TOXICITY AND MORTALITY OF SYNERGIZED CRUDE ESSENTIAL OIL EXTRACTS FROM *Tagetes minuta* (L.) ON *Periplaneta americana* (Blattidae)

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Abstract

Cockroaches are insects that belong to the order Blattodea, suborder Blattaria and family Blattidae. The species that is most common and wide spread in the world is *Periplaneta americana*(L.). They are increasing in number due to their resistance to synthetic insecticides that are in most cases used in their control. This has made them a public health problem due to their close association with human wastes and they are mechanical transmitters of human disease causing pathogens. Therefore there is need of coming up with an alternative insecticide. Although studies have shown that the *Tagetes minuta*(L.) has insecticidal properties, this property has not been tested on cockroaches. *T. minuta*'s insecticidal compounds have never been synergized with oil components of *Sesamum indicum*(L). In the present study, synergized crude essential oil extracts from *T. minuta* were tested as a potential fumigant and contact toxicant on *P. americana.* The study took place in Maseno Division where 35 households were interviewed using questionnaires and *P. americana* collected from their premise. The crude essential oil extracts were obtained from floral and folia parts of the plant by percolating with methanol and rotor-vaping, then synergized with oil components (methanol extract) from *S. indicum* seeds. The mixture of the two oils was tested for toxicity on the *P. americana* in the laboratory using bio-assay method; where cockroaches were exposed to insecticide. The tersi, tracheal and nervous systems of the *P. americana* were histologically examined to establish how the affected cells and tissues differ from the unaffected ones. Biopac MP35 unit was used to discern the physiological effect of the crude essential oil extracts from *T. minuta* by doing extracellular multiunit record on the *P. americana*. Data from questionnaires was converted into frequency counts and percentages using SAS statistical analysis software. Susceptibility of *P. americana* to *T. minuta*'s crude essential oil extracts was analyzed as a four factorial experiment. ANOVA and Duncan's Multiple Range test were used to test for significance of the experiments at $P \leq 0.05$, LC_{50} of *T. minuta*'s synergized and non-synergized extracts were determined using probit analysis method. From the questionnaires, 83% of the households admitted to cockroach menace; 54% use insecticides to control them and 91% said that not all cockroaches die after treatment. LC_{50} of synergized extract was 6.31 g/L while that for non-synergized extract was 8.91 g/L. Non-synergized extract had a mortality of 48 (n=60) while synergized extract had mortality of 60 (n=60). 120 g/L of both synergized and non-synergized extract had knockdowns and kills made within 20 and 25 minutes respectively. Oil components of *S. indicum* synergized *T. minuta*'s crude essential oil extracts thus reduced knockdown and kill times and increased the cockroaches' mortality. *T. minuta*'s insecticidal compounds did not cause anatomical changes on the tersi, tracheal and nervous tissue; it worked physiologically via impairing the function of Na^+ ion channels of the neurons thus killing *P. americana*. In conclusion, cockroaches are a menace to households of Maseno and they are susceptible to synergized and non-synergized extracts from *T. minuta*.

CHAPTER ONE

1.0 Introduction

1.1 Background information

Cockroaches are arthropods that belong to the class insecta, order Blattodea, sub order Blattaria and family Blattidae. They are household pests that are catholic in their feeding habit (Barbara, 2005). By this behavior and adaptation, they contaminate human food through feeding and spreading of pathogens from human wastes on to human food. Cockroaches have dorsoventrally flattened bodies which enable them to inhabit narrow crevices in walls (especially of semi-permanent or permanent but un-plastered walls) of houses and household furniture and thus makes them hard to deal with (Barbara, 2005). Cockroaches are public health problem due to their association with human wastes (e.g. kitchen remains and feces) and diseases (e.g. asthma, diarrhea and round worms); their movement from sewers and sewer lines into homes and commercial establishments bring about physical transportation of the pathogens from wastes to food. Cockroaches are found almost everywhere: in homes, caves, mines, privies, latrines, cesspools, sewers, sewerage treatment plants, and dumpsites and their presence in these places is of epidemiological significance (Bell and Adiyodi, 1981).

At least 22 species of pathogenic human bacteria, fungi, and protozoans have been isolated from common cockroaches (Rust *et al.,* 1991). Some of the bacteria isolated from cockroaches include *Shigella boydii*, *S. dysenteriae*, *Salmonella enteric serovar typhimurium*, *Klebseilla oxytoca*, *K. ozaena* and *Serratia marcescens* (Oothuman *et al*, 1989). Others are *Escherichia coli* and *Klebsiella pneumoniae* (Vythilingam *et al*, 1997); *Enterobacter* sp. and *Staphylococcus aureus* (Prado *et al,* 2002). Cockroaches also harbor fungi-Candida, Mucor, *Aspergillus fumigans*, *A. niger*, Rhizopus and Penicillium (Salehzadeh *et al*, 2007). In their study, Fakoorziba *et al*, (2010) found more than 18 different species of gram negative bacteria inside and on cockroaches found in hospitals; of which some are β-lactamase positive. Therefore, other than being pathogenic, they are also resistant to sulphonamide drugs and are very potent causes of nosocomial infections (Fakoorziba *et al*, 2010). This makes the "common" cockroach very important physical and mechanical transmitters of pathogens. They carry the pathogens from the sewers into

the human habitations. Cockroaches are also aesthetically displeasing because they can soil items with their excrement and regurgitation, not to mention the destruction through eating of paper documents and clothes amidst other problems.

P. americana are referred to as 'common' cockroaches because they are found nearly globally. This is so due to their dorsoventrally flattened body which is an adaptation that makes them ideal inhabitants of any crevices in the walls and furniture of households (Hoell *et al*, 1998). Currently, control of the cockroaches is mostly with pyrethroid insecticides (Milani, 1995); but cockroaches have developed resistance to these insecticides. The outcome of this is that 'common' cockroaches are drastically increasing in population and being mechanical transmitters of pathogens, they posse a public health risk (Miller and Koehler, 2003).

The weed plant, Mexican marigold, *Tagetes minuta* belongs to the family *Asteraceae*. This family encompasses the genus *Tagetes* that comprises of 36 species, of which the most widely known species are *T. erecta*, *T. patula* and *T. minuta* (Weaver *et al*., 1994). The three *Tagetes* species are best known because they thrive well in the tropics. Their essential oils have the following properties: anti-corns, anti-warts, anti-helminthic, carminative, diaphrotic, anti-spasmodic, bactericidal, insecticidal, insect repellence and nematocidal (Lawless, 1995). Weaver *et al.* (1994) reported that *T. minuta*'s essential oils have insecticidal and insect repellence properties but has not been tested against *P. americana*, and the mode of its insecticidal action was not known.

Sesame is a common name for plants found in the genus *Sesamum*. The most popular sesame plant is *Sesamum indicum* that produces edible oil. *Sesamum* that are of interest to this study include, *Sesamum angolense* (L.) and *S. indicum*. Oils produced by these two plants have synergistic properties to plant derived insecticides such as pyrethrum (Budowski and Markley, 1951; Eddy *et al*, 1954).

1.2 Problem Statement

The increasing resistance to synthetic insecticides by common cockroaches (Milani, 1995; WHO, 2006) has increased their population (Miller and Koehler, 2003) thus posing a higher public health risk to many households. Further the synergistic

toxicity of extracts of *T. minuta* and *S. indicum* has not been investigated against cockroaches, despite the potentials known to exist.

1.3 Justification of the Study

The control of common cockroaches is based on the use of synthetic insecticides, especially pyrethroids. The repeated use, and in many cases, sub-lethal doses of these substances through the years has caused resistance of the cockroaches and other arthropods of public health and veterinary importance to the insecticides (http://whqlibdoc.who.int/monograph/WHO_MONO_38_(2ed)_(chp1).pdf). An example of commonly used pyrethroid insecticide in Europe and USA is Fastac. Its' main active ingredient is alpha-cypermethrin having a 48h-LC_{50} for *Daphnia magna* estimated to be 0.8µg/l and 24h-LC_{50} for *Gammarus pulex* – 0.3µg/l (Yordanova *et al*, 2009). Pyrethroids and pyrethrins function by altering the voltage-gated Na^+ ion channels of the nerve cells making the nervous system functionless and resulting in death (Dorman and Beasley, 1991). Pyrethroid insecticides act by preventing transmission of nerve impulse, by blocking the passage of sodium ions through channels in nerve membranes, thus preventing signal passing down axons (Yordanova *et al*, 2009) and thereby causing a knockdown that culminates into death. The continuous use of sub-lethal doses of the pyrethroid-derived insecticides has led to a change in the amino acid sequence of the gated channel proteins found on the nerve cell, thus conferring resistance to the insecticides (Williamson *et al*, 1996; Martin *et al*, 2000). These problems have led to the need to develop alternative control methods, such as organic acids, essential oils and/or any of their components (Miller and Koehler, 2003). Essential oils are distilled from aromatic plants such as *T. minuta*; they possess intense smell, have less harmful effect over environment and have a wide public acceptance among producers (Isman, 2000).

T. minuta thrives well around the tropics, and its oils have insecticidal properties. These attributes make it suitable for controlling *P. americana*. Extracts from *T. minuta* can be effectively used against insect pests because the different components found in the essential oils act synergistically by enhancing the effect of one another (Krishna *et al*, 2005). By having the crude extract, it will minimize the cost of processing (Green *et al*, 1991). Crude extracts are cheaper than commercial pesticides, as they do not require a lot of investment (Krishna *et al*, 2005). The insecticide made will then be cost effective and

reliable in the eradication of cockroaches. The outcome of this would be an improvement of living standards.

1.4 Significance of the Study

The study established the synergizing effect of *S. indicum*'s oily extracts on insecticidal extracts from *T. minuta*. From this, an effective insecticide that can be used against the 'common' cockroach, *Periplaneta americana* can be formulated to commercial standards.

1.5 Hypotheses

i. Cockroaches are not pest menace in many households in Maseno Division.

ii. Synergized crude essential oil extracts from *T. minuta* is not effective as an insecticide against *P. americana*.

1.6 Main Objective

To develop an insecticide against the 'common cockroach', *P. americana*, using extracts from the weed plant Mexican marigold (*T. minuta*).

1.7 Specific Objectives

i. To determine incidence of cockroach menace in Maseno division and to identify the control measures used.

ii. To perform bio-assay tests for susceptibility of *P. americana* to synergized and non-synergized crude essential oil extracts from *T. minuta* with oils from *S. indicum*.

iii. To perform histopathological analysis of the affected tissues, cells and proteins of the *P. americana* by *T. minuta*'s crude essential oil extracts.

CHAPTER TWO

2.0 Literature Review

2.1 Cockroaches (Order Blattodea)

The sub order Blattaria of the order Blattodea encompasses organisms that are mainly cockroaches. They are primarily tropical insects (Borror *et al*, 1992) which are rather large. Most species are about the size of a thumbnail, but several species are bigger. The world's largest cockroach is the Australian giant burrowing cockroach, which can reach nine centimeters (3.5 in) in length and weigh more than 30 grams (Hoell *et al*, 1998). Cockroaches have a broad, flattened body and a relatively small head (Miall and Denny, 1986). They are generalized insects, with few special adaptations and may be the most primitive living neopteran insects. The mouthparts are on the underside of the head and include generalized chewing mandibles. They have large compound eyes, two ocelli, and long flexible antennae. The first pair of wings is tough and protective, lying as a shield on top of the membranous hind wings. All four wings have branching longitudinal veins, and multiple cross-veins. The legs are sturdy, with large coxae and five claws each. The abdomen has ten segments and several cerci (Hoell *et al*, 1998).

The classification of cockroaches follow the system first used by McKittrick (1964), who grouped the fifty or so North American species into five families as follows: Cryptocercidae (brown-hooded cockroaches); Blattidae (oriental and American cockroaches); Polyphagidae (sand cockroaches); Blattellidae (German and wood cockroaches) and Blaberidae (giant cockroaches). The genus *Periplaneta* is shared by several species of cockroaches, some of which are synanthropic. The synanthropic cockroaches are those found living in human habitations. They include *Periplaneta americana*; *P. fuliginosa*; *P. brunnea*, *P. australasiae*; *Blatta orientalis*; *Blatella germanica* and *Supella longipalpa*. As indicated by McKittrick (1964), *P. americana* (common cockroach) is, however, the most cosmopolitan of them all.

2.1.1 Distribution and habitation of synanthropic cockroaches

Synanthropic cockroaches are those found living in houses with humans. The genus *Periplaneta* is nearly worldwide in distribution. The most widespread species of the genus are *Periplaneta fuliginosa* (smoky brown cockroach), mainly in the United

States; *Periplaneta brunnea* (brown cockroach, native to African, but has been spread by human travel and commerce) and *Periplaneta australasiae* (Australian cockroach). It probably arose in Africa, spread throughout the tropics and subtropics, and is now circumtropical (Suiter, 1997). *Blatta orientalis* (oriental cockroach) probably originated in North Africa and has become a common pest in the southern, midwestern and northwestern United State but is known as far north as southern Canada, the Netherlands and Britain.

The German cockroach, *Blatella germanica*, originated in Africa and traveled first to Europe and from there to North America (Suiter and Koehler, 1991). *Supella longipalpa* (Brown-banded cockroach) is assumed to have originated in Africa (Suiter, 1997). Forty-seven species are included in the genus *Periplaneta*, none of which is endemic to the U.S. (Bell and Adiyodi, 1981). American cockroach, *P. americana*, was introduced to the United States from Africa as early as 1625. Since then, it has spread throughout the world through commerce (Bell and Adiyodi, 1981). They are often found residing indoors as well as outdoors. They are found mainly in basements, sewers, steam tunnels, and drainage systems (Rust *et al.*, 1991). *P. americana*, is readily found in commercial and large buildings such as restaurants, grocery stores, bakeries, and where food is prepared and stored. They can develop to enormous numbers, greater than 5,000 sometimes being found in individual sewer manholes (Rust *et al*, 1991).

2.1.2 Public health risk of *Periplaneta americana*

Cockroaches are commonly noted as household insect pests. They feed on human and pet foods, and can leave an offensive odor (Brenner *et al*, 1987). They passively transport microbes on their body surfaces including those that are potentially dangerous to humans, particularly in environments such as hospitals (Elgderi *et al*, 2006). At least 22 species of pathogenic human bacteria, fungi, and protozoans can be carried by field *P. americana* (Rust *et al*, 1991). Cockroaches produce proteins linked with allergic reactions in humans (Kutrup, 2003). One of the proteins that trigger allergic reactions associated with cockroaches has been identified as tropomyosin which is associated with muscle contraction (Santos *et al*, 1999). Allergens produced by cockroaches are a cause of asthma; saliva, droppings and decomposing bodies of cockroaches contain allergen proteins known to trigger allergies and increase the severity of asthma symptoms (Kang

et al, 1979). On this same note, in January 2010, The National Pest Management Association (NPMA), warned that cockroaches pose a big threat, during winter season, to those that suffer from allergies and asthma.

Development of resistance to pyrethroid insecticides is traced from the gene; as a result of amino acid substitution in a voltage-gated Na^+ channel (Chang *et al*, 2009). The commonly used insecticides are pyrethroid aerosols (Yordanova *et al*, 2009). The drastic increase in number of the common cockroaches, which are a public health risk, may be attributed to their resistance to pyrethroid insecticides. This is the reason why it is of great importance to formulate an insecticide that will help in controlling cockroaches whilst giving minimal room for resistance development.

2.1.3 Cockroach management strategies

Ways of controlling cockroaches include the use of insect growth regulators (IGRs), cockroach baiting, inorganic dusts, traps, biological controls and oothecal parasitoids (Miller and Koehler, 2003). These methods are referred to as least toxic cockroach management strategies (Miller and Koehler, 2003). Insecticide aerosols have also been used as alternative means of controlling cockroaches and are a more effective way of cockroach management. This has been effected by use of pyrethrum and pyrethroid insecticides (Miller and Koehler, 2003).

2.1.3.1 Least toxic cockroach management strategies

Least toxic cockroach management strategies include: Insect Growth Regulators (IGRs), cockroach baits, inorganic dusts, sticky traps and biological control (Miller and Koehler, 2003). IGRs function by mimicking the juvenile hormone analogues of insects and cause the nymphs to molt into "adultoids" (adults that are unable to reproduce). Cockroach baits consist of a toxicant mixed with a food source. Inorganic dusts such as silica gel and boric acid have been used frequently for indoor cockroach control; Silica gel is finely ground sand or glass that adheres to and absorbs the protective waxes on the cockroach cuticle resulting in cockroach death from dehydration while boric acid is a stomach poison that is picked up by cockroaches walking across dusted areas. Sticky traps are used indoors; another trapping method is the use of baited jars. Lastly, biological control involves the use of cockroach enemies including: wasps, nematodes,

spiders, toads and frogs, centipedes, birds, lizards, geckos, beetles, mantids, ants and small mammals such as mice.

2.1.3.2 Toxic cockroach management strategies

The well-known toxic management strategy for cockroach control is the use of pyrethrin and pyrethroid aerosols (Casida, 1980). Both work with a similar principle. Pyrethrin is a substance extracted from the floral part of a plant; pyrethrum. As reported by The National Chrysanthemum Society in 2002, Pyrethrum is a common name of the plants found in the genus *Chrysanthemum*; for example *Chrysanthemum cinerariifolium* and *Chrysanthemum coccineum* which are both old world plants. Pyrethrins have insecticidal properties and are used in the formulation of aerosol insecticides. They cause a knock down effect to insects by paralyzing the nervous system through altering the orientation of the voltage-gated sodium ion channel ligand receptor of nerve cells thus rendering them functionless (Martin *et al*, 2000).

Pyrethroid insecticides are synthetic and examples are alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin, permethrin and bifenthrin (WHO, 2006). They have the same mode of action as pyrethrin insecticides (neurotoxic), but the difference between the two is that pyrethrins occur naturally in floral part of a plant pyrethrum, while the latter is made in the laboratory. Both pyrethrin and pyrethroid insecticides are toxic ways of controlling cockroaches and can be used indoors and outdoors (Miller and Koehler, 2003).

2.2 Insecticide resistance

There are many definitions of insecticide resistance; however, the one promoted by the Insecticide Resistance Action Committee (IRAC) is the most pertinent to the management of pests and vector-control programmes. IRAC defines insecticide resistance as the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended (IRAC, 2010). According to this definition, differences in susceptibility apparent in laboratory bio-assays may not necessarily constitute resistance if the difference does not result in a change in the field performance of the insecticide.

Selective pressure of conventional insecticides is enhancing resistance of insects, mainly mosquito populations, at an alarming rate (Brown, 1986). Since then, this increased the demand for new products that are environmentally friendly, target-specific and degradable.

Currently, the control of the common cockroaches is based, mainly, on the use of synthetic insecticides, especially pyrethroid insecticides (Miller and Koehler, 2003). Nevertheless, the repeated use and in many cases sub-lethal doses of these substances throughout years has caused resistance of the common cockroach to pyrethroid insecticides (Milani, 1995).

Insecticide Resistance Action Committee (IRAC) journal gives mechanisms through which insects develop resistance to insecticides. The mechanisms are grouped into four distinct categories: metabolic resistance (involves the use of enzymes and is most common); target-site resistance (involves modification of specific ion channel of the neurons in nervous system and is the second most common); reduced penetration resistance (involves modification in insect cuticle and digestive tract linings and prevents or slow absorption or penetration of insecticide); and behavioral resistance (involves modification of insect behavior that help it avoid lethal effects of insecticides (IRAC, 2010).

A few cases of insecticide resistance were investigated at the molecular level during the 1990s using 'traditional' molecular techniques (Oakeshott *et al*, 2003). The number was limited because essentially only those cases involving known genes that could readily be cloned by heterologous PCR or reverse genetics were tractable (Oakeshott *et al*, 2003). Three types of mechanism were revealed by these early studies, two involving enhanced detoxification of the insecticide and one rendering the target site for the insecticide insensitive to its effects. One detoxification mechanism involving sequestration of the insecticide was seen in two cases of resistance to organophosphate and carbamate insecticides in aphids (Field *et al*, 1988) and culicine mosquitoes (Raymond *et al*, 1998). In these examples, carboxylesterases with high affinity for the insecticides but very low degradative activity were massively over-expressed as a result of 100-400 fold amplifications of the genes encoding them. The second detoxification mechanism, involving active degradation of the insecticide, was seen in two species of

flies in which structural mutations had arisen in specific carboxylesterases that converted them to kinetically inefficient - but apparently physiologically sufficient - organophosphate hydrolases (Newcomb *et al*, 1997; Campbell *et al*, 1998; Claudianos *et al*, 1999). The third mechanism, in which the target molecule mutates in such a way that it becomes insensitive to the insecticides, has now been found in several cases covering a range of species and types of chemical (Mutero *et al*, 1994; Williamson *et al*, 1996; Vaughan *et al*, 1997; Ffrench-Constant *et al,* 2000; Martin *et al*, 2000). The mutant target molecules include acetylcholinesterase for organophosphates, γ-aminobutyric acid (GABA) receptors for cyclodienes and voltage-gated sodium channels for the synthetic pyrethroids and dichlorodiphenyltrichloroethane (DDT) (Oakeshott *et al*, 2003).

2.3 *Tagetes minuta* (Family Asteraceae).

The genus *Tagetes* comprises 36 species belonging to the family Asteraceae. The genus contains several species that produce essential oils. Essential oils produced by plants have several applications (Weaver *et al*, 1994). *T. minuta* is native to the southern part of South America. Ever since Spanish colonization, it has been introduced around the world including Europe, Asia, and Africa (Soule, 1993). *T. minuta* may grow to between 0.6 - 1.2 meters tall. For some time people have used it as a flavorful tea for medical benefits such as a remedy for the colds, respiratory inflammations, or stomach problems (Soule, 1993). The leaves when dried may be used as a seasoning (Soule, 1993).

The species name, *minuta*, means small due to its flowers that are small. *T. minuta* has difficulty growing in a shade, and thrives well in open fields. The plant has little toleration for frost (Soule, 1993). *T. minuta* (Mexican Marigold), a tall upright marigold plant with small flowers, is used as a culinary herb in Peru, Ecuador, and parts of Chile and Bolivia, where it is called by the Incan term huacatay (Soule, 1993). In Chile and Bolivia, huacatay paste is used to make the popular potato dish called ocopa (Soule, 1993). The taste and odor of fresh *T. minuta* is like a mixture of sweet basil, tarragon, mint and citrus (Soule, 1993).

2.3.1 Insecticidal properties of *Tagetes minuta*'s essential oils.

Tagetes minuta produces essential oils that posses insecticidal properties (Maradufu *et* al, 1978; Arnason *et al*, 1986; Green *et al*, 1991; Wells *et al*, 1993; Perich *et al*, 1994, 1995 and MacEdo *et al*, 1997). The insecticidal compounds are 5-*E*-ocimenone (Maradufu *et* al, 1978) and thiophenes: 5-(but-3-ene-1-ynyl)-2,2'-bithiophene, 5-(but-3-ene-1-ynyl)-5'-methyl-2,2'-bithiophene, 2,2',5',2"-terthiophene, and 5-methyl-2,2',5',2"-terthiophene (Perich *et al*, 1995).

2.4 The Sesamum Plants

Plants of the genus *Sesamum* encompass several species. The most popular species are *S. angolense* and *S. indicum* because their seeds produce oils that have insecticidal synergism among other properties (CPRC, 1958). Seed oil of *S. indicum*, commonly known as sesame oil, contains two constituents namely sesamin and sesamolin, which have insecticidal synergism to pyrethrins (Budowski and Markley, 1951). Sesamolin and sesamol exhibit higher insecticidal synergism to pyrethrins. Unlike sesamin, sesamolin has not been found to occur in any genera other than Sesamum, nor has sesangolin been reported in any Sesamum species other than *S. angolense* (Jones *et al*, 1962). Moreover, sesangolin is a natural synergist that works with same strength as sesamin.

Synergy is the working together of two entities to achieve a common goal. One of the entities is the prime mover while the other provides energy to support the prime mover. In the present study, the prime mover was the crude essential oil extract from *T. minuta*. Sesamolin and sesamin provided the energy to boost or enhance the insecticidal action of the prime mover. Therefore, in the study, *S. indicum*'s sesamin and sesamolin were used to synergize crude essential oil extract from *T. minuta*.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Area

The study was conducted in Maseno Division of Kisumu County, Kenya. It is located about 25 kilometers from the Lake Victoria, Kisumu City on the Kisumu-Busia-Uganda road, North-west of the city. It is situated in Western part of Kenya at the GPS of 0° 10` 0`` South, 34° 36` 0` East (http://www.maplandia.com /Kenya/western/maseno/).

The inhabitants of Maseno Division are mainly peasant farmers practicing mixed crop farming. Climate of the area is equatorial, hot and wet with two dry and two rainy seasons (Kenya, 2007). According to 1999 census (Kenya) Maseno Division has a population of 65,304 people (http://www.cck.go.ke/html/final_annex1_ cover_status.pdf).

3.2 Inclusion criteria of Households

The inclusion criterion was that households had semi-permanent houses or permanent houses with walls having cracks. Assumption made was that the cockroaches collected had not been exposed to any insecticide in the recent past.

3.3 Sampling technique

Purposive sampling technique was used in this study to collect cockroaches that were not exposed to any insecticide in the recent past. Sampling was done by issuing out three 500 ml plastic sample collection bottles (Kenpoly-Kenya) to each of the thirty-five selected households. The sample collection bottles had perforated lids. Sample size of thirty is one of the standard sample sizes established by the World Health Organization in population health studies and epidemiology (Hoshaw-Woodard, 2001). The three sample collection bottles for each household were labeled appropriately. Each collection bottle was to hold a maximum of twelve cockroaches (Wang and Bennett, 2006). Cockroaches were trapped by the households and placed inside collection bottles that were then transported to the laboratory by the researcher. Growth stages of the common cockroaches collected were both nymphs and adults, alive and without any injury. The exercise took 48 hours (from the time sample collection bottles were issued to time when the samples arrived in the laboratory). Upon arrival at the laboratory, the cockroaches

were counted and their numbers recorded per specimen bottle and then introduced to a 30 liter capacity common pool plastic bucket (ACME Containers Limited, Kenya) for acclimatization. Three hundred adult naïve cockroaches (*P. americana*) were bought from the PBK (Pyrethrum Board of Kenya)-Nakuru, to be used in the experiments also; They were a yardstick for making conclusive inferences about the plant extracts. The adult naïve cockroaches were lab reared cockroaches and thus not exposed to any insecticides. All field collected cockroaches and the adult naïve cockroaches were kept in separate labeled common pools, at 24±1^{0}C and 60-70% humidity (Palacios *et al*, 2009).

The study needed over 20,000 cockroaches. Therefore traps were made improvised for this purpose. The trap constituted a bucket smeared with petroleum oil to the inside, towards the brim to prevent the trapped cockroaches from climbing out of the bucket. The bait used to draw the cockroaches into the bucket was bread soaked in beer (Miller and Koehler, 2003). Twenty households with highest cockroach catch, based on their specimen collection bottles (see appendix III), were issued with the traps. Two days later the cockroaches were taken from the twenty households and on arrival at the laboratory, the cockroaches were introduced in a common pool, one large bucket, for acclimatization at room temperature. Field collected cockroaches were kept in their common pool that was different from that of the naïve cockroaches bought from the Pyrethrum Board of Kenya. All the cockroaches were provided with water and fed a 1:1 (v/v; approximately) mixture of milk powder and wheat bran (Palacios *et al*, 2009) regularly in a time interval of 3 days before being used for experiments. Wheat bran and milk were prepared at a weight ratio of 1:3 and 200 g dough's of this mixture were placed on a plastic plate for the cockroaches to feed.

3.4 Administering the questionnaire

Thirty-five households were interviewed following the criterion shown earlier in section 3.3: sampling technique. The areas where the interviews were carried out include Sunrise-Maseno/Maseno mixed, Mabungo, Nyawita, Ebusembe, Esibembe, Eluambil'lo and Ebukhacha all of which are villages within Maseno. Five households in each area were interviewed. Copy of questionnaire used during the present investigations is given as Appendix I. The researcher assisted in asking the questions and filling in the questionnaires.

3.5 Plant material collection and preparation

Sesamum indicum (L.) seeds were bought from the nearby Luanda market and floral and folia parts of *T. minuta* (L.) were collected from farms in Maseno Division. Both of these materials were transferred to the Department of Botany Herbarium, Maseno University, for identification by comparison with authentic specimens. Five kilograms of *T. minuta* were first dried (at room temperature) out of sunlight in the laboratory and then chopped using a machete into small (around 1cm) pieces. The *T. minuta* plant materials were ground at Kenya Sugar Research Foundation (KESREF), Kisumu using mill plate (model CZ 04186-05; A.B.C. Hansen Co. A/S, Denmark) grinder.

3.6 Extraction processes

3.6.1 Fixed oil extraction from *S. indicum*

Sesamum indicum seeds (40 kilograms) were first roasted in an oven (WTBbinder-78532-Tuttlingen/Germany) at a temperature range of 70°C-80°C for 2 hours, then oil extraction was done at Bukura ATC where the seeds were put into a compressor-*Motorized oil extraction screw press* (made at The Jomo Kenyatta University of Agriculture and Technology) and squeezed to yield 1.5 liters of oil.

3.6.2 Sesamin and sesamolin extraction from sesamum oil

The method of Simanton (1949) was adopted. One liter of sesame oil was agitated eight successive times with equal volumes of absolute methanol (Kobian Kenya Limited). The eight extracts obtained were combined and the methanol removed under temperature below the thermal decomposition temperature of the oily extract 85°C in a vacuumed evaporator (N-1001S-W, 888-MY-EYELA) at a temperature of 60°C. The final product was an oily extract (about 9% of the original weight of the sesame oil) and a raffinate (about 89% of the original weight of sesame oil). The oily extract was left on shelf for 4 days to form crystals. The oily extract has higher synergy ability as compared to the raffinate because it contains a higher amount of sesamin and sesamolin as compared to the ruffinate (Simanton, 1949).

3.6.3 Extraction of crude essential oil from *T. minuta*

The method of Maradufu *et* al, 1978 was used, where one kilogram each of the ground floral and folia parts were transferred to five 5-litre conical flasks. Four litres of

methanol (Kobian Kenya Limited) were added to each of the five conical flasks. The mixtures were then put in a cabinet and left to percolate for a period of 168 hours (1 week). The mixture was filtered and the filtrate subjected to evaporation process using a vacuumed evaporator. Then the extracts were left in an open beaker in an incubator (Carbolite-7/98/1485-PIN30-201) at 55°C to facilitate evaporation of the remaining methanol to leave behind a dark green paste (methanol extract).

3.6.4 Synergized crude essential oil extract from *T. minuta*

A portion of the crude essential oil extracts from dried ground floral and folia parts of the *T. minuta* were synergized with the oily extract (sesamin and sesamolin). The ratio used in the preparation was 2 ml of oily extract from *S. indicum* to each of the different concentrations (depending on the experiment to be done) of crude essential oil extract from *T. minuta* to 1,000 ml of distilled water.

For comparison of knockdown versus kill time, the concentration used was 120 g/L, synergized with 2 ml of the oily extract from *S. indicum*. LC_{50} was determined using the following concentrations: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 g/L, each of which 2 ml of the oily extract from *S. indicum* was added as a synergizer.

3.6.5 Non-synergized crude essential oil extract from *T. minuta*

Another portion of the crude essential oil extracts from *T. minuta* was not synergized. It was prepared by the ratio of the different concentrations (depending on the experiment to be done) of crude essential oil extract from *T. minuta* to 1,000 ml of distilled water. The concentration of the crude essential oil extract used for comparison of knockdown versus kill time was 120 g/L. For determining the LC_{50}, the concentrations were as follows: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 g/L.

3.6.6 Synergized distilled water

Preparation of the synergized distilled water was done by adding 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0 and 4.4 ml of the oily extract from *S. indicum* to 1,000 ml of distilled water each. These mixtures were used for treatment to establish if the oily extracts have insecticidal property.

3.7 Experimental chambers

Exposure or experimental chambers were compartments made of transparent plastic (Lion Star Plastics-Indonesia), where the cockroaches were exposed to the plant extracts. The chambers measured 30x18x8 cm. Treatments of the exposure chambers was done by painting the extract on the sides and the floor for the case of contact toxicity. Without sex segregation, *P. americana* were introduced into the chamber immediately after applying the treatment. For fumigation toxicity, *P. americana* were introduced in exposure chamber just before spraying by use of a pressure hand pray (Lion Star Plastics-Indonesia). Immediately the cockroaches were exposed to the treatment, the chambers were covered and a 5 bar-digital-Casio stopwatch (HS-50W-IDF) was started to measure the time taken for the cockroaches to die. The knockdown and kill times were recorded for each run of the experiments. The numbers of dead cockroaches were also recorded. Eleven exposure chambers were used in the study. The naïve cockroaches were marked by tying a piece of string on their hind leg before introducing them into the exposure chamber whilst field collected cockroaches did not have a string tied. This was to differentiate between the field collected cockroaches and the naïve cockroaches. The chambers were cleaned with soap and water before using them in subsequent experiments.

3.7.1 Determining Lethal Concentration 50 (LC_{50})

The experiments for determining LC_{50} was a modification of the bio assays done by Palacios *et al*, 2009. They were done in two categories: (i) treatment with non-synergized crude essential oil extracts from *T. minutia* and (ii) treatment with synergized crude essential oil extracts from *T. minuta*. Each of the two categories had other two categories; (a) fumigation toxicity and (b) contact toxicity. For contact toxicity, treatment was done just before introducing the cockroaches into the experimental chambers while for fumigation toxicity treatment was done after introducing the cockroaches. The concentrations used were as indicated in sections 3.6.4 and 3.6.5. respectively. Thirty cockroaches (15 nymphs and 15 adults; in order to get unbiased results based on the age of the cockroach) were used in each exposure chamber for a given experiment. The number thirty was chosen for each experiment so as to be able to closely monitor each cockroach. For both synergized and non-synergized crude essential oil extracts from *T.*

minuta's experiment in chambers having 14 g/L, 5 adult naïve cockroaches were part of the 30 cockroaches in the chamber. After 72 hours, the numbers of dead cockroaches were recorded against their respective treatment concentrations. These experiments were repeated 4 times for both synergized and non-synergized crude essential oil extracts from *T. minuta.*

The same procedure was also done using synergized distilled water of the different concentrations as shown in section 3.6.6 for both contact and fumigation toxicity. Each chamber had 30 cockroaches (of which the chambers having 2 ml/L concentrations, for both fumigation and contact toxicity, had 10 adult naïve cockroaches each, as part of the thirty). This was done in one run and the results recorded after 72 hours.

3.7.2 Comparison between Knockdown and Kill times for *T. minuta*

In this section, experiments were done using the synergized and non-synergized crude essential oil extracts from *Tagetes minuta.* The concentration of 120g/L was prepared for the two extracts. Each chamber had 60 cockroaches of which 10 of them were adult naïve cockroaches. After treatment, the numbers of cockroaches' knockdown in each chamber were recorded after every 10 minutes for a period of 60 minutes. The recording was also done for deaths after every 10 minutes for a period of 60 minutes in same chamber. Treatments were repeated 4 times each having contact and fumigation toxicity.

3.7.3 Control

The control comprised 30 cockroaches (10 naïve adults, 10 field collected adults and 10 field collected nymphs) given no treatment. Cockroaches were introduced into the chamber and observations taken after 72 hours. This procedure was done once.

3.8 Histopathological analysis of the tissues

A total of 30 treated and untreated cockroaches were selected. The tissues from tersi, tracheal and nervous (nerve cords and tracts) systems were excised out under the view of dissecting microscope using scapel blades and forceps. The tissues were trimmed, on a dissecting board using a scapel blade, to the required orientations. Then

they were placed into 6 different vials and labeled. Trimmed tissues were processed for light microscopy by the following procedures (ICIPE's protocol):

i. Fixation; trimmed tissues were fixed in 2.3% glutaraldehyde overnight. This was primary fixation. The tissues were then washed twice in 0.1M PBS (phosphate, buffer saline). For secondary fixation, 0.25% aqueous osmium tetroxide was then added into the vials containing the tissues (this was done in a fume hood because the fumes of osmium tetroxide are very poisonous). The set up was left to fix for two hours, at room temperature (21°C), after which the fixed tissues were washed with distilled water (still in the fume hood) three times.

ii. Dehydration; was done in a series of treatment in ethanol, starting with 50% then 70%, 80% and 90% each for 30 minutes. Tissues were then treated with absolute ethanol, 3 times, and each taking 15 minutes.

iii. Clearing was done by pouring off the absolute ethanol and then treating the tissues with a 1:1 mixture of acetone and araldite (10ml of araldite cy212 + 10ml of DDSA (dodecyenyl succinic anhydride) + 0.4ml of BDMA (N-benzyldimethylamine). The set up was left overnight at room temperature.

iv. Infiltration of araldite was done by drained off the araldite-acetone mixture and pouring in araldite. The set up was then left for another 24 hours for infiltration to take place.

v. Blocking/Embedding was done by pouring hardened araldite (MNA (methyl nadic anhydride) + araldite in a ratio of 0.1MNA:1araldite) into a 24 well mould. Then four of each tissue from the six different vials was placed into a single well. The set up was then placed in an oven (WTBbinder-78532-Tuttlingen/Germany) at 60°C to polymerize for a period of 24 hours.

vi. Sectioning: The tissue blocks were trimmed using a clamp, hacksaw and scapel blade to sizes that could fit in the ultra-microtome (LKB-BROMMA-8800ULTROTOME) then Sectioned. As sectioning continued, the sections were lowered into a boat of water next to the glass-knife. A drop of water was placed on a clean glass slide and the sections placed using a chisel shaped

toothpick. This was done for each tissue. The glass slides were then labeled and dried on a rack.

vii. Biological staining. Toluidine blue in borax (5 g Toluidine blue: 25 g sodium borate: 500 cm^3 water) was dropped onto the tissues on glass slides for about 15 seconds. The stain was then washed off using distilled water, and the glass slides were placed on a rack to dry.

Examination of the tissue was done, to establish if there were injuries or morphological and color change of the cells, by the use of a digital compound light microscope (DM-B1, Motic USA; supplied by Wards Natural Science) and observations recorded.

3.9 Extracellular multiunit recording of nerve cells

Thirty-five adult cockroaches (*P. americana*) were selected (10 naïve and the rest were field collected). The cockroaches were subjected to extracellular multiunit recording, an in vivo study. Of these, 5 were used for calibration procedure of the instrument and were not treated with *T. minuta*'s extracts. The other 30 were treated: 15 (4 naïve and 11 field collected) with synergized crude essential oil extract from *T. minuta*, while the remaining 15 (4 naïve and 11 fields collected) with the non-synergized crude essential oil extract from *T. minuta*. Of the five cockroaches for calibration procedure, 2 were naïve while 3 were field collected.

A set up of Biopac MP35 equipment (MP35-WIN-BSLBME-W from Biopac Systems), a laptop computer and a printer were used for the analysis of the cockroach nervous system behavior before and after treatment with the crude essential oil extract from *T. minuta*. The Biopac MP35 unit was plugged as follows: electrode lead (SS2L) into channel one (CH1) to pick up electrical signals from the cockroach to the Biopac equipment. The Biopac MP35 data acquisition unit was turn on. Electrodes were connected to the cockroach via needles pricked into the cockroach's body parts as follows: red lead to anterior electrode, white lead to thoracic electrode and black lead (ground) to posterior electrode. The arrangement was left to stand for a period of two minutes for the cockroach to settle and before starting the Biopac student lab program.

Lesson three (LO3-EEG-1), a section of Biopac MP35 for acquiring data of extracellular multiunit recording, was chosen and the file name typed on the computer home page and set up calibrated by clicking on the calibrate button. This was preceded by

data recording section where recording was done for 840 seconds. This was done for the control cockroach that was not exposed to the plant extract from *T. minuta*. The rest of the cockroaches were injected with 0.5ml (0.2g/20ml H_2O) each, of the plant extract from *T. minuta*, followed by electrode connection for recording. Generated graphs were printed.

3.10 Data Analysis

Data from questionnaires was converted into frequency counts and percentages using SAS statistical analysis software. The time taken (in minutes) for the cockroaches to die and the number of dead cockroaches for each run of the experiment was recorded, both in a lab notebook and Microsoft excel worksheet. Susceptibility of *P. americana* to the synergized and non-synergized crude essential oil extracts from *T. minuta* based on action (knockdown and kill), synergy and method of application, the data was analyzed as a four factorial {i.e. 2 (synergized & non-synergized *T. minuta*'s extracts) by 2 (knockdown & kill) by 2 (contact & fumigation) by 6 (time intervals: 0, 5, 10, 15, 20 and 25 minutes)} experiment. ANOVA and a post-hoc test for comparison of means done using Duncan's Multiple Range test to test for significance of the experiments at $P \leq 0.05$. LC_{50} for both synergized and non-synergized *T. minuta*'s extracts were determined using probit analysis method. Probit analysis is a type of regression used to analyze binomial response variables. It transforms the sigmoid dose-response curve to a straight line that can then be analyzed by regression either through least squares or maximum likelihood. Probit analysis can be conducted using the following techniques: using tables to estimate the probits and fitting the relationship by eye; hand calculating the probits, regression coefficient, and confidence intervals; or having a statistical package such as SPSS to do it all (Vincent, 2009). In this study, probit analysis was used to determine the LC_{50} of synergized and non-synergized *T. minuta*'s extracts against *P. americana*. This was done by using tables to estimate the probits and a casio scientific calculator to get the logarithm of the different treatment concentrations after which probits were plotted against logarithm of concentrations to get a sigmoid curve. A regression line ($Y = a + bX + c$) was drawn from the sigmoid curve. LC_{50} was estimated at a probit value of 5.00 on the Y-axis to be the antilog of the corresponding log of concentration value on the X-axis

(Vincent, 2009). The data from histopathology and nerve behavior analysis were qualitative data; therefore analyzed by way of observation and making inferences.

CHAPTER FOUR

4.0 Results and Discussion

4.1 Cockroach menace in Maseno

Data from the questionnaires was analyzed. It was found that 100% (n=35) of the households reported being aware of the cockroaches, 82.86% reported problems caused by cockroaches; 54.29% reported insecticide use and 91.43% reported that all cockroaches do not die after treatment with insecticide (fig. 1).

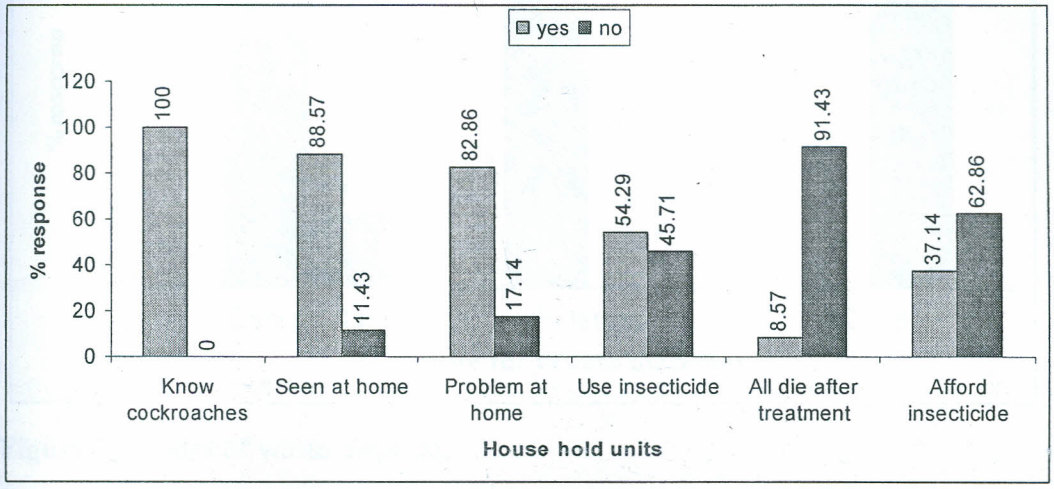


Figure 1: Cockroach awareness, menace and control.

Upon further interview on insecticide use, four common insecticides in the Kenya market that were considered in this study were Raid, Doom, Baygon and Ridsect. The household's response on their use was as follow: Raid (0% use), Mortein Doom (45.71% Yes & 54.28% No), Baygon (0% use) and Ridsect (5.71% Yes & 94.29% No). Responses on the preferred form of the insecticide were as follows: Spray (37.14% Yes & 62.86% No), Dust (17.14% Yes & 82.86% No), Paste (0% Yes & 100% No) and Chalk (8.57% Yes & 91.43% No). After treatment with insecticides, responses on the time taken for the cockroaches to reappear were as follows: Hours (2.86% Yes & 97.14% No), Days (5.71% Yes & 94.29% No), Weeks (31.43% Yes & 68.57% No), Months (8.57% Yes & 91.43% No) and Years (5.71% Yes & 94.29% No).

Other than using insecticides for cockroach control, there were also other methods used by the households. The responses were beating (20% Yes & 80% No), Biological (2.86% Yes & 97.14% No) and Nothing done (14.29% Yes & 85.71% No).

Demographically households that had permanent houses were 40% of the respondents while the other 60% had semi-permanent houses. All the households' houses had crevices on their walls. On modes of waste disposal, 77.14% of the households use compost pits while 22.86% did not use compost pits. Figure 2 shows information on waste disposal.

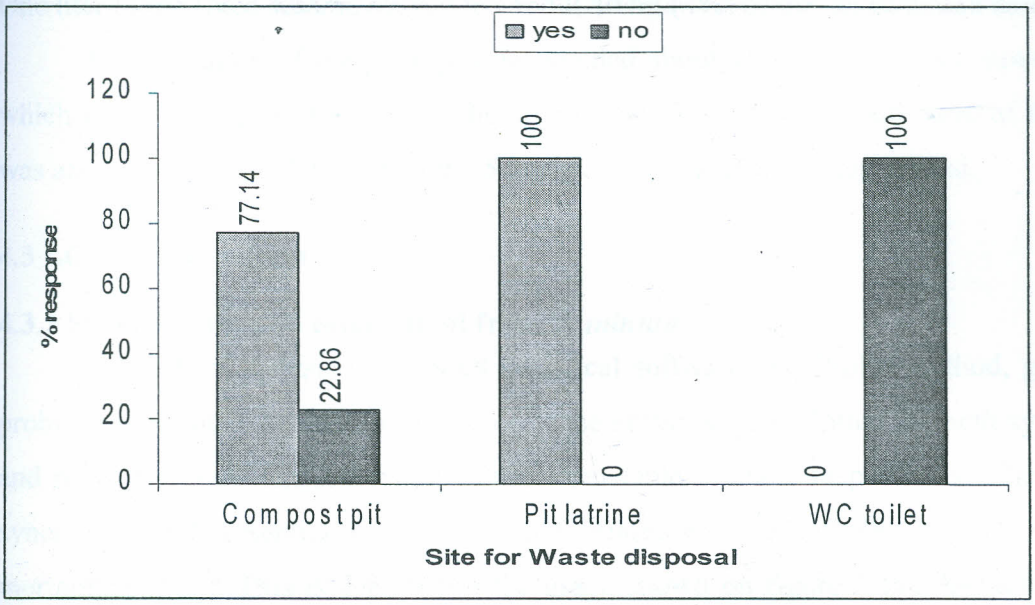


Figure 2: Modes of waste disposal.

The distances between the houses and the compost pit were 2 to 15 meters (65.71% of the households); 8.57% had their distance being 20 meters and above; while 25.71% did not use compost pits. All the households used pit latrines and the distance between the houses and the pit latrines were in the range of 2 to 15 meters for 74.29% while 25.71% had their distances at 20 meters and above.

When interviewed, 82.86% of the households embraced the fact that cockroaches are a menace. Some of the mentioned cockroach related problems were cockroaches eat clothes, books and food stuff; they contaminate water, milk and other food stuff thus spread diseases; they soil clothes, furniture, utensils and other household items; bad smell from their excrement; destroy electronic gadgets and their site is a nuisance.

4.2 Yields from the extraction processes

After passing the processed 40 kilograms of *S. indicum* seeds through the motorized oil extraction screw press machine, one and a half liters of the fixed sesame oil was yielded. This was used as raw material for the extraction of sesamin and sesamolin. One liter of the fixed sesame oil yielded about 40 ml (4%) of the sesamin and sesamolin.

One kilogram of the plant powder yielded about 18 grams of crude essential oil, which after leaving on the shelf in the lab, a thick black paste. Total yield of the paste was about 105 grams (2.1%) from the 5 kilograms of the plant material used.

4.3 LC_{50} determination

4.3.1 Synergized crude essential oil from *T. minuta*

After data analysis using SAS statistical software and Probit method, graphs of probit of mortalities against logarithm of concentrations were plotted for both synergized and non-synergized extracts using values from table 1 and 2 respectively. The LC_{50} of synergized crude essential oil extract from *T. minuta* was 6.31 g/L ($P<0.0001$) at a Probit mortality of 5.00. This is depicted in the graph shown on figure 3 that has a trend line [linear (series1) with the equation: $y = 3.920x + 1.823$] that was used in establishing the LC_{50}, by tracing the corresponding number of Probit mortality 5.00 on the X-axis (log [c]). The number is 0.8. Therefore the antilog of 0.8 is 6.31 g/L. This is the LC_{50} value. Figure 3 shows a graph that was plotted with values from table 1 which gives the Probit values of mortality and logarithm values of the different treatment concentrations that we used in the LC_{50} experiments.

Table 1: Probit of mortality and log of concentration calculated from % mortality and concentrations for the treatment with synergized extract respectively.

% Mortality	Probit of mortality	Conc. (g/L)	log [c] (g/L)
100	8.09	22	1.34
99	7.33	20	1.30
96	6.75	18	1.26
95	6.64	16	1.20
82	5.92	14	1.15
67	5.44	12	1.08
53	5.08	10	1.00
49	4.97	08	0.90
40	4.75	06	0.77
22	4.23	04	0.60
08	3.59	02	0.30
00	0.00	00	0.00

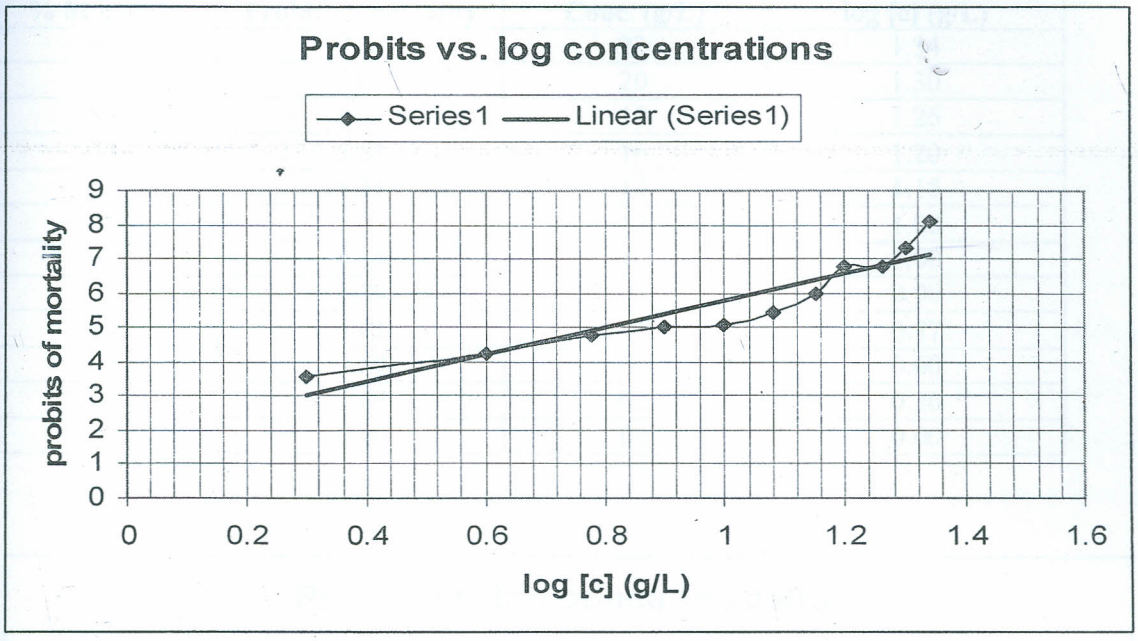


Figure 3: LC_{50} of synergized crude essential oil extracts from *T. minuta* for *P. americana*.

4.3.2 Non-synergized crude essential oil from *T. minuta*

Probit analysis of data from the treatment with non-synergized crude essential oil extract from *T. minuta* gave LC_{50} of 8.91 g/L ($P<0.0001$) at a probit mortality of 5.00. Figure 10 shows a graph with a trend line [linear (series1) with the equation: $y = 4.166x + 0.969$] that was used to establish the LC_{50} of non-synergized crude essential oil extracts from *T. minuta* by tracing the corresponding number of probit mortality 5.00 on the X-axis (log [c]). The number is 0.95. Therefore the antilog of 0.95 is 8.91 g/L. This is the LC_{50} value. Figure 4 shows a graph that was plotted with values from table 2 which gives Probit values of mortality and logarithm values of the different treatment concentrations that we used in the LC_{50} experiments.

Table 2: Probit of mortality and log of concentration calculated from % mortality and concentration for the treatment with non-synergized extract respectively.

% Mortality	Probit of mortality	Conc. (g/L)	log [c] (g/L)
99	7.33	22	1.34
98	7.05	20	1.30
89	6.23	18	1.26
73	5.61	16	1.20
62	5.31	14	1.15
51	5.03	12	1.08
44	4.85	10	1.00
27	4.39	08	0.90
16	4.01	06	0.77
08	3.59	04	0.60
01	2.67	02	0.30
00	0.00	00	0.00

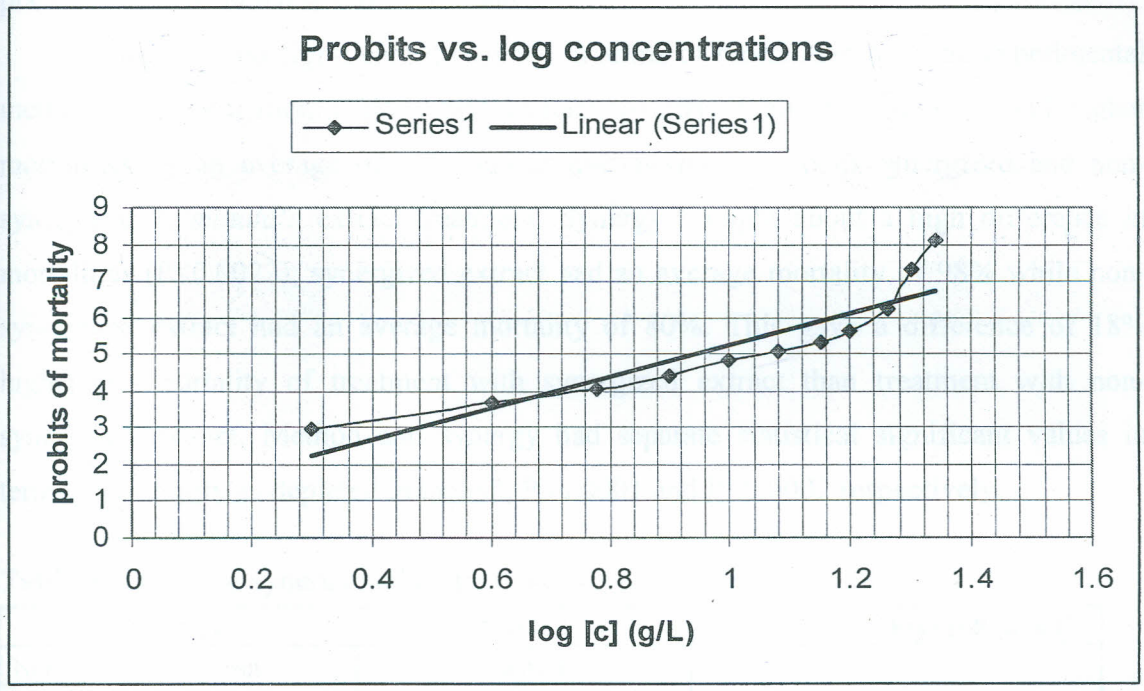


Figure 4: LC_{50} determination of non-synergized crude essential oil extracts from *T. minuta*.

As depicted in appendix II, both method of application (fumigation and contact toxicity) and synergy (synergized and non-synergized extracts from *T. minuta*), the concentrations that had statistically significant effects (deaths) on the cockroaches were 4, 6, 8,.... and 22 g/L ($P<0.0001$). Concentration of 2 g/L and below had statistically insignificant effect on the cockroaches.

4.3.3 Synergized distilled water

There were no deaths recorded for all of the 11 chambers after 72 hours, except for the chamber that had fumigation application method with a concentration of 3.2 ml/L. This chamber had 1 adult cockroach dead out of the 30 cockroaches which was statistically insignificant.

4.3.4 Comparison of Synergy versus Method of treatment

Table 3 shows the mean mortalities of the different treatment methods and synergies (n=60). The variables were synergy and method of treatment. Pertaining susceptibility, all the categories of cockroaches responded in a similar manner to both synergy and method of treatment. The field collected cockroaches (nymphs and adults) and the adult naïve cockroaches subjected to the experiment were all susceptible to the plant extracts as shown in tables 3, 4 and 5.

There was no significant difference in terms of mortalities with the experimental methods i.e. fumigation toxicity and contact toxicity; fumigation toxicity had higher mortalities by an average of two than contact toxicity for both synergized and non-synergized *T. minuta*'s extract treatment. Synergy brought about a high difference in mortalities ($P<0.0022$); synergized extract had an average mortality of 98% while non-synergized extract had an average mortality of 80%. This gave a difference of 18% higher for mortality of treatment with synergized extract than treatment with non-synergized extract. Method and synergy had separate statistical significant values in terms of mortality as depicted in table 3, $P<0.0001$ and $P<0.0002$ respectively.

Table 3: Effects of synergy and method on mortality.

Synergy	Method	%mean mortality±2SE (n=60)
Non-synergized extract	Contact	78
Non-synergized extract	Fumigation	80
Synergized extract	Contact	97
Synergized extract	Fumigation	100

Treatment with synergized crude extracts from *T. minuta* reduced the time taken for deaths to occur from 25 to 20 minutes ($P<0.0012$); all the 60 cockroaches died within 20 minutes after treatment as depicted in table 4. Treatment with non-synergized crude

extracts from *T. minuta* had the deaths occurring within 25 minutes after treatment as shown in table 5.

Table 4: Effect of synergy on time taken for deaths to occur.

	Treatment with synergized extract (%mortality±1SE: n=60)			
Time(min)	Contact (No. dead)	Cumulative deaths	Fumigation (No. dead)	Cumulative deaths
0	0	0	0	0
5	25	25	32	32
10	18	43	15	47
15	27	70	50	97
20	30	100	3	100

Table 5: Effect of non-synergy on time taken for deaths to occur.

	Treatment with non-synergized extract (%mortality±1SE: n=60)			
Time(min)	Contact (No. dead)	Cumulative deaths	Fumigation (No. dead)	Cumulative deaths
0	0	0	0	0
5	20	20	27	27
10	18	38	17	44
15	19	57	30	74
20	33	90	26	100
25	10	100	0	100

4.4 Knockdown and kill

4.4.1 Synergized & Non-synergized *T. minuta*'s crude essential oil

The knockdown and kill times for treatments of 120 g/L concentrations of both synergized and non-synergized crude essential oil extracts from *T. minuta* are depicted in tables 6 and 7 (both with $P<0.0001$). Table 6 shows knockdown and kill times for treatment with synergized crude essential oil extracts from *T. minuta*. More than 85% of the cockroaches were knocked down within the first 15 minutes after treatment; while after 20 minutes all the cockroaches (100%) were dead. Table 7 shows knockdown and kill times for treatment with non-synergized crude essential oil extracts from *T. minuta*. More than 65% of the cockroaches were knocked down within the first 15 minutes after

treatment; while after 25 minutes all the cockroaches (100%) were dead. All the cockroach categories (adult naïve and field collected cockroaches) were indifferently susceptible to the plant extracts as shown in tables 6 and 7.

Table 6: Knockdown and kill time for synergized crude essential oil extracts from *T. minuta.*

	Treatment with synergised extract (n=60)			
Time(min)	**% knockdown**	**Cumulative knockdown**	**% dead**	**Cumulative deaths**
0	0	0	0	0
5	28	28	27	27
10	17	45	15	42
15	42	87	36	78
20	13	100	22	100

Table 7: Knockdown and kill time for non-synergized crude essential oil extract from *T. minuta.*

	Treatment with non-synergized extract (n=60)			
Time(min)	**% knockdown**	**Cumulative knockdown**	**% dead**	**Cumulative deaths**
0	0	0	0	0
5	25	25	20	20
10	17	42	18	38
15	25	67	22	60
20	28	95	32	92
25	5	100	8	100

4.5 Control

The 30 cockroaches in the test control chamber were alive after 72 hours. This served as the negative control of the bio-assay experiments.

4.6 Histopathological analysis

The treated and untreated tissue (from treated and untreated cockroaches) from the Tersi, Tracheal and Nervous systems were passed through histology procedure and mounted on to glass slides. Toluidine blue in borax stain was used for both the treated and untreated tissue.

Upon examining the stratified squamous epithelia of the tersi, for both untreated and the treated cockroaches (plate 1), had cells that looked similar in shape (polyhedral);

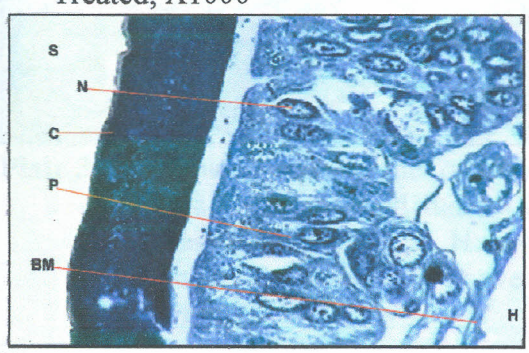
color (picked the color of the stain used (toluidine blue in borax)) and size (as viewed under X1000 magnification). No cell crenated or lysed. The cell shape of the simple squamous epithelia of the tracheal system of both the treated and untreated cockroaches (plate 2) were similar in appearance (squamous shape); both had the color of the stain used (toluidine blue in borax) and were similar in size as viewed under X1000 magnification. None of the squamous cells of the tracheal system was crenated or lysed.

As of the nervous tissue, both the treated and untreated sections (plate 3) were similar. Neurons depicted by FNs in plate 3 were similar in shape (spherical). They also had the color of toluidine blue in borax as the stain. Both treated and untreated tissue from the nervous system had similar sizes of their cells as viewed under X1000 magnification. The cells were not lysed or crenated and their connective tissue wrappings (perineuria and epineuria) were not damaged or deformed in any way. They looked similar for both treated and untreated tissue.

The tissues looked similar for both categories: treated & untreated and naïve & field collected cockroaches. Therefore, the photomicrographs shown in plates 1, 2, and 3 are representative of all the categories. Each of the tissue was photographed at X1000 magnification. Photomicrographs for each of the tissues are as follows:

(i) Tersi tissue:

Treated; X1000



Untreated; X1000

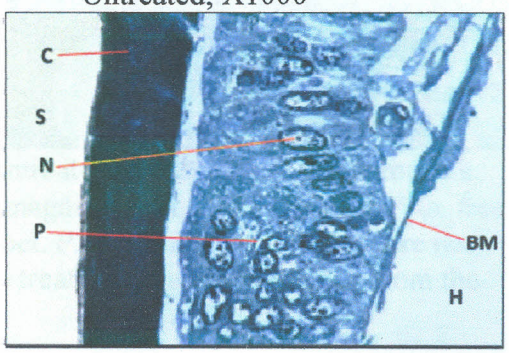


Plate 1: Photomicrographs of the treated and untreated tissue, from the cockroach's tersi, viewed under X1000 magnification. BM: basement membrane. C: thick layer of squamous cells. H: Haemocoel. N: nucleus of epithelial cell. P: polygonal shaped epithelial cell. S: external surface. Notice no morphological differences between the treated and untreated tissue.

(ii) Tracheal system's tissue:

Treated; X1000 Untreated; X1000

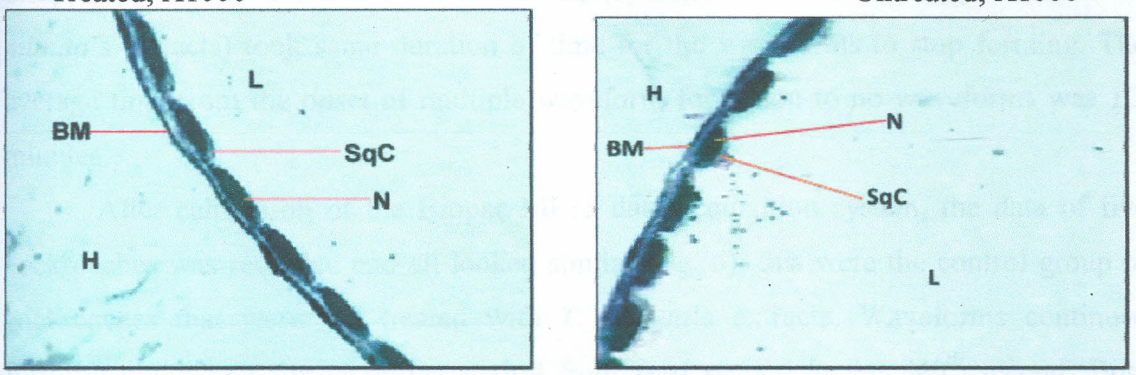


Plate 2: Photomicrographs of the treated and untreated tissue, from the cockroach's tracheal system, viewed under X1000 magnification. BM: basement membrane. H: haemocoel. L: lumen. N: nucleus of epithelial cell. SqC: squamous epithelial cell. There are no morphological differences between the two tissues.

(iii) Nervous system's tissue:

Treated; X1000 Untreated; X1000

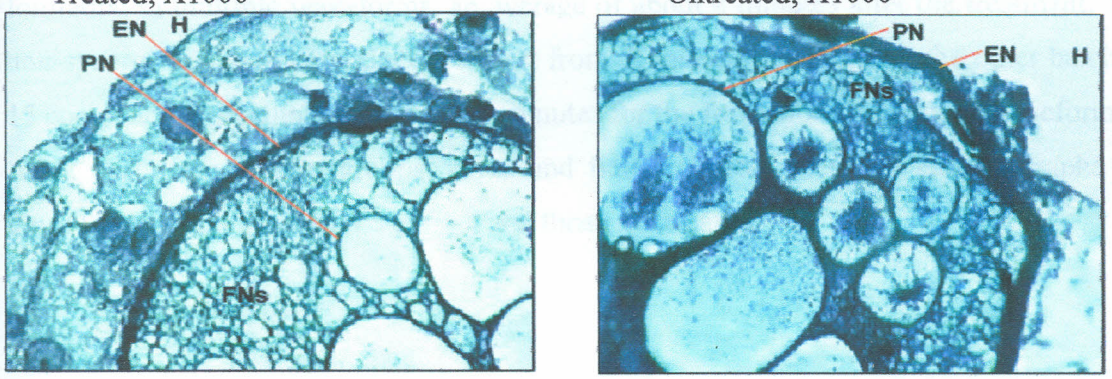


Plate 3: Photomicrographs of the treated and untreated tissue, from the cockroach's nervous system, viewed under X1000 magnification. EN: epineuria. FNs: free neurons (not in a fascicle). H: haemocoel. PN: perineurium. There were not morphological differences between the treated and untreated tissue from the nerve cord.

4.7 Cockroach nerve behavior

The graph on figure 5 is extracellular multiunit recordings that show the EEG, alpha, beta, delta and theta waves of the untreated cockroaches. The waves continue to form up to the time that the experiments ended (14 minutes). The graph on figure 6 also

shows extracellular multiunit recordings depicting the EEG, alpha, beta, delta and theta waves of the treated cockroaches. The waveforms continue to form normally up to 4.7th minute and increase in intensity up to 6.3th minute, after which they diminish and disappear at the 8th minute. Both treatments (synergized and non-synergized crude *T. minuta*'s extracts) took same duration of time for the waveforms to stop forming. The average time from the onset of multiple waveform formation to no waveforms was 1.3 minutes.

After calibration of the Biopac MP35 data acquisition system, the data of five cockroaches was recorded and all looked similar (fig. 5); this were the control group of cockroaches that were not treated with *T. minuta*'s extracts. Waveforms continued forming throughout the recording period from zero second to the 840th second. Both groups treated with synergized and non-synergized crude essential oil from *T. minuta* had similar data (fig. 6). The difference between the data for synergized and that for the non-synergized crude essential oil extract treatment was that one took longer time than the other, for the reaction to begin. *Tagetes minuta*'s synergized crude essential oil extract treatment on first batch of 15 cockroaches took a shorter time for the onset of the formation of multiple waveforms, an average of about 4 minutes after the treatment. The non-synergized crude essential oil extract from *T. minuta* treatment on the other batch of 15 cockroaches took an average of 5.3 minutes for the formation of multiple waveforms. Adult cockroaches from groups, naïve and field collected recorded similar graphs for those treated with *T. minuta*'s extract and those not treated.

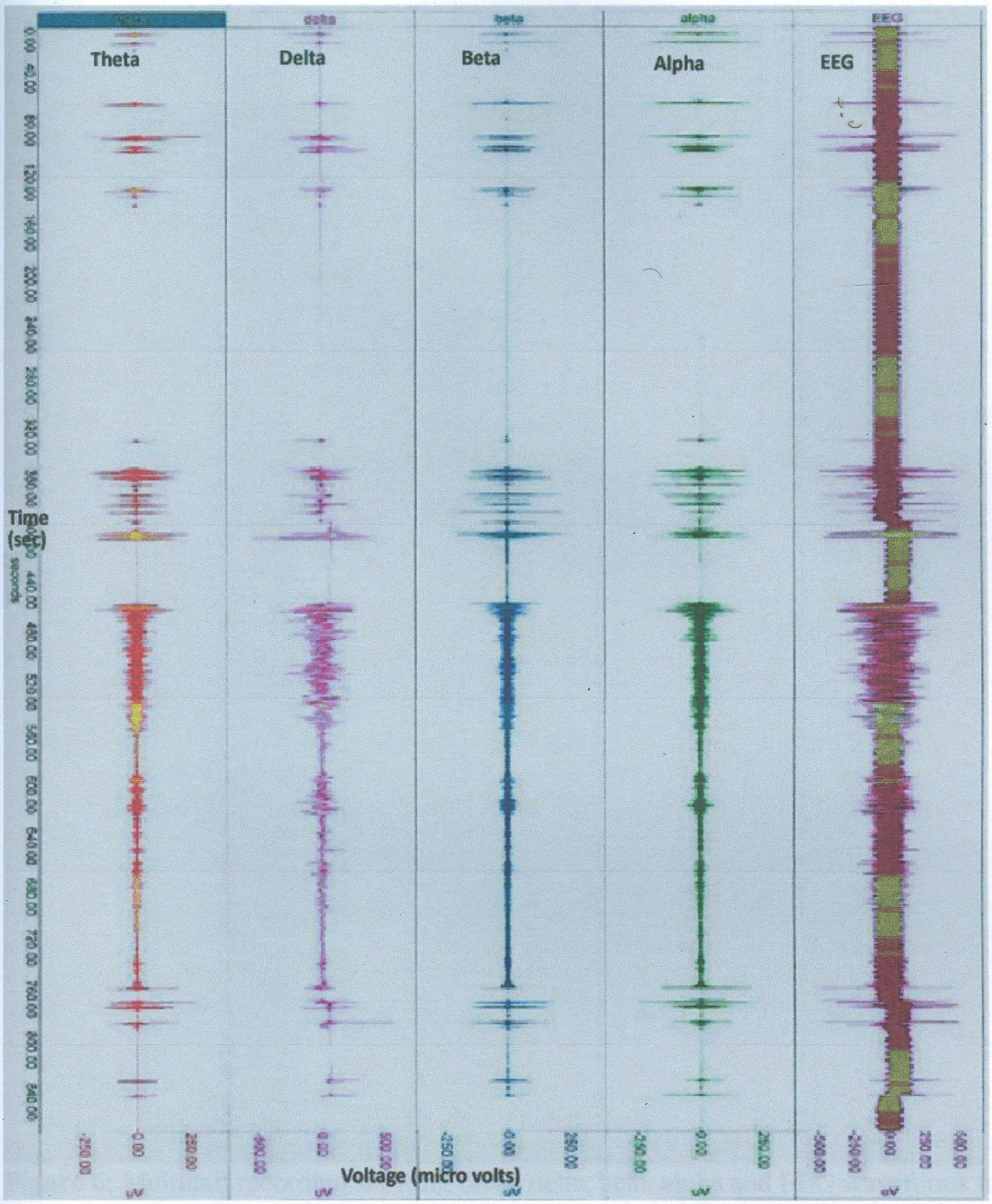


Figure 5: Multiunit recording showing theta, delta, beta, alpha and EEG (Electroencephalogram) waveforms for cockroach not treated with *T. minuta*'s extract. There is continuous formation of waveforms throughout the recording period (neurons were firing nerve impulses normally).

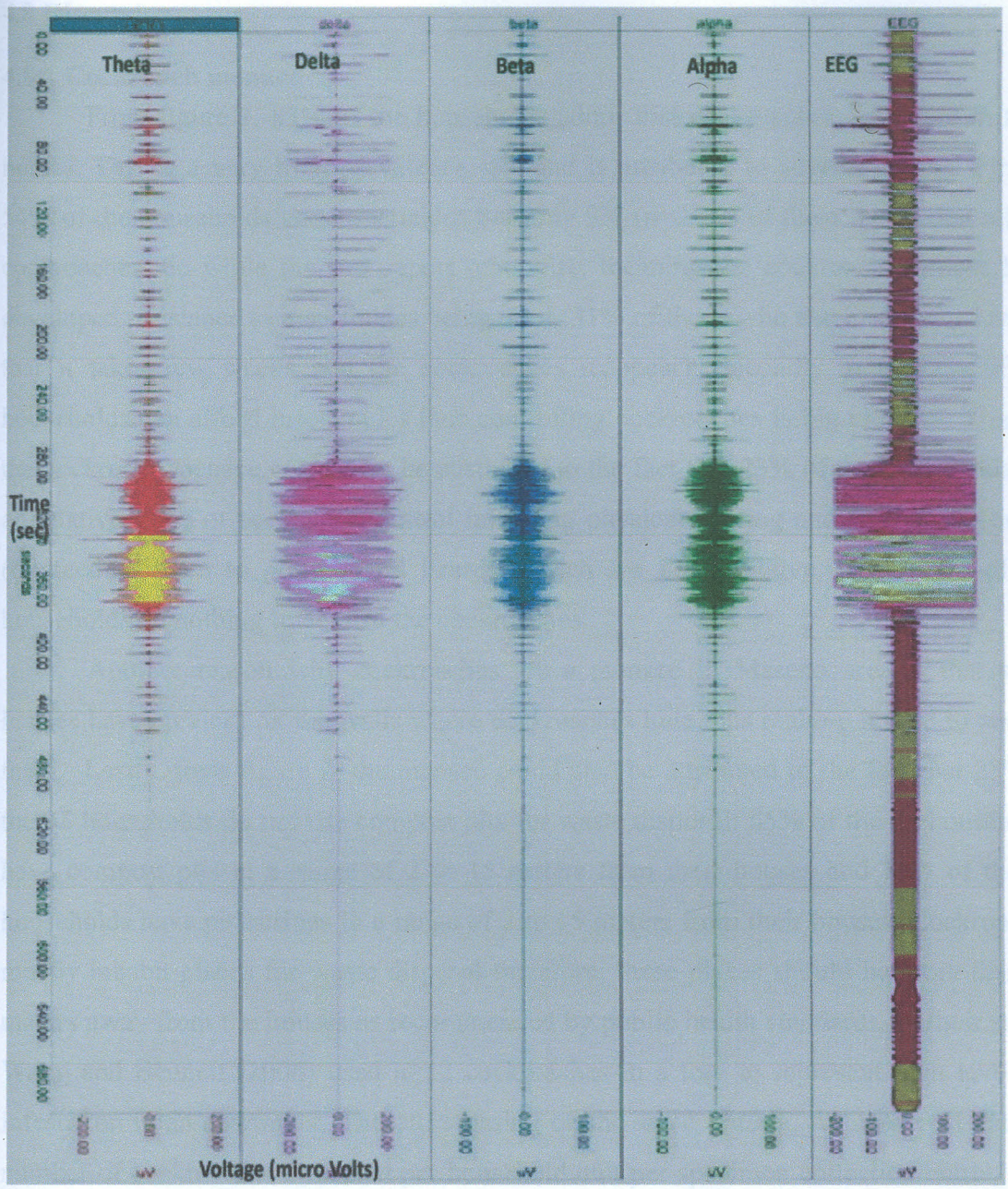


Figure 6: Multiunit recording showing theta, delta, beta, alpha and EEG waveforms for cockroach treated with *T. minuta*'s extract. Notice the dense waveforms around 5 minutes and the absence of waveforms at 8minutes' mark onwards (neurons were neither firing nor conducting nerve impulses).

4.8 Discussion

4.8.1 Cockroach menace

From figure 1, 83% of the households admit that cockroaches are found in their homes. This is a very high prevalence rate and is attributed to several factors. Firstly, 54% of the households use insecticides but only 9% (n=2/19) of them report that all the cockroaches die while the rest report otherwise, meaning the cockroaches might have developed resistance to insecticides being used (31% of those who use insecticides report that it takes few weeks and the cockroaches reappear). Secondly, only 37% of the households can afford insecticides thus controlling cockroaches is big problem. Thirdly, the cockroach menace could also be attributed to the fact that 23% of the households use alternative ways of cockroach control including physical beating and biological control (by feeding them to poultry and lizards) which are not effective, while 14% of the households do nothing to control the cockroaches.

Another reason why cockroaches are a menace in Maseno area is that some houses have crevices on the walls where cockroaches hide thus making it hard to control them. Lastly, from figure 2, the menace could also be attributed to the fact that 25% of the 35 households do not use compost pits for waste disposal: 65% of the 35 households have compost pits in a range of 2 to 15 meters from their houses and 75% of the 35 households have pit latrines in a range of 2 to 15 meters from their houses. Cockroaches mostly inhabit places for waste disposal therefore; these places should be more than 20 meters away from the houses as recommended by public health standards. In their study, Wang and Bennett (2006) used $n \geq 12$ cockroaches in a trap to represent high levels of infestation in an apartment. Therefore basing on the same criteria, Appendix III data on number of cockroaches collected per household unit per specimen collection bottle, 91% of the households had the number of cockroaches collected per specimen collection bottle well above $n \geq 12$. This is a clear indication that there is a high cockroach infestation in households of Maseno Division, thus being a menace to the households.

If the stated reasons for cockroach menace are not corrected, then pathogens will be transported from the waste disposal areas to houses by the cockroaches and there will be disease out-breaks. The pathogens spread by cockroaches range from bacteria, fungi,

protozoa and viruses (Oothuman *et al.*, 1989; Rust *et al.*, 1991; Vythilingam *et al.*, 1997; Prado *et al.*, 2002; Salehzadeh *et al.*, 2007).

4.8.2 Bio-assays

As shown in figures 3 and 4, the LC_{50} for synergized and non-synergized crude essential oil extracts from *Tagetes minuta* were 6.31 g/L and 8.91 g/L respectively. Synergized distilled water had no statistical significance on kill.

The combination of synergy and method of treatment, as shown in table 3, had a difference of 2 % on the mean mortalities of fumigation and contact toxicity; meaning that fumigation toxicity works better than contact toxicity method of treatment. On the other hand, synergy produced a significant difference of 20% mean mortality between synergized and non-synergized *T. minuta*'s extract meaning that synergized extracts of *T. minuta* work better than the non-synergized extract. Tables 4 and 5 show further details about method of treatment where the synergized extracts had deaths recorded within 20 minutes (97% deaths recorded within 15 minutes for fumigation toxicity treatment as compared to 70% deaths within 15 minutes for contact toxicity treatment) while the non-synergized extracts had deaths record with 25 minutes (100% deaths recorded within 20 minutes for fumigation toxicity treatment as compared to 90% deaths within 20 minutes for contact toxicity treatment). This was an indication that the method of treatment matters because for both synergized and non-synergized extracts, fumigation toxicity treatment had a higher number of deaths recorded as compared to contact toxicity treatment.

Synergized and non-synergized crude essential oil extracts from *T. minuta* had their actions starting within the first 5 minutes for both knockdown and kill, as shown on tables 6 and 7 respectively. But the synergized extract's knockdown and kill happened within 20 minutes after treatment, while that of the non-synergized extract happened within 25 minutes after treatment. On average, knockdown and kill for synergized and non-synergized crude essential oil extracts from *T. minuta* happened within a short range of time for each cockroach. Figure 6 shows the duration from knockdown to when the cockroach dies (knockdown happening at 4.7 minutes after treatment and the cockroach died at 8 minutes after treatment).

All these happenings (synergy and method of treatment) had the same mode of behavior observed for all the cockroach categories. There were adult field collected cockroaches, adult naïve cockroaches and nymph field collected cockroaches. All these were subjected to the same experiments. There was no segregation based on sex of the cockroach. And therefore all the cockroach categories gave results that were similar in terms of their susceptibility to the treatments applied with synergized and non-synergized extracts from *T. minuta*.

4.8.3 Histopathological analysis

From the photographic plates shown in section 4.6, it is clear that the treated tissues appear to be similar with untreated or normal tissue. The similarity here was in terms of color, shape and size of the cells. A close examination was done to check whether some cell had undergone crenation or lysis. There were no morphological changes for the three tissue types from both treated and untreated cockroaches. The expected reaction from the treated tissues was, severe injury that ruptures and atrophys the epithelial cells (Konar, 1969), enlargement of epithelial cell (Ahmed, 1995) or loss of tissue identity via crenation, change in shape, disruption of epithelia and their basement membranes and overal tissue disruption leaving gaps and lacunae (Khan *et al*, 2011). But none of the mentioned expected changes were observed on the three treated tissue types.

This meant that the crude essential oil extract from *Tagetes minuta* did not cause any anatomical changes to the tissues suspected to be the portals of entry into the *Periplaneta americana* or the suspected cells that confer susceptibility to the insecticidal compounds. Therefore, penetration into the insect may be attributed to diffusion of the insecticidal compounds across epithelial cells into the haemolymph.

4.8.4 Nerve cell behavior

From the extracellular multiunit recording results, figure 5 show waveforms of the extracellular multiunit recordings. Throughout the period, from 0^{th} to 14^{th} minute, the waveforms continued forming. This showed that nerve cells continued firing and conducting electric impulses. The cockroaches for this case were untreated thus no disruption of their normal nerve cell functions. On the contrary, figure 6 (treated

cockroaches) had waveforms that were sparse from 0^{th} to 5^{th} minute, dense from 5^{th} to 6^{th} minute, sparse from 6^{th} to 8^{th} minute and no waveforms from 8^{th} to 11^{th} minute. The change from sparse to dense to sparse and finally no waveforms clearly show that the *T. minuta*'s extracts affected the neurons normal nerve impulse firing and conduction.

The alpha, beta, delta and theta waves look alike to each other in both figures 5 and 6 because the cockroach's nervous system lacks specialized compartments. This similarity is due to cockroach's primitive (simple) nervous system; lower organism have rather simple nervous system made up of neurons, nerve tracks and nerve cords. Their brains are made up of nerve cords and ganglions; a case of all arthropods and other invertebrates (Borror *et al*, 1992). The lower organisms' simple nervous systems have fewer neurons as compared to the higher organisms (Purves *et al*, 2001). Neurons are the functional units of the nervous system and they fire nerve impulses that are very minute electrical currents or potential differences (Holmes, 1993).

Biopac MP35 picked up and amplified the minute potential differences that became the extracellular multiunit recording data. Figure 5 showed continuous firing of nerve impulses for the untreated cockroaches meaning the neurons were functioning normally. Figure 6 depicted normal firing of nerve impulses for the first 5 minutes which is suggestive of time taken for the *T. minuta*'s extract to diffuse to the neuron, immediately after treatment. During the 6^{th} minute, there was a sharp increase in the intensity of waveforms suggesting that the insecticidal compounds had bound to the neurons' voltage-gated ion channels which are responsible for initiation of action potentials (giving rise to waveforms). The 7^{th} and 8^{th} minutes had the intensity of the waveforms reduce drastically and from the 9^{th} to 11^{th} minute the waveforms ceased. This is suggestive that the voltage-gated ion channels responsible for the formation of action potentials were getting affected by the plant extract and thus became malfunctioned.

Neurons have mainly three trans-membrane protein ion channels on their axonal membrane. These are channels for Na^+, Cl^- and K^+ ions (Lodish *et al*, 2000). Purves *et al* (2001) indicate that the extracellular environment has a lot more Na^+ ions than in the cytosol. On the other hand, K^+ and Cl^- ions are more in the cytosol than the extracellular space. The extracellular portion of the membrane has an overall positive charge while the intracellular portion has a negative charge.

For a nerve impulse to be generated and transmitted, the stimulus must be strong enough to cause an action potential. Action potential occurs in 3 phases; depolarization, hyperpolarization and repolarization (Tortora and Grabowski, 1996). In the depolarization phase, a neurotransmitter causes Na^+ channels to open thus Na^+ ions rush into the cell down the concentration gradient to bring the membrane potential to 30 mV. This action triggers opening of K^+ and Cl^- ion channels that cause hyperpolarization where the membrane potential shoots down from 30 mV to -91.1mV. Repolarization is a function of voltage-gated K^+ ion channels. They open and thus cause increase in permeability to K^+ ions. This causes an increased efflux of K^+ ions from the cytosol thus repolarization occurs (Lodish *et al*, 2000).

For there to be onset of an action potential, the Na^+ ion channels must be activated first before any other ion channel. Depolarization is a function of P_{Na}. This is followed by repolarization and hyperpolarization, a function of P_K and P_{Cl}. Therefore this clearly shows that the active ingredients found in the *Tagetes minuta*'s crude essential oil work via stimulating the Na^+ ion channels to open thus creating action potentials; dense concentration of waveforms during the 6^{th} minute (fig. 6). Restoration of the nerve cell membrane back to resting potential is very important to facilitate conduction of subsequent nerve impulses. After a neurotransmitter has finished its work it is done away with either by recycling it using an endocytic vesicle or degrading it by use of an enzyme, for example, acetylcholinesterase degrading acetylcholine (Purves *et al*, 2001). In a case where the enzyme is malfunctioning then there will be continuous binding of acetylcholine on to its receptor on the post-synaptic membrane. This will lead to irreversible opening of the Na^+ ion channels thus the membrane would not be restored to resting membrane potential. In the same way, the insecticidal compounds in *Tagetes minuta* bind to and cause opening of Na^+ ion channels. Nevertheless, after this, there is no enzyme to degrade the compound. The resultant effect is that the neurons could not be restored to resting membrane potential thus are rendered functionless. The cockroach then dies. These applied to both adult naïve and adult field collected cockroaches.

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

'Common' cockroaches, *P. americana* have increased in number due to their resistance to synthetic insecticides. Therefore, their close association with humans and human wastes posse a public health risk. The present study was to investigate a potentially new effective and reliable insecticide for control of *P. americana*. The study took place in Maseno Division where 35 households were selected, interviewed and cockroaches collected from there. Adult naïve cockroaches were used in comparison with field collected cockroaches for the bio-assays. Crude essential oil extracts from *T. minuta* were synergized with oily extracts from *S. indicum* and tested for fumigant and contact toxicity against *P. americana*. Tersi, tracheal and nervous systems' tissues were histologically analyzed to establish effect of *T. minuta*'s crude essential oil extracts. The mode of action of *T. minuta*'s insecticidal compounds was discerned by doing extracellular multiunit recording for the nervous system of the cockroach.

Data analysis was done using SAS statistical analysis software where by ANOVA and Duncan's Multiple Range test were used to test for significance of the experiments at $P \leq 0.05$ and establish the LC_{50} of *T. minuta*'s synergized and non-synergized extracts using probit computational method. Number of households which admitted to cockroach menace was 83%, 54% use insecticides to control them and 91% said that all cockroaches do not die after treatment. This is an indication that cockroaches are a menace to households in Maseno Division. LC_{50} of synergized and non-synergized *T. minuta*'s extract were 6.31 g/L and 8.91 g/L, respectively. Synergized and non-synergized *T. minuta*'s extract had mortalities of 100% and 80% respectively. Synergized and non-synergized *T. minuta*'s extract of 120 g/L each had knockdowns and kills within 20 and 25 minutes, respectively.

The reduction of LC_{50} of the synergized extract, increased mortality of cockroaches after treatment with the synergized plant extract and reduction in knockdown and kill times by 5 minutes show that *T. minuta*'s extracts were synergized by sesamin and sesamolin. *T. minuta*'s insecticidal compounds did not cause anatomical changes or injuries to the tersi, tracheal and nervous tissue. It seems to work physiologically via impairing the function of Na^+ ion channels of the neurons thus killing

P. americana. By these results, it will be of great importance that specificity and side-effects of the new insecticide be established and addressed, before the commercial sector starts production of the insecticide for sale so as to be used by the public in fighting the cockroach menace.

Conclusion

Although more than half of the population of Maseno uses synthetic insecticides to control cockroaches, not all cockroaches die. Therefore, it is concluded that *Periplaneta americana* are a menace in households of Maseno.

This study also established that *Tagetes minuta*'s crude essential oil extract has insecticidal property. Upon addition of *Sesamum indicum*'s sesamin and sesamolin there was synergy to the insecticidal property in *T. minuta*'s extracts leading to increased mortality and reduced knockdown and kill time. LC_{50} of both synergized and non-synergized crude essential oil extracts from *T. minuta* were 6.31 g/L and 8.91 g/L respectively. There was no death recorded for the treatment with synergized distilled water. These are indicative of sesamin and sesamolin do not have insecticidal property but act as synergist to insecticidal compounds from *Tagetes minuta.* Therefore, *P. americana* are susceptible to kill by synergized and non-synergized *T. minuta*'s extracts.

The mode of action of the crude essential oil extracts from *T. minuta* was found to be physiological because the cells of the tissues were neither injured nor destroyed. This is suggestive that the portal of entry was through the epithelial linings of the tersi and the tracheal system; then diffusion of the plant extract through the haemolymph to the neurons. The insecticidal compounds work via binding to the Na^+ ion channels of neurons. Continuous binding of these compounds to the Na^+ ion channels cause action potential but the neurons could not be brought back to the resting membrane potential. Therefore the neurons were rendered functionless thus death of the cockroach.

Recommendations

The following recommendations were made on the findings of this study:

- The study established that cockroaches are a menace in Maseno Division. Therefore, it is recommended that proper hygiene procedures of waste disposal and sanitation be practiced in order to keep cockroaches away from homes.
- From the study, it has been established that *Tagetes minuta*'s crude essential oil extracts can be synergized with sesamin and sesamolin and improve its' insecticidal property. Therefore it is recommended that this formulation be developed to commercial standards and be used as an alternative for the control of cockroaches.
- Studies must be carried out to establish if there are any side effects of the insecticide on human beings and their remedies, before releasing it to the public.
- For the future, Patch clamp studies must be done to establish behaviors of Na+ channels in relation to insecticidal compounds of *T. minuta*.

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