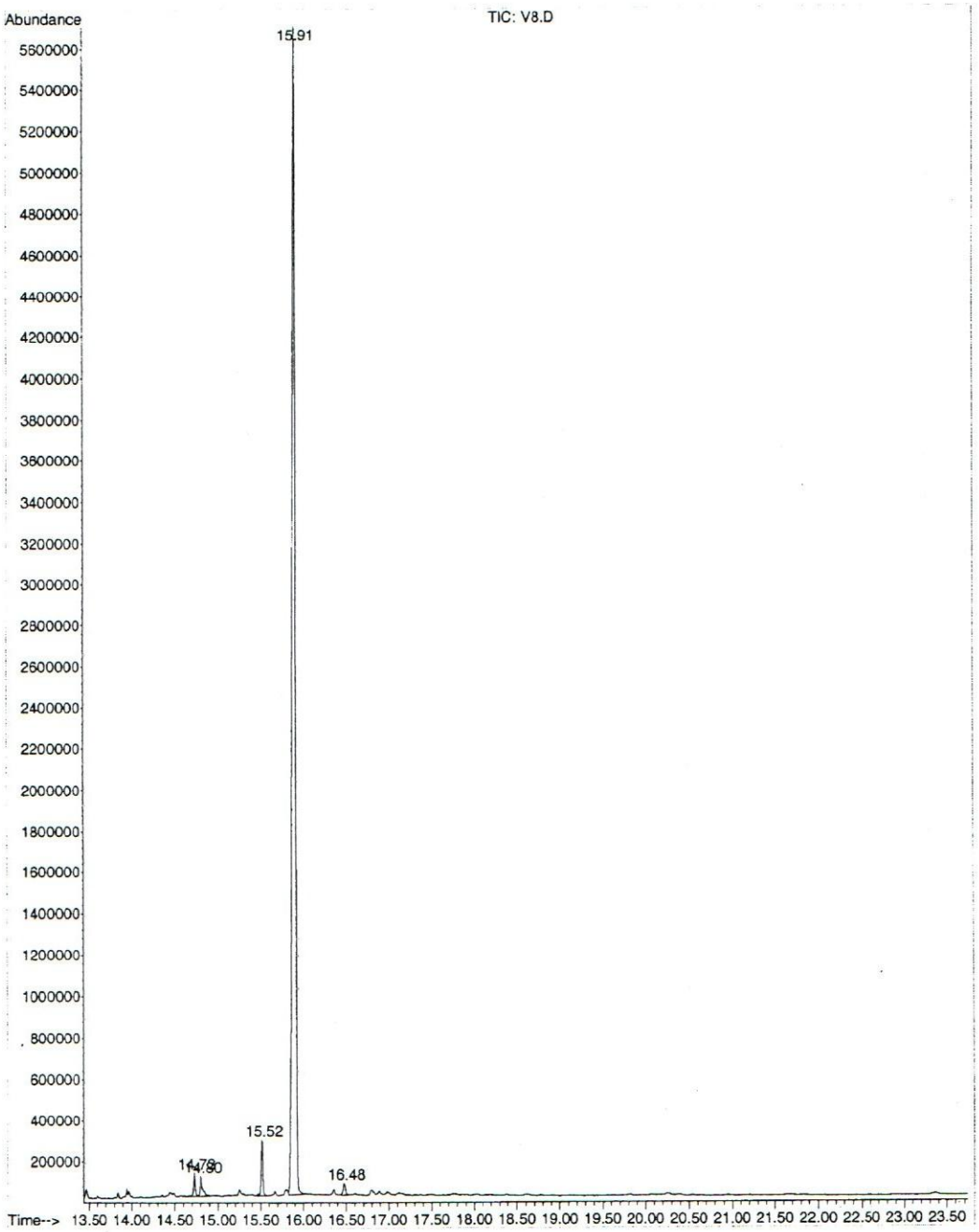
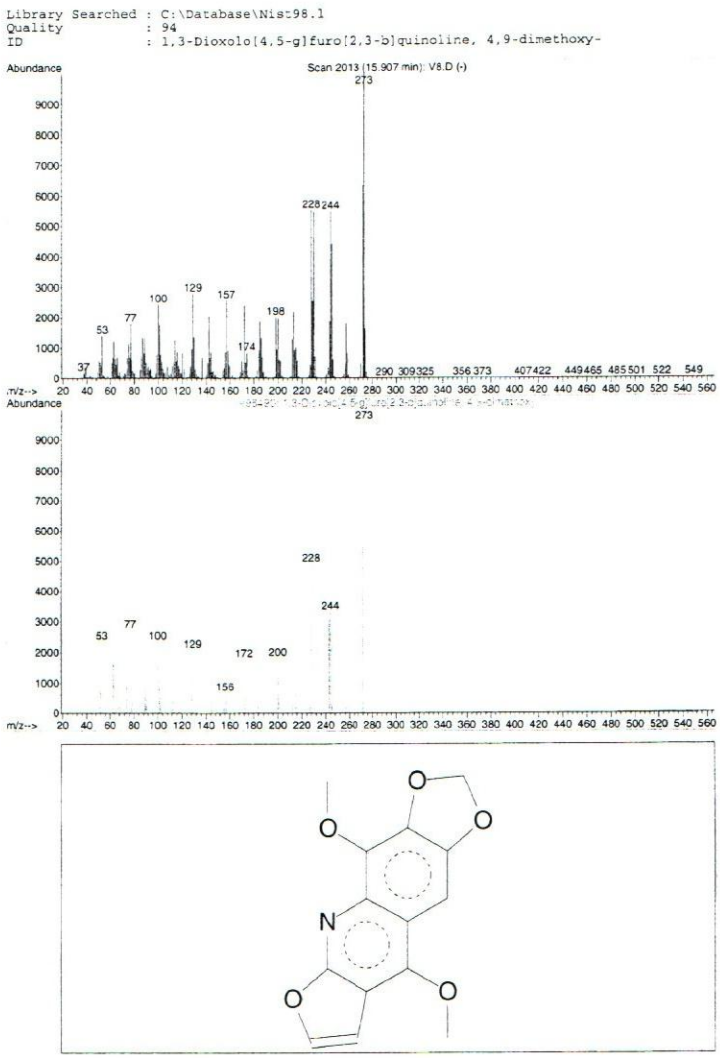


Figure 14: The MS spectra of flindersiamine




The comparisons of the MS spectrum of the extracted compound to the one obtained from the Aldrich Library confirmed the structure of flindersiamine (Aldrich, 1985).

## 6.5 Discussion

Following the synergistic activity exhibited by the crude $CHCl_3$/MeOH extract of *V. uguenensis*, the compound responsible for the potency in synergism was isolated. After fractionating the extract into 6 fractions that were assayed for synergism to pyrethrins, fractions $V_1$ and $V_2$ were the best in synergising pyrethrins as killing agents but not as a knockdown agents (Table 12). It is interesting to note that synergism exhibited by the extract could not be narrowed down to a specific compound since the plant has not been cited to contain any chemically or biologically active compounds. The suspicions that the compounds with the 3,4-methylenedioxy phenyl moieties are responsible for synergising pyrethrins (Beroza and Barthel, 1952) was confirmed when fraction $V_1$ was purified to yield flindersiamine, an alkaloid possessing the 3,4-methylenedioxy phenyl group. Flindersiamine whose structure was confirmed by its $^{1}$H-NMR, $^{13}$C-NMR, DEPT and IR spectra has been reported to occur in other members of the Rutaceae family (Vaquette *et al.*, 1976).

Flindersiamine did not increase the efficacy of pyrethrins during the first 10 min. after treatment but it doubled the toxicity of the extract 24 h after the treatment. Therefore this work does not give new information on the way flindersiamine synergises pyrethrins in solutions but indicates that flindersiamine is a synergist for insecticidal activity.

# CHAPTER SEVEN

# GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

## 7.1 Discussion

At the beginning of the study it was hypothesized that compounds present in the selected plants could synergise pyrethrins. Bioassay guided fractionation led to isolation of piperine, from *P. nigrum* L. and flindersiamine from *V. uguenensis* as the consistent synergistic agents to pyrethrins. The presence of the 3, 4-methylenedioxy phenyl moiety in the elucidated compounds ascertained the arguments by Hopkins and Maciver (1965) that the compounds possessing this group synergise pyrethrins. The comparative extents of synergism by these two compounds vary extensively. Piperine was a more potent synergist than flindersiamine an indication that there are aspects beyond the presence of the 3, 4-methylenedioxy phenyl group in the phenomenon of synergism that is yet to be known. The elucidation of the structure-activity relationship will be key in understanding the art of synergism.

Investigations of the pyrethrins and the five potent synergistic materials showed clearly that the ethanol extract of *P. nigrum* retained its superior performances both as a knockdown and a killing agent. However, it is irrational to compare the synergistic effects of the crude extracts when the effects of the solvents, the active ingredients and the control were unknown. Since, the investigation that is qualitative whereby only intrinsic toxicities of the synergised pyrethrins were being measured, therefore, a solvent that is non-toxic to the housefly was necessary. Screening various solvents showed that ethanol and isopropyl alcohol were the best since they had minimal effect on the test fly. However, isopar M and SST were not good at all for qualitative investigations.

For this study, very low concentrations of pyrethrins were selected in order to lower the cost of formulations and maintain their effecacy using crude extracts. Pyrethrins 0.2% was very effective by itself. Pyrethrins 0.15% had average performance in terms of kill yet very good knockdown agent. It is pointless to enhance these doses. At pyrethrins 0.1%, which recorded a good knockdown effect but a below average mortality performance, was chosen. This performance necessitated the need to synergise it since pyrethrins 0.05% showed an insignificant mortality effect yet improved knockdown effects. Pyrethrins 0.05% does not perform well probably due to its very low quantities of the actives.

The Kenya Bureau of Standards advocates that an insecticide qualifies to be good if it attains percentage mortality of 95% after 24 h and therefore if the synergised pyrethrins meet this specification they could be classified as potent insecticides (Kenya Standard, 2000). The screenings done in this study showed that only one sample; ethanol extract of *P. nigrum* qualifies at this stage of evaluation as a potential booster. However, since the $CHCl_3$/MeOH extract of *V. uguenensis* exhibited slight synergism, yet the plant had not been cited for any biologically or chemical activity but it was found necessary to investigate the compound present as well.

### 7.2 Conclusion

The results of the tests to determine the synergistic effects of the fifteen crude extracts from the various plants upon the pyrethrins showed that:

a) The ethanol extract of *P. nigrum* L. provided a superior performance both as a knockdown and killing agent and piperine is responsible for this observed performance.

b) The hexane extracts of *B. napus*, *S. indicum* and *P. americana* were effective as knockdown agents but not good killing agents

c) The $CHCl_3$/MeOH extract of *V. uguenensis* gave average performance as a knockdown as well as a killing agent and flindersiamine compound was responsible for this observed performance.

d) The hexane extracts of *Z. chalybea* and *A. vera*, the methanol extracts of *S. indicum*, *B. napus, P. americana, Z. chalybea, P. longum* and *A. vera* as well as $CHCl_3$/MeOH extract of *A. vera* were potent as knockdown agents but very poor killing agents.

### 7.3 Recommendations

The search for an environmentally safe, cheap and locally available alternative to piperonyl butoxide has yielded some positive results since various aspects of the combination of the pyrethrins and these potent synergistic agents are not known.

a) The immediate bioefficacy evaluations confirmed compatibility of pyrethrins to these synergists. However, long term stability of pyrethrins in these alkaloids should be studied.

b) In unraveling various aspects of the synergists as well as the mixture of pyrethrins and synergists, a detailed material safety data sheet should be documented. This sheet will seek to clarify several factors such as toxicity, solubility and the optimum quantity required to invoke an economical and efficient amount of the synergist.

c) Action to lower the pungency associated with piperine without compromising its quality should be evaluated.

### 7.4 Policy

There is no concrete statement of intentions and principles in relation to the adoption of the plant based synergists to pyrethrins by the Pyrethrum Board of Kenya. The study of using plant based extracts as synergist was a long term project. However, the immediate success of the study calls for an immediate drafting of a policy that will observe the substitution of piperonyl butoxide by the compound obtained in this study. The policy should center on the profitability and competitiveness of the pyrethrum enterprise across the major producing regions and the synthetic analogs in the market. It is clear that the existence and the level of the domestic market for pyrethrum and pyrethrum products rely heavily on the use of the synergists, therefore official regulations concerning the production of the synergistically active plant extracts, incorporating them in pyrethrum based products and marketing the new products should be instituted. This expression of incorporating the new synergist should precede a clear environmental policy.

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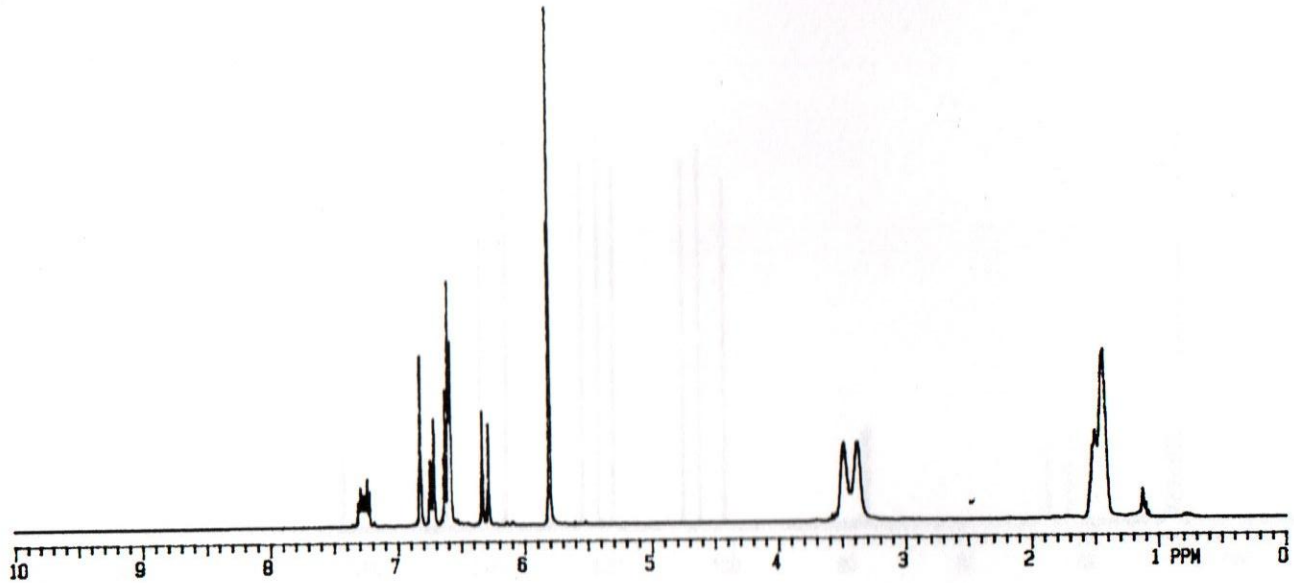
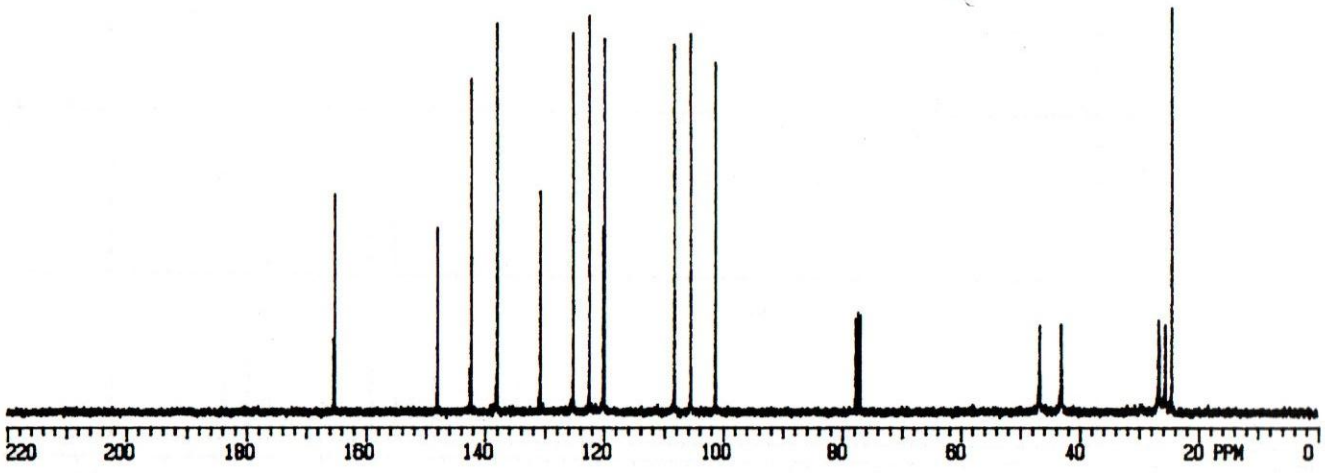
**Appendix I: Performance rank of the extracts as synergists to pyrethrins 0.1% against houseflies**

| Sample | $KD^{t}_{50}$ | Factor | Rank | Order | Sample | % Kill | Factor | Rank | Order |
|---|---|---|---|---|---|---|---|---|---|
| 0.1% Pys | 3.09 | 1 | 11 | E | 0.1% Pys | 40.46 | 1 | 6 | P |
| I | 6.06 | 0.51 | 15 | A | I | 73.6 | 1.82 | 2 | STD |
| VC | 6.03 | 0.51 | 15 | H | VC | 18.58 | 0.46 | 12 | I |
| VM | 5.37 | 0.58 | 14 | F | VM | 15.43 | 0.38 | 13 | D |
| VH | 4.63 | 0.67 | 13 | D | VH | 21 | 0.52 | 11 | U |
| U | 4.17 | 0.74 | 12 | G | U | 58.96 | 1.46 | 4 | F |
| G | 2.11 | 1.46 | 6 | C | G | 24.4 | 0.6 | 10 | PYS |
| B | 2.48 | 1.25 | 9 | P | B | 29.43 | 0.73 | 8 | C |
| P | 2.35 | 1.31 | 8 | B | P | 100 | 2.47 | 1 | E |
| C | 2.33 | 1.33 | 7 | STD | C | 32.46 | 0.8 | 7 | B |
| F | 1.59 | 1.94 | 4 | PYS | F | 44.4 | 1.1 | 5 | A |
| H | 1.46 | 2.12 | 3 | U | H | 21.23 | 0.52 | 9 | H |
| E | 1.39 | 2.22 | 1 | VH | E | 32.17 | 0.8 | 7 | G |
| D | 1.66 | 1.86 | 5 | VM | D | 60.23 | 1.49 | 3 | VH |
| A | 1.44 | 2.14 | 2 | I | A | 32.1 | 0.79 | 8 | VC |
| STD | 2.89 | 1.07 | 10 | VC | STD | 100 | 2.47 | 1 | VM |

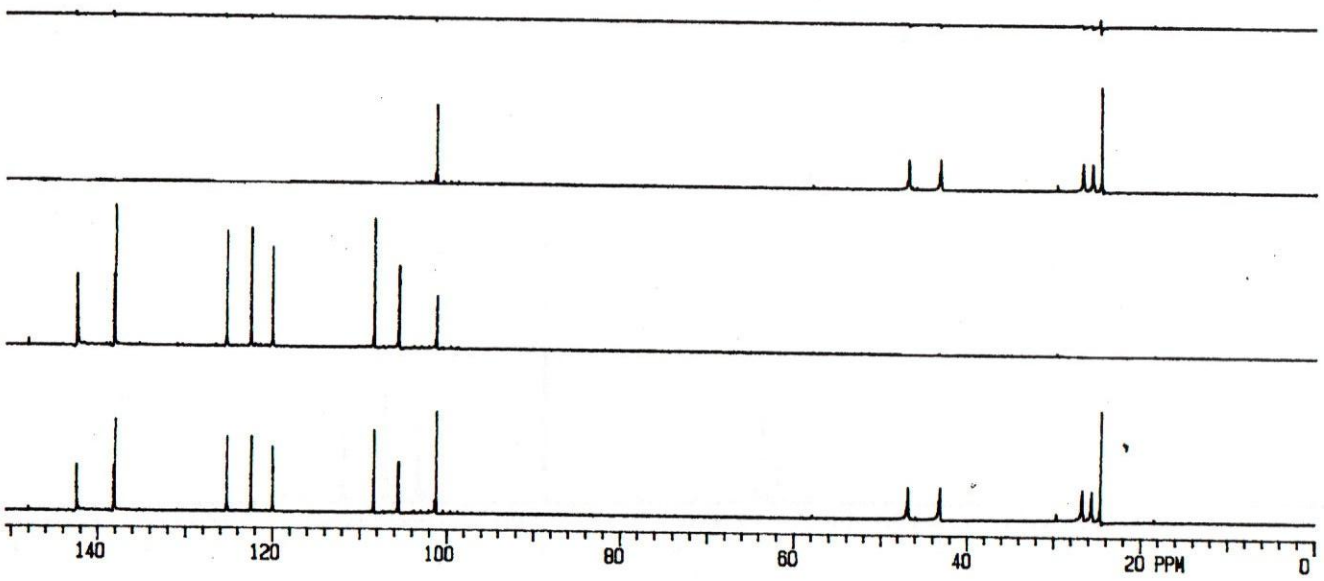
**Appendix II: $^{1}$H-NMR spectra for piperine**



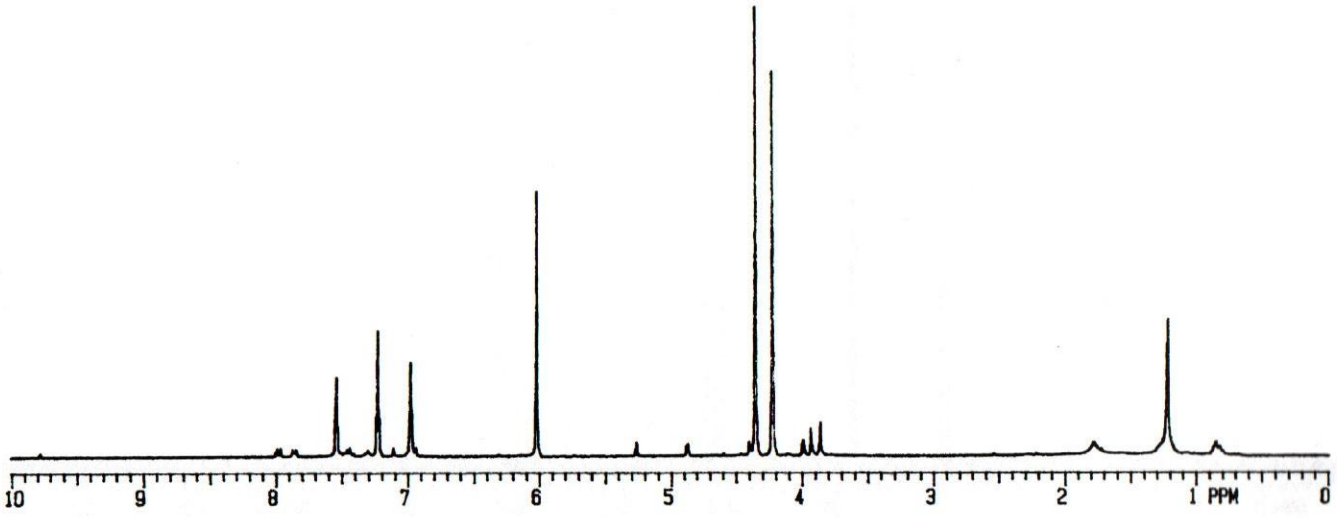
**Appendix III: $^{13}$C-NMR spectra for piperine**



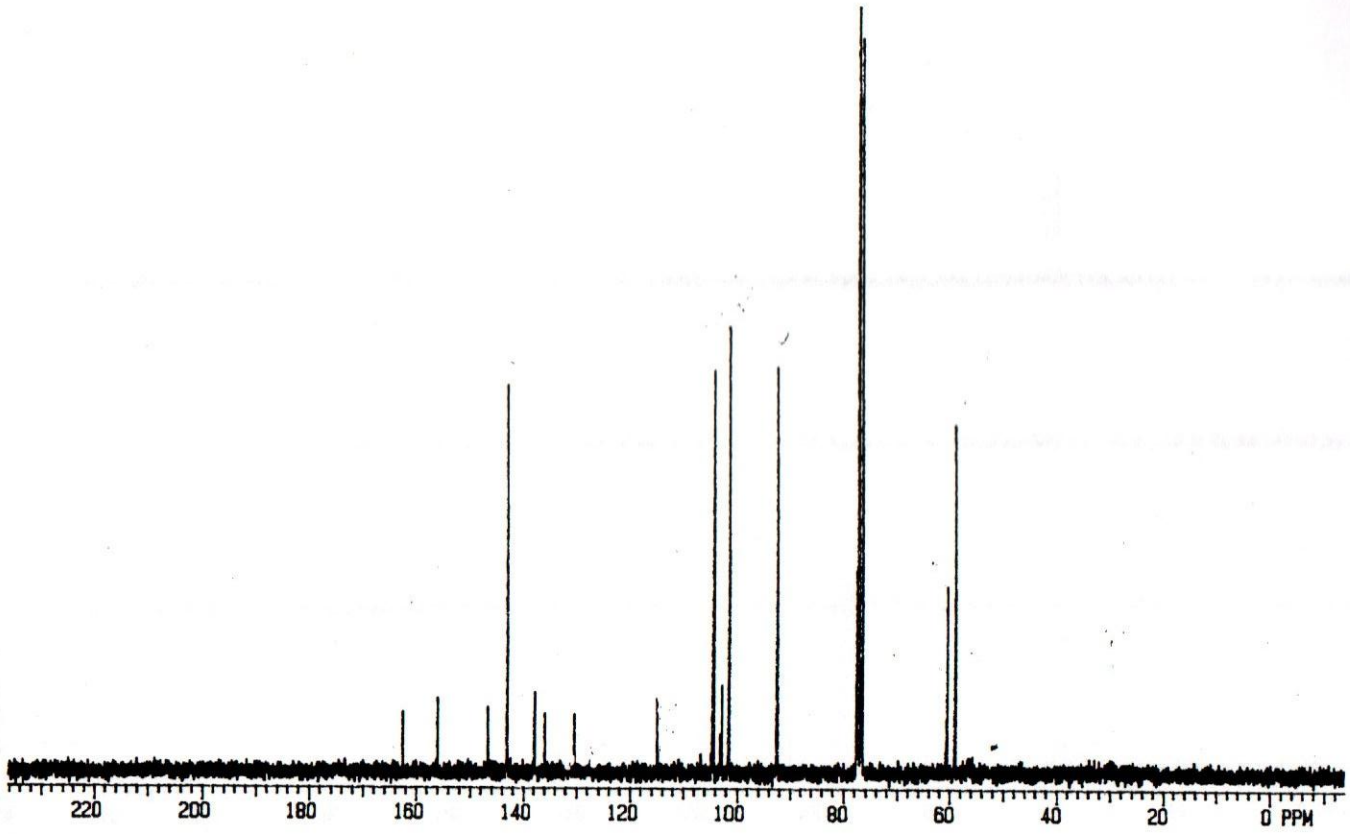
**Appendix IV: DEPT spectra for piperine**



**Appendix V: $^{1}$H-NMR spectra for flindersiamine**



**Appendix VI: $^{13}$C-NMR spectra for flindersiamine**



**Appendix VII: DEPT spectra for flindersiamine**