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Comparative insecticidal properties of Basotho medicinal plants against *Culex quinquefasciatus* (Diptera: Culicidae) mosquitoes from the Eastern Free State Province of South Africa

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DECLARATION

I, Serero Abiot Modise, hereby declare that this research project is my original work and has not been presented for a degree in any other university.

MODISE SA

This dissertation has been submitted for examination with our approval as the university supervisor.

Dr. ASHAFA AOT

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ABSTRACT

Culex quinquefasciatus is a vector of human and animal disease causing pathogens that are of socioeconomic problem in developing countries. The mosquitoes have developed resistance against synthetic insecticides, hence the search for natural botanical insecticides. The present study was aimed at investigating the secondary plant metabolites, cytotoxicity, larvicidal, pupicidal and insecticidal potential of *Artemisia absinthium, Artemisia afra, Cosmos bipinnatus, Foeniculum vulgare, Mentha longifolia* and *Tagetes minuta* against *C. quinquefasciatus*. The leaf extracts contained mostly saponins, alkaloids, terpenoids, steroids and flavonoids. Plant aqueous and ethanol extracts exhibited cytotoxic effects for *T. minuta* (LC_{50} = 0.10 mg/ml; LC_{50} = 3.16 mg/ml), *A. absinthium* (LC_{50} = 2.89 mg/ml), *C. bipinnatus* (LC_{50} = 5.66 mg/ml; LC_{50} = 4.81 mg/ml), and *A. afra* (LC_{50} = 5.39 mg/ml) against brine shrimp nauplii. Ethanolic extract mortality and concentration doses had was significant difference ($F_{5,5}$ = 13.69; P < 001) towards nauplii mortality. Most larvicidal bioactivities were observed in ethanolic and hexane extracts for *F. vulgare* (LC_{50} = 0.10 mg/ml; LC_{50} = 1.03 mg/ml), *M. longifolia* (LC_{50} = 1.05 mg/ml; LC_{50} = 0.10 mg/ml), *T. minuta* (LC_{50} = 1.17 mg/ml; LC_{50} = 1.01 mg/ml) and *A. afra* (LC_{50} = 1.02 mg/ml; LC_{50} = 1.14 mg/ml), and while larvae mortality and extract concentrations showed significant difference ($F_{5,5}$ = 9.95; P < 0.01). Pupicidal bioactivity was displayed by both ethanolic and hexane extracts of *A. afra* (LC_{50} = 1.10 mg/ml; LC_{50} = 1.04 mg/ml), *T. minuta* (LC_{50} = 1.11 mg/ml; LC_{50} = 1.12 mg/ml), *C. bipinnatus* (LC_{50} = 1.14 mg/ml; LC_{50} = 1.16 mg/ml) and *M. longifolia* (LC_{50} = 1.13 mg/ml; LC_{50} = 1.21 mg/ml). The extract concentration level were directly proportional to pupa mortality percentage with *M. longifolia* (R^2 = 0.85) and *A. afra* (R^2 = 0.74). The aqueous extracts had no fatal effect on larvae and pupa at all the concentrations tested. The rate of knock-down was highest for *M. longifolia* (KD_{50} = 4.91 min^{-1}) followed by *F. vulgare* (KD_{50} = 9.87 min^{-1}), *T. minuta* (KD_{50} = 12.39 min^{-1}), and *A. afra* (KD_{50} = 19.02 min^{-1}). The insecticidal activity was greater in *M. longifolia* (LD_{99} = 0.25 g) followed by *F. vulgare* (LD_{99} = 0.25 g), *T. minuta* (LD_{99} = 0.25 g) and *A.*

afra (LD_{99} = 0.25 g). The insecticidal mortality ratio between evaluated plants had significant difference ($F_{5,4}$ = 283.11; $P < 0.01$). In this study, ethanolic and aqueous extracts had more cytotoxic activity against *A. salina* nauplii than the hexane extracts, whereas, ethanolic and hexane extracts exhibited stronger larvicidal and pupicidal activities than the aqueous extracts. The selected Basotho medicinal plants possessed convincing insecticidal, pupicidal and larvicidal activities and therefore can be recommended for mosquito control at Kroonstad as well as in nearby communities of the eastern Free State Province.

Keywords: Basotho medicinal plants, botanical insecticides, *Culex quinquefasciatus*, dose-response analysis, insecticidal, larvicidal, pupicidal

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CHAPTER 1

INTRODUCTION

1.1. Medically important insect overview

Insects are known to be one of the world's most diverse and abundant living organisms, with a fair distribution but are found to be rare in the Antarctica region (Cranston & Gullan. 1994; Hunter *et al.* 2008). Many insects have become specialised to occupy particular niches and take part in ecosystem services like pollination, decomposition, disease vectors, food chain and natural products (Daly *et al.* 1998). Many insects are found to be beneficial to both human and animal, but some are also known to pose health threats. The most well-known insect group are the true flies (Diptera) where some members are of medical and veterinary importance. According to Goddard (2008) the Culicidae (mosquitoes) family can directly or indirectly affect human or animal health. The mosquitoes can show impact on animal health from bites during probing, secreted salivary toxins, or secondary infection on previously bitten tissue sites, as well as transmitted pathogens (Foster & Walker 2009). Mosquitoes according to Mullen and Durden (2009), are among major insect groups that are significantly involved in the transmission or cause the development of illnesses in humans and animals.

The Diptera (true flies) is among the largest insect orders with over 85 000 described species and with only two suborders, namely Nematocera and Brachycera (Triplehorn & Johnson 2005; Scholtz & Holm 2008). In Nematocera group, the Culicidae family is divided into three subfamilies on which only two are of medical and veterinary importance in southern Africa, and these include Anophelinae as well as Culicinae (Service 1993; Barraclough & Londt 2008; Foster & Walker 2009). The Culicinae subfamily is the largest mosquito group with about 2,925 described species in 33 genera (Service 2012). The *Culex* genera is by far the largest, commonest and most important genus of the group with about 751 species arranged in 22 sub genera (Harbach & Kitchin 1998;

Harbach 2007). According to Service (1993), most *Culex* adult female mosquitoes mainly bite at night, and prefer to feed predominantly on birds, a few amphibians and reptiles, as well as several mammals (including man). The *Culex quinquefasciatus* (Say 1823) which is commonly known as southern house mosquito, (synonym *Culex fatigans, C. pipiens fatigans*, or *C. p. quinquefasciatus*), larvae is found in organically polluted waters that are closer to human settlement (Service 1993; Foster & Walker 2009). The females of *C. quinquefasciatus* mosquito have estimated densities of about 15 million per square kilometre, and can render about 80 thousand bites to residents in poor districts per year in Myanmar situated Southeast Asia, whereas in West Africa of Burkina Faso, residents of cities have been estimated to experience 25 thousand bites per year (Foster & Walker 2009).

1.2. Mosquito: *Culex quinquefasciatus*

Generally, adult mosquitoes are slender, with body surface covered with scales, setae, and fine pile creating the characteristic marking and light brown body colours (Figure 1.1 & Figure 1.2) (Triplehorn & Johnson 2005; Scholtz & Holm 2008). Figure 1.3 shows the piercing-sucking mouthpart type forms an elongated proboscis which assists them to suck up liquid food source (Foster & Walker 2009). The female's hind leg femur is pale to the tip except for dark scales found dorsally along the leg length, and also has pale scaling at knee spot and apical hind tibial spot, while the rest of the leg is with dark scales (Snell 2005). The head and thorax of pupa are fused to form cephalothorax, while the respiratory organs are the tympana cavity connected to the anterior ends of the dorsal longitudinal tracheal trunks (Wallage 2008). The abdomen projects from the cephalothorax and the presence of tail fins (paddles) assists in movement (Service 2012). Head of the larvae is triangular-shaped, sclerotized and often darker in colour than rest of the body (Foster & Walker 2009). Larvae mosquito mouthparts are composed of lateral brushes with pectinate hairs that serve as combs (with 50-60 setae) for retaining particles filtered form water (Wallage 2008; Service 2012). The terminal abdominal ending siphon (respiratory tube) has fours pair of ventolateral setae turfs (hair 1a-S usually above pecten teeth level), with distinct swell in lower half to middle and 8-12 pectin teeth while the lateral comb has a patch of 30-40 scales (Snell 2005; Service 2012).

Classification

Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Family	:	Culicidae
Genus	:	*Culex*
Species	:	*C. quinquefasciatus* (Say 1823)

Figure 1.1 Male of *Culex quinquefasciatus* (Service 1993).

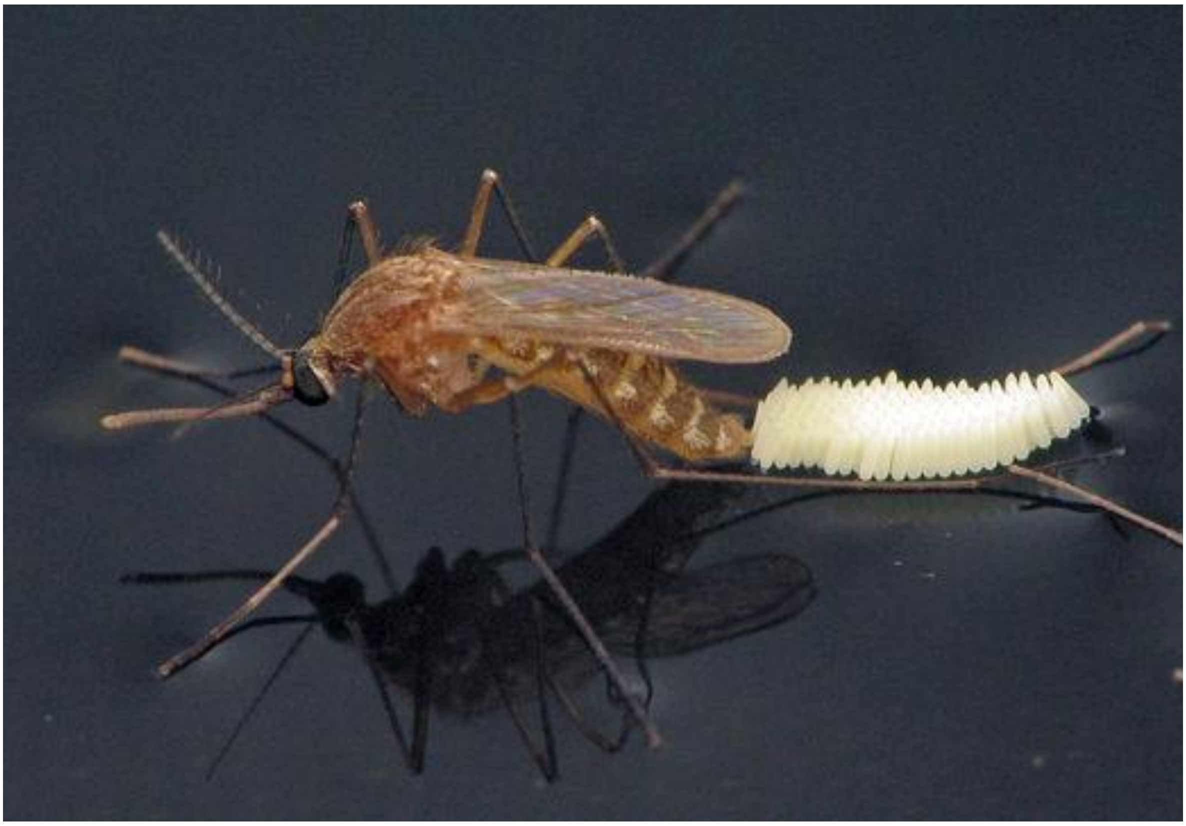

Figure 1.2 Female of *Culex quinquefasciatus* laying singular egg rafts on water surface (Russel 1999).

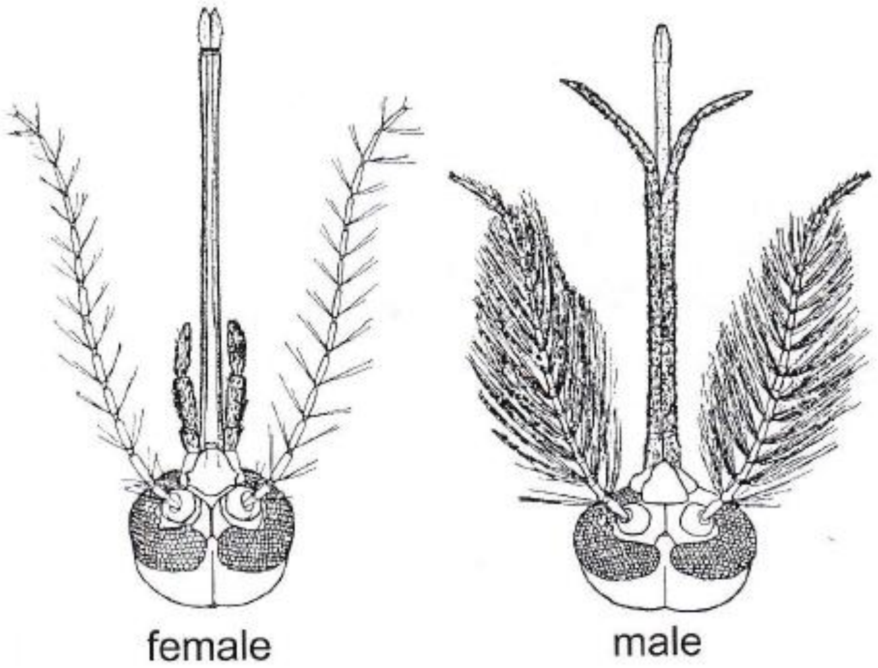

Figure 1.3 Adult head region of female and male of *Culex quinquefasciatus* with mouthparts and antennae type (Foster & Walker 2009).

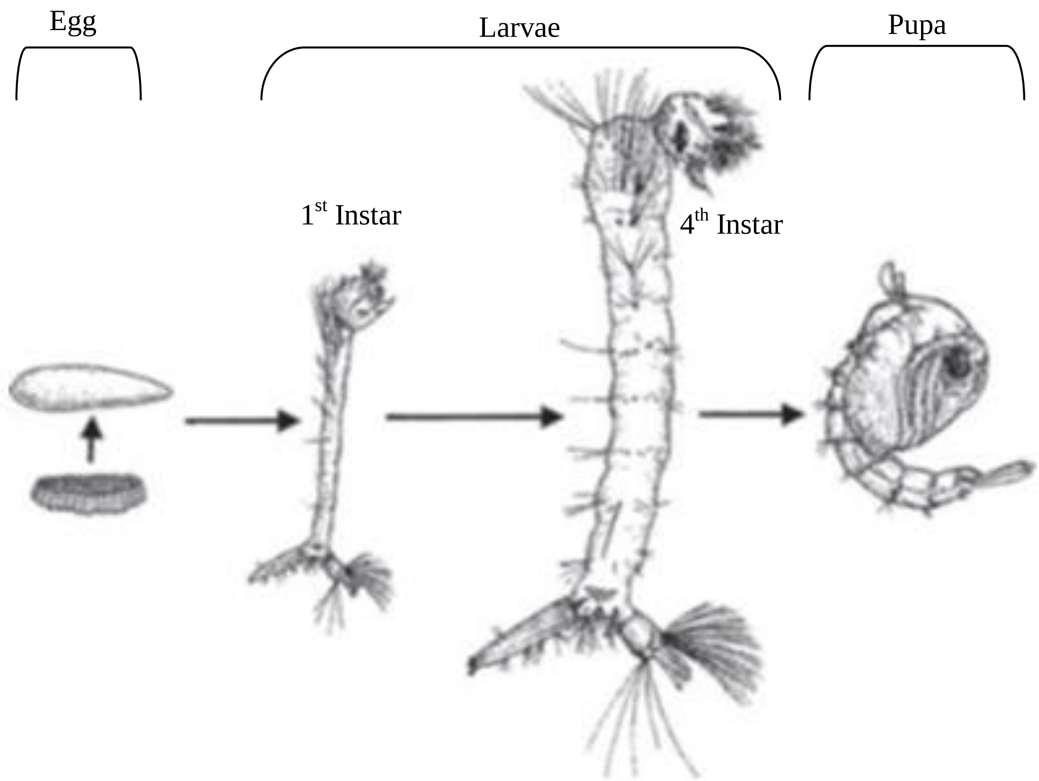

Figure 1.4 Immature aquatic life stages of *Culex quinquefasciatus* from egg, larvae to pupa stages (Service 1993).

The adult mouthparts of some predatory Culicidae are adapted for piercing and sucking that form a proboscis which usually allow them to feed on fluids such as blood, plant sap and nectar (Barraclough & Londt 2008). The *Culex* mosquitoes undergo a complete metamorphosis which consists of the egg, larvae, pupa and adult developmental stages. The different mosquito developmental stages occur and dwell in different mediums, whereby the immature stages (larvae and pupa) are aquatic, while the adult is terrestrial (Figure 1.4). The amphibious lifestyle of mosquitoes poses a challenge in the application of control measures, and thus contributes to create management inconsistency in medicine, veterinary sciences, agriculture and horticulture were both larvae and adults stages gets tricky to control (Crosskey 1993; Foster & Walker 2009). Their great diversity of habitats and life-history strategies has allowed them to colonise many contrasting environments, e.g. larvae living on a variety of habitats such as ponds, swamps, salt-waters, rock-pools, tree-holes, plant-axils, pitcher plants, polluted water and artificial structures (Service 2012). The Culicidae have had the most impact on human welfare, colonisation and development by being involved in the transmission of disease causing pathogens in Africa (Harbach 2007; Barraclough & Londt 2008).

1.3. Mosquito vector competence

1.3.1. Mosquito behaviour: *C. quinquefasciatus* female feeding

According to Service (2012), during the first to three days of adult life, both mosquito sexes must obtain sugar meal for sexual maturation, to acquire energy to find mates, search for food, dispersal and finding vertebrate blood (for females). Adult mosquitoes get their natural sugar meal from plant nectar or honeydew repeatedly throughout their life stage. The hosts of female mosquitoes include all classes of vertebrates namely mammals, birds, reptiles, amphibians and even fish (amphibious fish) (Powers *et al.* 2008). The mosquito's host specificity is a function of both the mosquito's innate host preference and the host available to the mosquito when and where it is active. The mosquitoes use volatile chemical compounds to locate and find host for blood meal. Main factors that play a role in host attraction and finding are odour created volatile aromatic, carbon dioxide, heat released and humidity (Rutledge 2008; Foster & Walker 2009). After the female has landed on its preferred or available host, feeding and probing are stimulated by chemical compounds such as fatty acids on skin surface and adenosine triphosphate (ATP) in adjacent blood vessels (Service 2012). The chemical receptors on the antennae, proboscis and tarsi play a role in detection of host and to begin ingestion. The females ingest as well as digest vertebrate blood to initiate production of eggs by stimulating a cascade of hormones from brain and ovaries (Rutledge 2008; Foster &

Walker 2009). During blood feed, mandibles are drawn back and maxillary stylets pierce small vertebrate vessels, the blood enters the food canal through suction force by cibarial and pharyngeal pump, while saliva is secreted with anticoagulant and digestive enzymes from hypopharynx (Powers *et al.* 2008).

1.3.2. Public and animal health: disease transmission

Female mosquitoes bite is able to cause the development sickness in various ways, both directly and indirectly, that includes: from actual bite's tissue piercing, salivary secretions released prior to feeding, and transmitted of pathogens. Mosquito bites can inflict pain on host skin. The proteins and foreign agents in saliva secreted into the host's blood stream during feeding can cause the development of immune response in the host. The immunity can lead to immediate or/and delayed responses which result in allergic reactions such as skin burning, itching, decolouration and swelling due to hypersensitive responses (Foster & Walker 2009). The three mammal (including humans) pathogenic groups known to mosquito borne diseases include viruses, nematodes and protozoans (Goddard 2008; Powers *et al.* 2008). The transmission of disease causing pathogens in female mosquitoes can be achieved either by mechanical or biological transmission routes. Mechanical transmission occurs when mosquito physically carries pathogens from one host to another, provided that the pathogen undergoes no development or significant multiplication (Goddard 2008). Biological transmission happens when the ingested pathogen undergoes further developmental stages or multiplication within mosquito vector into infective stages (Foster & Walker 2009; Service 2012). According to Powers *et al*. (2008), the evolutionary relationship between pathogen, mosquito vector and host is dependent on the spatial and temporal ecological factor such as host availability, vector competence, and pathogenic success, for illness to develop in humans.

The ability of arthropod vectors to acquire, maintain and transmit pathological agents is referred to as vector competence (Goddard 2008; Powers *et al*. 2008). It is important for the female mosquito to detect, find and feed on suitable host for blood feeding (haematophagous) mosquitoes, and not all of them are vectors of disease agents. An ideal vector according to Goddard (2008) would then be the one to provide a suitable internal environment for the pathogen, be long lived, have a feeding host preference pattern that matches the host range of the pathogen, feed often for an extended period and disperse readily.

1.3.3. Distribution

The *C. quinquefasciatus* is widely distributed across Africa and in southern Africa it is found in South Africa, Lesotho, Swaziland and Mozambique (Service 1993; Harbach & Kitchin 1998), as shown in Figure 1.2. The range of possible pathogens that mosquitoes of *C. quinquefasciatus* are associated with depends on the location, climate, topography, ecotype, and communities of recipient ecosystems. The spread (mobility) and mortality rates of the mosquito borne diseases depend on the transmitted pathogen, female *C. quinquefasciatus* vector competence, infected animal hosts, host feeding range and environment that it occurs. The transmitted infectious diseases represent a heavy burden on the social and economic improvement in most developing countries (WHO 2002).

Figure 1.5 The global distribution of *Culex quinquefasciatus* modified from Harbach 1981.

1.3.4. Mosquito-borne diseases: Southern Africa

The Culicidae mosquitoes are of public health and veterinary concern around the world, by being involved in the transmission of disease causing pathogens. Mosquitoes cause and transmit disease causing agents, and any illness developed from this is therefore said to a mosquito-borne disease. Mosquitoes are known to be involved in the transmission of infectious diseases such as avian malaria, lymphatic fillaria, and arthropod borne viruses. The *C. quinquefasciatus* mosquito is known to be the primary vector of avian malaria and elephantiasis in Southern Africa (Service 2012). The avian malaria is known to be caused by Apicomplexa protozoans of *Plasmodium ovalle,* *P. malariae and P. vivax*, which has cause a great decrease in abundance and diversity of inland as well as coastal birds (Powers *et al*. 2008). The WHO (2010) report has indicated that about 120 million people in tropical, subtropical, and temperate regions of the world are infected with lymphatic filariasis. The *Wuchereria bancrofti* nematode parasites are responsible for lymphatic filariasis disease called elephantiasis. The WHO (2012) reported that there is estimation of about over 50 million dengue infections worldwide annually, that pose health risks in many developing countries. In southern Africa, *C. quinquefasciatus* mosquito is suspected to transmit rift valley fever, a haemorrhagic fever which claims economic losses of over 1 million cattle and sheep during outbreaks (Service 1993). The sporadic rift valley fever is caused by *Phlebovirus* of a virus family Bunyaviridae that are able to infect and spread to a range of animals including humans, and are called epizootic (Powers *et al*. 2008). A few human death cases have been reported, whereby during the outbreak of 2005 and 2010 in Free State province about 10 farmers were killed by haemorrhagic rift valley fever in South Africa (WHO 2005).

1.4. Integrated Vector Management (IVM)

1.4.1. Mosquito control overview: New approach

In many developing and poorer countries, the frequent occurrence of mosquito borne disease is seen as a problem for social and economic improvement (Service 1993; Mullen & Durden 2009). Many methods that have been introduced and some improved to minimise or stop mosquito borne diseases failed, because of the complications encountered during the control of mosquitoes, transmitted pathogens as well as treatment of the resulted illness (Foster & Walker 2009). The majority of mosquito control strategies are intended on preventing mosquito biting irritation, keeping mosquito populations at acceptable densities, minimising mosquito-vertebrate contact, reducing the longevity of female mosquitoes, and also preventing or stopping disease transmission (Rutledge 2008; Foster & Walker 2009). The WHO (2004) indicated that up to now, there is no effective medication available for a number of mosquito borne diseases. Therefore, prevention of the diseases through vector control is an important component of disease control. According to WHO (2012), integrated vector management (IVM) is one of the management approached that aims to provide strategies for vector control in preventing an controlling vector borne diseases. The holistic approach of the IVM involves vector control strategies that are integrative and inclusive, in order to improve the efficacy, cost-effectiveness, ecological soundness and sustainable mosquito control techniques.

1.4.2. Mosquito control: techniques and encountered problems

The complication with mosquito vector control is their amphibious life of immature (larvae and pupa) states being aquatic and adults live on land. The *C. quinquefaciatus* larvae prefer polluted waters that are situated near or around human settlements (Service 1993; Foster & Walker 2009). The *C. quinquefaciatus* larvae might have developed some resistance to assist in tolerating toxins in polluted water to some degree. Service (1993) pointed out three larval control methods that involve physical control, genetic control, biological control and insecticidal control. The physical control approach includes mechanical or environmental, structure or source reduction, that include, filling up of small pools and ground borrow pits or removing artificial structures which creates temporary pools (Service 1993). The genetic control approach is seen at times as another biological control strategy of reducing mosquito fecundity that might help lower larval counts, for instance, introducing sterile adult male mosquitoes into the natural population (Goddard 2008). Another biological control is achieved by the introduction of natural enemy agents to reduce mosquito larvae populations, for example, infecting larvae with infectious fatal viruses, bacteria or protozoans, and predacious organisms that have all proven to be effective for long-term control (Foster & Walker 2009). Spraying oily chemicals such as kerosene (paraffin) and other petroleum oils on the water surface, was among effective oldest mosquito control strategies that assisted to either suffocate or poison the mosquito larvae as well as pupa (Yu 2008).

The search for chemical insecticides that are more effective and specific saw the introduction of synthetic larvicides such as dichlorodiphenyltrichloroethane (DDT) or 1,1,1-trichhloro-2,2-bis(4-chlorophenyl)ethane. The DDT products were used as contact poison for most arthropod pests and products were developed by Paul Hermann Muller during the Second World War 1939, which saw the establishment of DDT greatly reducing the use of mineral oils (Yu 2008). However, wrongful and the abundant use of DDT both in the control of agricultural and medical pests later showed that DDT can persist for longer periods in the environment (not biodegradable) (Hoy 2008), and was able to accumulate in both animal and plant tissues. The use and production of DDT as well as other related organochloride chemical pesticides was stopped, because of its extreme environmental persistence, general invertebrate contact poison, high toxicity, and bioaccumulation (from prey to predator). The Environmental Protection Agency (EPA) banned the agricultural usage of DDT in the USA in 1972 (Klassen 2008). The elimination of DDT led to the development of safer, specific and effective synthetic pesticides (insecticides), such as organophosphates, carbamate and pyrethroids. The organophosphates fenthion or chlorpyrifos are recommended for larval control dwelling in highly polluted waters (Service 1993).

Currently, the insecticide resistance of Culicidae mosquitoes poses a threat in the control of vector-borne disease. The major factors that contribute to the development of resistance to insecticides include the method of chemical application, period of chemical exposure and the nature of targeted insect. Resistance in these instances is seen as a genetic change that enables insects to withstand toxins and, it's inherited by future generations to survive better (Yu 2008). Other insects are able to tolerate effects of toxins but only for the ambient exposed population at that period, and if the insects are no more exposed to the toxin, the tolerance wears off (Powers *et al*. 2008). The resistance mechanisms differ in nature of action. Behavioural resistance helps vector to avoid contact with toxins by relying on response of chemical receptors to external stimuli, while physiological resistance are metabolic responses after contact with toxins. Georghiou and Wirth

(1997) reported that the mosquito of *C. quinquefasciatus* has developed multiple resistance towards insecticidal classes of organophosphates, carbamates and organochlorines.

The adult mosquito control strategies are employed to deter female egg oviposition, repel to minimise bites, or eradication using insecticides (Klassen 2008). The chemical substances that are used in formulating insecticide mixtures involve the active ingredient and inert ingredients, to make an effective and safer combination. The important factors that need to be considered during the formulation of insecticides includes, chemical and physical properties of active ingredients and inert ingredients (including ingredient compatibility), the toxicant properties, nature action of targeted insect, chemical residuals (derivatives) and hazard to users (Yu 2008). The insecticides chemical structure is mostly used to categorise insecticides.

1.5. Insecticides: Classification (Yu 2008)

The chemical name of an insecticide is assigned according to the International Union of Pure and Applied Chemistry (IUPAC) rules. The chemical name should describe the chemical composition and structure of the active insecticide ingredient. The WHO (1984) recognises only four chemical pesticides namely organophosphates, carbamates, pyrethroids and organochlorides that are authenticated for public usage. More products have being developed and some newly formulated, therefore the range and usage has increased ever since. The agricultural based integrated pest management (IPM) is among well-developed pest control approaches, and recognise about thirteen pesticide classes. According to Yu (2008), each pesticide (insecticide) class share chemical structure and function. The insecticide classes that are recognized include organochloride and organophosphates, carbomate and pyrethroids, insect growth regulators and fumigants, and botanical insecticides. The classification of insecticides includes the following:

1.5.1. Organochloride and Organophosphates

The class organochlorine insecticides is the first modern commercial chemical insecticide e.g. dichlorodiphenyltrichloroethane (DDT) which majorly contain chlorine, carbon and hydrogen molecules. The DDT analogue and other related isomeric derivatives are highly toxic to a broad range of arthropods and, can persist for a very ~~longer~~ period in the environment (not degradable). Furthermore, these pesticides are highly photostable, lipophilic as well as metabolically stable. As a result, they tend to biologically accumulate in the food chain from one organism to another. The organophosphates (OP) insecticides are a large class of phosphoric acid derivatives. The chemical structure of derivatives replaces the hydrogen-atom of phosphoric acid with organic radical such as methyl, ethyl, or phenyl, and whereas the oxygen-atom can be replaced by sulphur or nitrogen. The phosphate subclass include chemicals such as dichlorvos which is primarily used to control adult mosquitoes, blackfly and flea populations. Most organochlorines (such as DDT) systemically function to bind to the sodium channel active site and disrupt the neurotransmission in the central nervous system (CNS) of insects that result in hyper-excitation, tremors that lead to total paralysis and death. Currently, mosquitoes of *C. quiquefasciatus* have developed resistance against organochloride of DDT group (Thanispong *et al*. 2008).

1.5.2. Carbamate and Pyrethroids

The carbamates insecticides are characterised by ester bonds of carbamic acid. The carbamate derivatives are formed by replacing the hydrogen-atom (acid side) with aliphatic or aromatic radicals. The pyrethroids insecticides consist of natural and synthetic pyrethrum analogues. The active ingredients of pyrethrum are pyrethrins of a dried flower solvent extract from *Chrysanthemum cinerariaefolium* plant. The pyrethrum insecticides contain alcohol groups of pyrethrolone or cinerolone with either chrysanthemic acid or pyrethric acid. The biological ingredients of pyrethrum are not stable in direct sunlight. However, the synthetic pyrethrum are relatively more stable and are recommended for use in agriculture, forestry and public health insect control. The carbamates toxins bind to calcium channel active site, which influences the permeability of synaptic membrane by inhibition of acetylcholineasterase enzyme that mediates neuron transmission in CNS, resulting in hydrolysis of acetylcholine neuron messenger. The hydrolysis of messenger acetylcholine neuron prolongs synapse and leads to neuron hyper-excitation, and the affected insects exhaust energy, tremors, muscle convulsions, flaccid paralysis and later death (Hoy 2008; Klassen 2008).

1.5.3. Insect growth regulators and Fumigants

The insect growth regulator insecticides are chemical substances that play a role in disrupting the growth and development of insects, after which the insect is eventually killed. The juvenoids analogues are chemicals which imitate juvenile hormones in structure and function, for instance methropene resemble juvenile hormone (III) and is mostly used during mosquito larvae control. The juvenoids insecticides alters development and growth of the immature stages (egg, larvae and pupa), that may lead to rapid uncontrolled impaired growth, sterilisation, unsuccessful larvae or pupa stages, deformed aquatic respiratory organs and thereafter death. The fumigants insecticide class are composed of small molecular compounds, very volatile, capable of penetrating through large mass layers, and are known to interfere with octopamine neurotransmitter. These small compounds inhibit secretion of cyclic adenosine monophosphate messenger (cAMP) affecting nature of insect response to stimuli, resulting in delayed responses latter paralysis and death follows.

1.5.4. Microbial and Miscellaneous insecticides

The microbial insecticides functions as a combination of both the insect pathogenic bacterium and chemical toxins it releases, for example *Bacillus thuringiensis* releases ʒ-endotoxins which are species (or genus) specific that bind to epithelium layer in the midgut, increasing haemolymph (blood) pH to be more alkaline, then result in paralysis and followed by death. The *C.*

quinquefasciatus mosquitoes have cross and multiple resistance towards toxins releases by *B. thuringiensis* subsp. *israelensis* (Georghiou & Wirth 1997). The miscellaneous insecticides are all recently developed insecticides that differ structurally from previously described insecticide classes, e.g. neonicotinoids, furmamidines, and inorganic insecticides. The miscellaneous insecticides play a role in disrupting chitin synthesis of insect making it more vulnerable to death (Yu 2008).

1.5.5. Botanical insecticides

The natural botanical insecticides are plant derived chemical substances that are produced during photosynthetic reactions. Plants produce biologically active secondary metabolites that play a role majorly in defence and chemical communication (Hopkins & Hüner 2009). Plant secondary (natural) compounds are divided into three categories namely terpenes, phenolic and nitrogen containing compounds (Taiz & Zeiger 2010). Terpene family is chemically and functionally diverse classes of compounds. Terpenoids and their derivatives share a basic five carbon isoprene unit (2-methyl-1,3-butadiene) and are water soluble. According to Lambers *et al.* (1998) the majority of terpenoids are involved in plant herbivore repellence and deterrence, for instance, monoterpene esters of pyrethroids found in *Chrysanthemum cineraiifolium* with pyrethrin as active ingredients used insecticides products, some plants contain mixture of volatile monoterpenes and sesquiterpenes called essential oils that play a role as insect repellent. However, other plants contain non-volatile terpenes like liminoids that deter away herbivore feeding from citrus fruits. The azadirachtin which is ~~a~~ composed of tetremortiterpanoid extracted from *Azadirachta indica* seed oil, is known to affect the secretion of insect brain hormone (Prothoracicotropic hormone). Effects of the insect brain hormone include inhibiting the moulting process causing development and growth abnormalities that eventually cause death (found as ingredient of some insect juvenoid insecticides). Lastly, glycosides and saponins are toxic to insects in significant concentration and are also known to affect growth and development of insects (Yu 2008; Hopkins & Hüner 2009; Taiz & Zeiger 2010).

Phenolic compounds and their derivatives have a hydroxyl group on their aromatic ring. Most of phenolic compounds are water insoluble namely carboxylic acid, glycosides, flavonoids and are soluble in organic solvents (Lambers *et al.* 1998). Flavonoid groups of flavones such as retenone are found in roots of *Derris* and *Lonchocarpus* species which inhabits the NADH dehydrogenase enzyme complex in the mitochondrion electron transport chain, to influence insect cellular respiration (Yu 2008). Other cardiac glycosides according to Hopkins and Hüner (2009) are very toxic in lower concentration for example, oleandrin from *Nerium oleander* ~~which~~ is used as an active ingredient in some insecticides. The nitrogen containing secondary metabolite family consists of alkaloids, cyanogenic glycosides, glucosinolates and non-protein amino acids which are water soluble (Taiz & Zeiger 2010). The toxic alkaloids are readily available from plant extracts in large or small quantities that include nicotine from tobacco plants (*Nicotiana rustica*) and quinine from *Cinchina officinalis* that interfere with the GABA-gated chlorine channels to stimulate neuron hyper-excitation, and flaccid paralysis as well as death follows (Yu 2008; Taiz & Zeiger 2010). The sabadilla that is extracted from South American lily seeds of *Schoencaulon officinale*, with active alkaloids, namely cervadine and veratridine that are involved in the disruption of neuron transmission by binding to sodium channel sites causing delay in transmission, hyper-excitation of neurons resulting in total paralysis and death (Yu 2008). The ryania extract from ground roots of a tropical shrub of *Ryania speciosa* with alkaloids like ryanodine which induce paralysis in insects by direct action on the muscles, resulting in sustained contraction and paralysis. The documented active plant extracts are known to be more efficient in controlling agricultural, veterinary and public health insect pests in relative to synthetic insecticide classes (Yu 2008; Hopkins & Hüner 2009).

Secondary metabolites have an uneven distribution in a plant and hence the compounds are not present in every plant part (Salisbury & Ross 1992; van Wyk *et al*. 1997). Most secondary compounds have an evolutionary relationship with certain plants which leads to compounds beingspecific and restricted to one species or among related species (Lambers *et al*. 1999). Plants parts that are mainly used for medicinal practices include roots, bulbs, rhizoids, tubers, barks, leaves, stem, flowers, fruits, seeds, nectar gum and wax secreted (van Wyk *et al*. 1997; van Wyk *et al*. 2002).

1.6. Role of secondary compounds

The secondary metabolites have a restricted distribution in plant diversity, in that, certain secondary metabolites are found in one plant type or related species (Taiz & Zeiger 2010). The uneven distribution of secondary metabolites give plants specific abilities to render ecosystem services and functions that others cannot. The main functions of secondary metabolites include the assistance during interactions or symbiotic relationships, reproduction, defence against animal herbivores, and chemical communication (Salisbury & Ross 1992; Lambers *et al*. 1998). Plant derived secondary metabolites are found not only to benefit plant survival but can also be used by other organisms survival or pest control (van Wyk *et al*. 2002). Plant natural products (secondary metabolites) contain chemical compounds that are able to exert biological activities on other organisms (Colegate & Molyneux 2008). The comprehensive use of traditional Chinese medicine, Indian Ayurveda, Arabic unani and South Africa homeopathic medicine dates back to centuries (WHO 2002). Many indigenous cultural groups have been using plant natural products for animal and human primary herbal therapy. The local people understood better the use of plant diversity to sustain their living, by exploiting plant natural products for food and medicine purposes (van Wyk *et al*. 1997; Shale *et al*. 1999; WHO 2002). In South Africa, the majority of local Free State communities are dominated by Sesotho speaking tribe, and mainly found in the higher altitude sandy grassland biome of Free State. They mainly use grass, sedge and herbs for cultural and traditional medicinal practices e.g. grass species that contain hydrocyanic acid, *Eragrostis plana* is used as a tonic and for ailment treatment, while *Eleusine coracana* is used to treat leprosy and liver related diseases (Moffett 1997).

The plants with medicinal properties have active ingredients that give plants their ecological service (Taiz & Zeiger 2010). The active ingredients are natural (secondary) compounds that are able to elude activity in another organism in lower or higher concentration (Hopkins & Hüner 2009). The plant secondary metabolites are categorised according to shared distinct structural structure (Lambers *et al.* 1998; Hopkins & Hüner 2009; Taiz & Zeiger 2010). The categories might have derivatives made up by simple structural unit and some composed of complex molecules compiled by larger numbers of simple units (Salisbury & Ross 1992; Hopkins & Hüner 2009). A few groups of unique secondary compound structural units are shared between groups by having characters from two secondary metabolite groups, for instance, glycosides with an isoprene unit and an aromatic phenol character (Taiz & Zeiger 2010). The biological activities of the natural ingredient might be from a single secondary compound, combination of two or multiple holistic contributions from either a single or between secondary metabolite classes (Yu 2008). The secondary metabolites from one class share biological synthetic pathway, and are either produced as by-products during or after the photosynthetic metabolisms (Hopkins & Hüner 2009). Figure 1.6 indicates that of secondary compounds shared between classes might differ in their biosynthesis pathway from parent secondary class (Lambers *et al*. 1998; Hopkins & Hüner 2009).

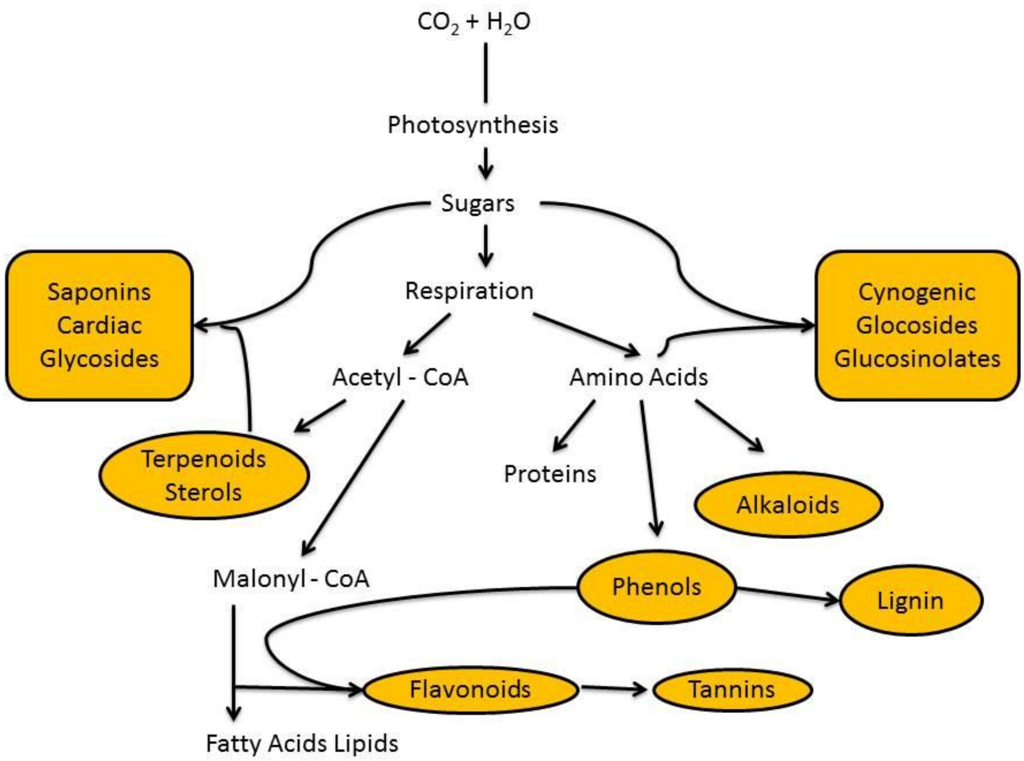

Figure 1.6 Molecular pathways for synthesis of plant secondary compounds, modified from Hopkins & Hüner (2009).

1.7. Ethnobotany Practices

Plants form part of the primary producers in the ecosystems life hierarchy, in which their photosynthetic ability make the flowering plants to be major source of food to many living organisms. The ecological functions and services of flowering plants include the conversion of solar energy into chemical-metabolic energy, nutrient cycles, consumption of carbon dioxide and to make oxygen available (Salisbury & Ross 1992; Lambers *et al*. 1998) for other organism to use. To other ecological extend, plants have been a major sources of medicine to public and animal health. Many developing countries still depends on plant natural products for traditional medicinal use as a primary health care (Shale *et al*. 1999). The cultural and traditional customs in medicinal plants practices for herbal therapy were not scientifically documented but have been passed on from one person or generation to the next by word of mouth (van Wyk *et al*. 1997; Shale *et al*. 1999). Currently Africa is still lagging behind with validation of some medicinal plant uses to document their biological active ingredient activities (WHO 2002). The plant natural products and their derivatives contribute to almost 50% of clinically used drugs in the world (Balandrin *et al.* 1993). Plant derived products have contributed to day to day health care and the developed plant extracts are sold informal and commercial herbal markets (van Wyk *et al*. 1997). The documentation of indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources (Muthu *et al*. 2006).

The medicinal plants play a major in cultural heritage which is brought by plant biodiversity found in Southern Africa. There are over 300 000 flowering plants and 9 000 of are endemic, whereas 3 000 are used for medicinal uses in South Africa (van Wyk *et al*. 1997). The climate and ecological variations found in South African biomes makes plant flora to be among the world's most diverse, abundant and species rich regions (Mucina & Rutherford 2006).

1.8. Alternative mosquito control

There is currently about six plant derived chemicals that are registered as insecticides in the USA, Southern America and India, whereas there are no registered plant derived chemicals from plants in Southern Africa (Hoy 2008.). Many ethnobotanical surveys have shown that local inhabitants have ample knowledge and usage custom of traditional insecticide plants (WHO 2002). The majority of plants with insecticidal properties have been used also as medicine to treat a variety of illnesses by the local community, whereby some of these plants are selected to prevent and minimise bites from mosquitoes and other blood feeding insects (Karunamoorthi *et al*. 2009). The traditional and scientific practices in pest control have been aimed for insects with medical, veterinary and economic importance (WHO 2002). In the control of insects such as mosquitoes, the methods in controlling mosquito populations to manageable sizes must include the control of aquatic larvae and pupa as well as terrestrial adults. Several plant bioactive compounds used during mosquito control must be specific to select only mosquito larvae or pupa in a water body and either repel or reduce mosquito bites.

The WHO (2010) listed Culicidae as among major insects that are involved in vector-borne diseases in Africa and the newly implemented integrated vector management focuses more on mosquito control. The ability of *Culex* mosquitoes to have developed resistance genes over time, urges the call for other efficient and effective alternative mosquito control strategies. In South Africa, the Department of Agriculture, Forestry and Fisheries as well as the South African Bureau of Standards (SABS) support the development of new trends in mosquito control strategies (SANPRA 2000; South African Government 2004). The majority of natural botanical insecticides have been shown to be more effective and efficient than synthetic insecticides. The South African indigenous plant flora holds some of the known global biodiversity hotspot and with about eight described vegetation types (biomes) (van Wyk *et al.* 2003). The secondary metabolites produced during photosynthetic reactions have medicinal properties for pest control. The abundance of *Culex* mosquito in some rural regions of the Free State province poses a public health threat, whereby the mosquito might spread as well as establish vector-borne diseases. The wide pathogen (viral, bacterium, protozoans) transmission range and a variation of preferred feeding hosts (humans, rodents, livestock) of *Culex* mosquitoes influence the urgency of developing alternative control strategies. Most of the arthropod borne viruses appears sporadically like rift valley fever, which does not allow local animals including humans enough time to acquire immunity (Klassen 2008). The medicinal plants found in the Free State province might have insecticidal properties towards *Culex* mosquitoes, and help provide better, easy accessible, affordable, safer to use, efficient and biodegradable active compounds.

The mode of action of plant bioactive compound differs between insecticide classes. The current study investigates the natural botanical insecticide properties of Basotho medicinal plants, and aims to evaluate the insecticidal, , pupicidal and larvicidal activities of *Artemisia absinthium, Artemisia afra*, *Cosmos bipinnatus, Tagetes minuta*, *Foeniculum vulgare* and *Mentha longifolia*, against mosquito of *Culex quinquefasciatus* (Diptera: Culicidae) from the eastern Free State Province of South Africa.

CHAPTER 2

OBJECTIVES

2.1 Problem statement

The Culicidae mosquitoes are involved in the transmission of medically and economically important disease causing pathogens. The *Culex quiquefasciatus* mosquitoes are vectors of Rift Valley Fever, Yellow Fever, West Nile Virus and Equine Encephalomyelitis in southern Africa (Foster & Walker 2009; WHO 2009). The viral infections of rift valley fever can cross hosts to infect even humans during mammalian blood feeding by female *Culex* mosquitoes in southern Africa (Service 2012). The WHO (2009) reported that rift valley fever outbreaks that dated from 2006 to 2009 claimed lives of 50 people, of which about 60% of the deaths is from Free State Province communal farmers. According to Powers *et al*. (2008) most of the viral pathogens (for instance rift valley fever, yellow fever, west nile virus) appear sporadically and create uncertainty of when would the outbreak be, and leads to great economic loss when animals are exposed to the transmitted viral pathogens. The major factors that play a role in the success of these vector borne diseases is *Culex* vector competence, effective detoxification metabolism, rapid resistance development, wrongful use of applied insecticides, climate change and the ability of *C. quinquefasciatus* larvae to tolerate aquatic pollutants (Goddard 2008; Foster & Walker 2009).

The wrongful use and high toxicity of chemical insecticides during control or eradication of *Culex* mosquitoes, has led to negative impacts on biological accumulation of heavy metals in both animals and humans (Zahran & Abdelgaleil 2011). In recent studies, the *Culex* mosquitoes are documented for their ability to develop resistance to most applied synthetic chemical insecticides, and that creates problems in controlling both the mosquitoes (larvae & adult stages) and the transmitted disease causing pathogens in South Africa (Gerritsen *et al*. 2008; Read *et al.* 2009). The majority of currently used commercial synthetic insecticides are chemically stable and can persist for longer period in the environment, and therefore biologically accumulate in organism's food chain. Again, commercial synthetic insecticides are highly toxic and are not species specific (Yu 2008). The integrated vector management approach calls for other alternative methods that are safer for public use, efficient and effective, environmentally friendly, easily assessable and species specific. The local medicinal plants possess biologically active compounds which are recognised to offer better insecticidal properties than synthetic insecticides.

2.2 General objective

The aim of the present study was to evaluate and document the insecticidal properties of Basotho medicinal plants that include *Artemisia absinthium* (Asteraceae), *Artemisia afra* (Asteraceae), *Cosmos bipinnatus* (Asteraceae), *Tagetes minuta* (Asteraceae), *Foeniculum vulgare* (Apiaceae), and *Mentha longifolia* (Lamiaceae) against mosquito *Culex quinquefasciatus* (Diptera: Culicidae) from the eastern Free State Province of South Africa.

2.3 Specific aims

- To quantify phytochemical constituents present in organic solvent extracts of these Basotho medicinal plants.
- To investigate the cytotoxicity activities of these Basotho medicinal plants against brine shrimps (*Artemia salina*) nauplii.
- To evaluate the larvicidal activities of the extracts from these Basotho medicinal plants towards aquatic mosquito larvae.
- To investigate the pupicidal activities of the extracts from these Basotho medicinal plants towards aquatic mosquito pupae.
- To assess the insecticidal activities of these Basotho medicinal plant leaf powder
- To validate the traditional usage of selected Basotho medicinal plants as insecticides by the Basotho tribe from the eastern Free State province.

CHAPTER 3

CHOICE OF PLANT

3.1. Natural botanical insecticides

The six selected Basotho medicinal plants include Asteraceae family species namely *Artemisia afra, Artemisia absinthium, Cosmos bipinnatus* and *Tagetes menuta*, Apiaceae family representative *Foeniculum vulgare*, and lastly Lamiaceae family species *Mentha longifolia*. The main selection criteria for these Basotho plants included their aromatic properties, traditional use as insecticide, easily accessible, and plant relatively not infected by insect pests in natural habitat. The aromatic properties of plants are due to volatile compounds that play a role in repelling and deterring pests. Most plants that emit strong odour are used traditionally as insect repellents by the Basotho tribe in the Free State province. These selected Basotho medicinal plants are suspected to contain biologically active volatile compounds and other anti-insect phytochemicals hence no insect pests were found in their natural habitat.

The biological availability and quantity of active secondary compounds in a plant depends on the species, age, targeted plant part, geographical location, environmental conditions, growing season, and symbiotic associations (Colegate & Molyneur 2008; Sasidharan *et al*. 2011). The type of collection, storage and preparatory methods used during extractions of active ingredients from plants also affect the quantity of compounds (Colegate & Molyneur 2008). Plants extracts are preparations that contain the active ingredients of a medicine and are isolated using suitable solvents such as water and alcohol (van Wyk *et al*. 1997). Other plant extracts perform better due to active ingredients or more efficient as a mixture with co-extractants (Colegate & Molyneur 2008; Sasiharan *et al*. 2011). The clues in the detection of bioactive compounds are usually led forward by traditional knowledge built from past experience (WHO 2002). Most bioactive compounds that are suspected to contain insecticide properties were screened using biological assays for their bioactivity and availability as insecticides (Yu 2008). The interesting part of monitoring the presence of active ingredient(s) using biological assays during isolation of insecticide agents, are the process of the bioassay guided fractionation (isolation) of testing all fractions for insecticidal activities until the bioactive agent is obtained in a pure form (Colegate & Molyneur 2008). The detection and isolation of bioactive agents also play a role in finding insecticide compounds that are specific to a certain insect species or range of insects with similar targeted compounds, for better insect pest control strategies.

3.2 Basotho medicinal plants

3.2.1. Family Asteraceae

The family name Asteraceae (Compositae) was deduced from their star-like (aster) inflorescence which is among the largest flowering groups (Bremer 1994). Asteraceae has a worldwide distribution with about 12 subfamilies that are represented by 1620 genera with 23 000 species (Merman *et al*. 2000). The distinct character on the inflorescence of the family is the shape of capitulim (flower head) with numerous sessile florets which all share a receptacle (Bemer 1994; Merman *et al*. 2000). The traditional medicine practices of most indigenous communities around Africa are in fact driven from plants of Asteraceae (Salie *et al*. 1996). There are about 246 genera that are represented by 2300 species in South Africa, and the majority of the 17 known tribes are used for medicinal purposes (van Wyk & Tilney. 2003). The Asteraceae are commercial and traditionally known to produce pesticides, essential oils, ailment medicine, edible food, and some species are used as ornamentals. The family is among the most commonly used in traditional medicine in South Africa, and has received much scientific attention in search of biologically active compounds (Patil *et al*. 2011).

Most of the aster medicinal plants fall under the tribes Anthemideae, Gnaphalieae and Helenieae (van Wyk & Tilney. 2003). The genus of *Artemisia* is well known in traditional and medical practises, and it is mostly used for treatment of of reducing phlegm, relieving cough, invigorating blood circulation, stopping pain, inducing sweat, diuresis (van Wyk *et al.* 1997). Over 260 species of *Artemisia* genus have been investigated for bioactive compounds showing antimalarial, antiviral, antitumor, antipyretic, antioxidant, antihepatitis, antihemorrhagic, anticoagulant, antianginal, anticomplementary, antiulcerogenic, antispasmodic, and interferon-inducing properties (Tan *et al*. 1998; Liu *et al*. 2009; Patil *et al*. 2011). The bioactive constituents were isolated from different species of *Artemisia* genera, they include mono- (23-25) and sesquiterpenoids (1-22), flavonoids (26-57), coumarins (59-64), isoprenylcoumaric acid derivatives (65-68), caffeoylquinic acids (70-73), acetylenes (74-77), sterols (78-79), a phenoxychromene (57), an acetophenone glucoside (58), a henylpropene (69), methyl jasmonate (80), and y-tocopherol (81), respectively (Viljoen *et al*. 2006; Liu *et al*. 2009; Patil *et al*. 2011).

3.2.1.1. *Artemisia absinthium* L.

3.2.1.1.1. Classification

Class: Magnoliopsida (Angiospermae)

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *A. absinthium* L.

Figure 3.1. *Artemisia absinthium* L. shrub growing in its natural habitat around Qwaqwa area, eastern Free State Province, South Africa.

3.2.1.1.2. Description and Ecology

The stems of *A. absinthium* are branching, grooved, with height ranging from 0.8-1.2 m around and have a silverfish colour. The leaves are spirally arranged around each stem, with a greenish-grey colour dorsally (above) and a fading white colour ventrally (below). The leaf surface is covered with silky silvery trichomes that bear small oil producing glands. The basal leaves vary from being bipinnate to tripinnate and are about 25 cm in lenth with long petiole, while the cauline leaves which are directly on the stem are about 5-10 cm smaller, less divided and with shorter petiole. The flowers are pale yellow in colour, tubular and clustered in spherical bent down capitula (flower head) and branched as panicles. *A. absinthium* starts flowering from late summer to late autumn, and is wind pollinated (anemophilous), whereas seeds are dispersed by gravity. The simple bearing dry, small, indehiscent, one seed fruit develop into a simple ovary (van Wyk *et al.* 2003).

The perennial herbaceous woody shrub prefers loamy sandy rocky areas and has a fibrous root system. In Southern Africa, *A. absinthium* L. is distributed in higher altitudes regions that range between 2000-2890 m above sea level, namely Free State, Lesotho, Eastern Cape, Mpumalanga, Gauteng and KwaZulu-Natal (Herman *et al*. 2003).

A. absinthium L. is known to produce allelochemicals that inhibit growth of other plants near its range. It is used traditionally as a pest repellent and deterrent in gardens against snails and beetles, while the infusion extracts of leaves and flowers are used as insecticides for human and animal louse (ref). It has possible active pesticide ingredients named absinthe (phenolic compound) and thujone (terpenoids ketone), that have been synthetically integrated in commercial pesticides (Lanchenmeier *et al*. 2006). Previous insecticidal studies on *A. absinthium* were on essential oil and aqueous extract activities towards pest of stored products namely the common bean weevil *Acanthoscelides obtectus* and two-spotted spider mite *Tetranychus urticae* (Chiasson *et al*. 2001; Derwich *et al*. 2009). The ability to cause deaths at lower concentrations of *A. absinthium* to such pests and the traditional usage, has led to the investigations of insecticidal (larvae, pupae, adult) properties against *Culex quinquefasciatus* mosquito in this study.

3.2.1.2. *Artemisia afra* Jacq.

3.2.1.2.1. Classification

Class: Magnoliopsida (Angiospermae)

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *A. afra* Jacq. ex Willd.

Figure 3.2. *Artemisia afra* shrub growing in its natural habitat around Qwaqwa in the Eastern Free State Province.

3.2.1.2.2. Description and Ecology

Artemisia afra Jacq. is one of the most commonly used medicinal plants in traditional practices in Southern Africa (van Wyk 2008). The species is a perennial herbaceous woody shrub, which can grow up to 2 m in height. The branched stems are thick and woody at the base, while they become thinner and softer towards the tip. The fine like-leaves are arranged spirally along the stem, and contain oil hairs on the leaf surface (van Wyk *et al*. 1997). The leaves have a soft texture, are dark green on the adaxial surface and light green on the abaxial surface, and can reach a length of 8 cm and a width of 4 cm in maturation stages. The nodding yellow flowers are 3-4 mm in diameter and with crowded bracts at the tip of the branches furthermore, the growing period of *A. afra* is from early September and flowers late in summer from March to May (van Wyk et al. 1997; Liu *et al*. 2009).

A. afra is a common species that is fairly distributed in all provinces of South Africa, except the Northern Cape. The wild species prefers to grow in mountainous areas that range from 2000-2400 meters above sea level, on damp slopes, along river and stream edges. In terms of continental boundaries, *A. afra* is the only species among 400 species of genus *Artemisia* that is indigenous to Africa. It also growsin Lesotho, Swaziland and northwards into tropical Africa (van Wyk *et al*. 1997; Liu *et al*. 2009).

The most common traditional medicine uses of *A. afra* are treatment of cough, fever, influenza and cold related ailments, as well as colic, and kidney disorders (van Wyk *et al*. 1997; Liu *et al*. 2009). The dried stem and leaves are burnt to repel insect pests in traditional practices for pest control (Liu *et al*. 2009; Brendler *et al.* 2010). There is currently no scientific validation (research) done that confirms the insecticidal properties from traditional insect control claim, and hence the study was aimed at investigating the insecticidal activities of *A. afra* extracts against *Culex* mosquitoes.

3.2.1.3. *Cosmos bipinnatus* Cav.

3.2.1.3.1. Classification

Class: Magnoliopsida (Angiospermae)

Order: Asterales

Family: Asteraceae

Genus: *Cosmos*

Sepcies: *C. bipinnatus* Cav.

Figure 3.3. *Cosmos bipinnatus* shrub growing in its natural habitat around Qwaqwa area in the eastern Free State Province.

3.2.1.3.2. Description and Ecology

Cosmos bipinnatus Cav. is half-hardy annual herb with an erect stem (main stem) reaching a height from 0.4-2.5 m. The individual plants usually tend to extend their branches out about as wide as their height. The stem is dressed with pinnatus, sectum (feathery), green leaves that are cut deeply into threadlike segmented. The leaves are orientated to sprout at a right angle on the stem. The leaf base surface is usually light green and distal surface is reddish with a pointy ending. A single petal ray florets are about 2-4 m in diameter, with yellow capitula (centre), while, the rays colour range from white, red and pink. In temperate regions, *C. bipinnatus* flowers from late spring until early autumn periods (Herman *et al*. 2003).

C. bipinnatus Cav. was introduced into South Africa from Mexico, and it is now used for medicinal practices. The plant is found in areas that are above 2000 m in altitude, and it is common in Free State, Gauteng, Mpumalanga, North West and KwaZulu-Natal provinces (Herman *et al*. 2003).

The traditional medicinal uses of *C. bipinnatus* Cav. flower include treatment of various diseases like jaundice, intermittent fever, and splenomegaly, while its triterpene alcohols such as helianol were reported to show anti-inflammatory activity (Jang *et al*. 2008). Traditionally, *C. bipinnatus* is used in gardens to attract pollinating insect .e.g. butterflies, bees, and other pollinating flies. (Vánky *et al*. 2005). The insecticidal properties of *C. bipinnatus* have not yet been reported. Therefore, the current study intended to evaluate the insecticidal properties of *C. bipinnatus* leaf extracts against a *Culex* mosquito.

3.2.1.4. *Tagetes minuta L.*

3.2.1.4.1. Classification

Class: Magnoliopsida (Angiospermae)

Order: Asterales

Family: Asteraceae

Genus: *Tagetes*

Sepcies: *T. minuta* L.

Figure 3.4. *Tagetes minuta* shrub growing in natural habitat around Qwaqwa eastern Free State Province.

3.2.1.4.1. Description and Ecology

Tagetes minuta L. is an annual hard-woody shrub, with an erect stem which can get to a height of 3 m, and is supported by a tap root system. The foliage is pungent, globrous, and dotted with embedded translucent glands. The compound pinnate leaves have an opposite to alternate arrangement along a firm stem. The leaves are about 5-15 cm in length, the narrowly lanceolate leaflets are 11-17 on compound leaf and with length of about 2-4 cm of sharply serrate leaflets. The panicle-like (cymes) inflorescences consist of numerous narrow cylindrical flower heads (capitulum) that are about 7-10 mm long and 3-4 mm in diameter. There are 3-4 phyllaries in a single series, fused into a tube dotted with glands. The pale yellow petal rays range from 1-5 and the orange-yellow disk florets (3-5) barely extend beyond the phyllaries. The receptacle lack chaffy bracts (Vasudevan *et al*. 1997).

T. minuta L. is found in areas elevated between 1400 and 2125 m of altitude above sea level. *T. minuta* L. is wide spread in South Africa and found in all provinces except the Northern Cape. The *T. minuta* L.is listed as an invasive weed plant that competes to grow on waste damps and abundant cultivated agricultural sites. It is found from east Africa to southern Africa (Herman *et al*. 2003).

The allelopatric behaviour of *T. minuta* L. has contributed to its success as an invasive alien species (Vilâ & Weiner 2004). The secreted secondary allelopatric compounds play a role to inhibiting the growth of parasitic root nematodes and other microbes in the soil (Hopkins and Hüner 2009). Fraction extractions and essential oil of *T. minuta* revealed the larvicidal and insecticidal against Indo-Pakistan Malaria Mosquito (*Anopheles stephensi*) and (Yellow Fever Mosquito) (*Aedes aegypti*) (Green *et al*. 1991; Perich *et al*. 1994; Perich *et al*. 1995). The use of *T. minuta* for the control of medically and veterinary important insects has been limited, and hence the current study intended on evaluating the insecticidal (cytotoxicity, larvicidal, pupicidal & insecticidal) properties of *T. minuta* in South Africa.

3.2.2. Family: Apiaceae

The Apiaceae also commonly called Umbelliferae is widely distributed across temperate regions in the world (van Wyk & Tilney 2003). There are currently about 78 genera with 368 species in the sub-Saharan region, of which 60% genera and 90% species are endemic to Africa (van Wyk & Tilney 2003). The Apiaceae family is well represented with 38 genera that are indigenous to southern Africa (Liu *et al*. 2007). Apiaceae medicinal and economic importance is well known in three of its subfamilies, and the taxonomically complex Apoiedeae is the largest subfamily. (Pimenov & Leonov 2004; Zhou *et al*. 2008). The previous taxonomical studies had shown to support subfamily Apioideae as a monophyletic group, and whereas the many of the tribes and subtribes are traditionally not recognised the subfamily as a monophyletic group (Downie *et al*. 2010). Members of the Apioideae subfamily contain compound umbels, characteristic fruits that possess two one-seeded mericarp suspended from a free carpophore, and a well-developed vittae (Zhou *et al*. 2008).

The genus *Foeniculum* is found worldwide and adds to the 19 endemic Apiaceae genera in southern Africa (Liu *et al*. 2007). Although the *F. vulgare* species is endemic to southern Europe and the Mediterranean region, currently it is cultivated throughout the temperate and tropical regions mainly for medicinal and economic importance (He & Huang 2011). The major biologically active compounds include *trans*-anethole, fenchone, estragol (methyl chavicol) and α-phellandrene that vary in level of concentration due to method of extraction and geographical origin (Rather *et al*. 2012). Previous studies on Apioideae have shown that there is higher chemical diversity and level of biological activities from the subfamily, and hence is widely used for culinary and medicinal use (van Wyk & Tilney 2004).

3.2.2.1. *Foeniculum vulgare* Mill.

3.2.2.1.1. Classification

Class: Magnoliopsida

Order: Apiales

Family: Apiaceae (Umbelliferae)

Genus: *Foeniculum*

Sepcies: *F. vulgare* Mill.

Figure 3.5 *Foeniculum vulgare* shrub growing in its natural habitat around Qwaqwa area of the eastern Free State Province.

3.2.2.1.1. Description and Ecology

Foeniculum vulgare Mill. is an herbaceous, perennial, ambelliferous plant that can reach heights of 3m. The stem is thick, hollow, striated and can spread to 1.5 min width . The leaves are soft, sectum (feathery) and segments are deeply cut (1-5 cm in length). The leaves have basal sheath that is about 10 cm in length, alternate arrangement around stem and are tripinnate. The flower are yellow, with about 5-30 petal rays that are 1-6 cm in length, and produce large terminal flat tooped umbels. *F. vulgare* Mill. is fairly distributed in the eastern parts of South Africa up to 2000 m altitudes, in areas of Gauteng, Mpumalanga, KwaZulu-Natal, Free State, Eastern Cape and Western Cape provinces (van Wyk & Tilney 2003).

F. vulgare Mill is commonly used for culinary and traditional medicine practices . Most parts of the plant such as, young shoots, leaves and fully ripened and dried fruits are commonly used for homemade remedies. Its aromatic fruits have been used as a culinary spice in many countries (He & Huang 2011). *F. vulgare* Mill. is commonly used for gastrointestinal disorders, relief of abdominal pains, cramps, stomach gas, indigestion and bloating. It is an appetite suppressant (Raffo *et al*. 2011).

The essential oil extracted from leaves of *F. vulgare* possess deterrent and repellence activities towards silver leaf white flies (*Bemisia tabaci*) which is a pest of tomatoes (*Solanum lycopersicum* L.) by affecting settlement and oviposition of *B. tabaci* (Baldin *et al*. 2013). Also the essential oils from seeds showed larvicidal activities against mosquitoes of *Anopheles stephensi* and *Culex pipiens* (Zoubiri *et al*. 2010; Sedaghat *et al*. 2011). However, some studies have focused on agricultural pest control using essential oil from *F. vulgare*, a few studies show mosquitocidal properties, and hence the plant was selected to investigate the larvicidal, pupicidal and insecticidal activities of leave extracts from *F. vulgare* in South Africa.

3.2.3. Family: Lamiaceae

The mint family Lamiaceae (Labiatae) is the sixth largest family of flowering plants and has a worldwide distribution (Drew & Sytsma 2012). The plant family Lamiaceae is further classified into 7 subfamilies, with 240 genera that comprises of more than 7200 species (Bräuchler *et al*. 2010). The subfamily division are classified based on morphological and molecular data (Drew & Sytsma 2012). Nepetoideae is the largest subfamily comprising of 105 genera with 3600 species, and are recognised mainly for their medicinal and economic importance (Retief & Herman 2003; Drew & Sytsma 2012). Characters that distinguish members of subfamily Nepetoideae into a monophyletic group include a hexacolpate, three nucleated pollen, an investing embryo and presence of rosmarinic acid (Bräuchler *et al*. 2010). The Nepetoideae is further classified into three tribes, with the largest being Mentheae tribe which is the most diverse in terms of distribution, floral forms, breeding system and habit (Bräuchler *et al*. 2010; Drew & Sytsma 2012).

The *Mentha* genus with 25 species (of which 13 are natural hybrids) that are known as common herbs for medicinal, culinary and aromotherapeutic properties, among the Mentheae tribe. (Kumar *et al*. 2011). The *Mentha* genus is found most in temperate regions such as Eurasia, Australia, and South Africa (Khani & Asghari 2012). *Mentha* species essential oil is generally composed of the following; monoterpenoids namely menthol and menthone, (*cis*/*tans*)-carvone and pulegone / pipertone rich *Mentha* oil (Viljoen *et al*. 2006; Khani & Asghari 2012).

3.2.3.1. *Mentha longifolia* Huds.

3.2.3.1.1. Classification

Class: Magnoliopsida

Order: Lamiales

Family: Lamiaceae

Genus*:* *Mentha*

Sepcies: *M. longifolia* Huds*.* subsp. *polyadena* Briq.

Figure 3.6 *Mentha longifolia* shrub growing in its natural habitat around Qwaqwa area in the eastern Free State Province.

3.2.3.1.1. Description and Ecology

Mentha longifolia Huds. subsp. *plolydena* Briq. is a perennial herb that is common in wet areas, with creeping rhizomes and an erect stem of up to 0.8 m in height. The stem is square in cross section and finely procumbent. The stem and leaf structures have oil glandular trichomes on their surface. The leaves are oppositely arranged, with an oblong-elliptical, that are 5-10 cm long and 1.5-3 cm in with. The leaves are dense tomentose (turfs), with dorsal glabrous surface of leaves being green and ventral glabrous surface is greyish-green. The flowers are about 3-5 mm in length, lilac, and the dense clusters (verticellasters) produces a purplish colour on tall branched tapering cylindrical raceme (100 mm long, 14 mm wide) (Viljoen *et al*. 2006).

The distribution of *M. l.* subsp. *polydena* is restricted and disjunct, and occurs in areas of altitudes up to 2135 m, namely Mpumalanga, Gauteng, Free State, KwaZulu-Natal, Eastern Cape and North West (Retief & Herman 2003). This plant is mainly used for coughs, colds, fever, asthma, and other bronchial relates ailments. It is also used to treat headaches, indigestion, flatulence, hysteria, and painful menstrual cycles (van Wyk *et al*. 2003). There are three recognised subspecies of *M. longifolia* in Southern Africa that are distinguished by floral parts, location and chemical composition. These are namely *M. longifolia* subsp. *wissii* (Namibia & Namaqualand) and *M. longifolia* subsp. *capensis* (throughout South Africa), and the above mentioned *M. longifolia* subsp. *polydena* (Viljoen *et al*. 2006).

There is no reports on the insecticidal properties of essential oil from *M. longifolia* include the toxicity and repellence of two stored products pests namely the flour beetle (*Tribolium castaneum*), cowpea weevil (*Callosobruchus maculatus*) (Khani & Asghari 2012). Furthermore, the essential oil had shown acaridical effects towards the two spotted spider mite (*Tetranychus urticae*) (Motazedian *et al*. 2012). The aerial parts ethanolic extracts of *M. longifolia* were reported to possess larvicidal activities against mosquitoes of *Culex pipiens* in Turkey (Cetin *et al*. 2006). *M. longifolia* was selected because there has been no report on the insecticidal activities of *M. longifolia* extracts for *Culex* mosquito control in South Africa.

CHAPTER 4

MATERIALS AND METHODS

4.1. Plant collection and identification

The plants were collected from multiply population groups around the eastern Free State Province during their growing seasonal period (February to May 2013). Some of the plants occur and grow in local communal illegal dumping sites, unwanted agricultural lands, gardens, road sides and mostly on disturbed areas whereby they do not depend on human intervention for their reproduction and survival. The selected plants are represented by *Artemisia absinthium* (Asteraceae), *Artemisia afra* (Asteraceae), *Cosmos bipinnatus* (Asteraceae), *Tagetes minuta* (Asteraceae), *Foeniculum vulgare* (Apiaceae), and lastly *Mentha longifolia* (Lamiaceae). The main selection criteria for these Basotho plants included their aromatic properties, traditional uses as insecticides, easily accessible and plant relatively not infested by insect pests in their natural habitat.

The collected plants were taxonomically identified and authenticated by Dr. AOT Ashafa of the Plant Science Department at the University of the Free State (UFS) Qwaqwa campus, while further identification was done by Dr. CJ Potgieter at Bews Herbarium at Pietermaritzburg campus of the University of KwaZulu-Natal. The voucher specimens, with reference numbers *Artemisia afra* (ModMed.2013/1), *Artemisia absinthium* (ModMed.2013/2), *Cosmos bipinnatus* (ModMed.2013/3), *Tagetes minuta* (ModMed.2013/4), *Foeniculum vulgare* (ModMed.2013/5), and *Mentha longifolia* (ModMed.2013/6), were prepared and deposited at the UFS Qwaqwa campus herbarium accordingly. The photographs of plant species during their matured flowering developmental stages were taken, to assist during identification processes and be kept as digital referencing at the UFS Qwaqwa campus herbarium.

4.2. Plant preparation: Dried powder or crude material

The fresh plant leaves were separated, rinsed under running water to remove foliage debris and then dried for 7 days at room temperature as well as using an Ecotherm oven (Laboratory Consumables Pty, RSA) at a temperature of 40°C until a constant weight was reached. The dried leaf material was crushed, ground into fine or coarse powder using an electric blender (Nanning Mainline Food Machinery Company Ltd, China) (Figure 4.1). The powder material was kept in a refrigerator at 4°C in an air tight container until extraction.

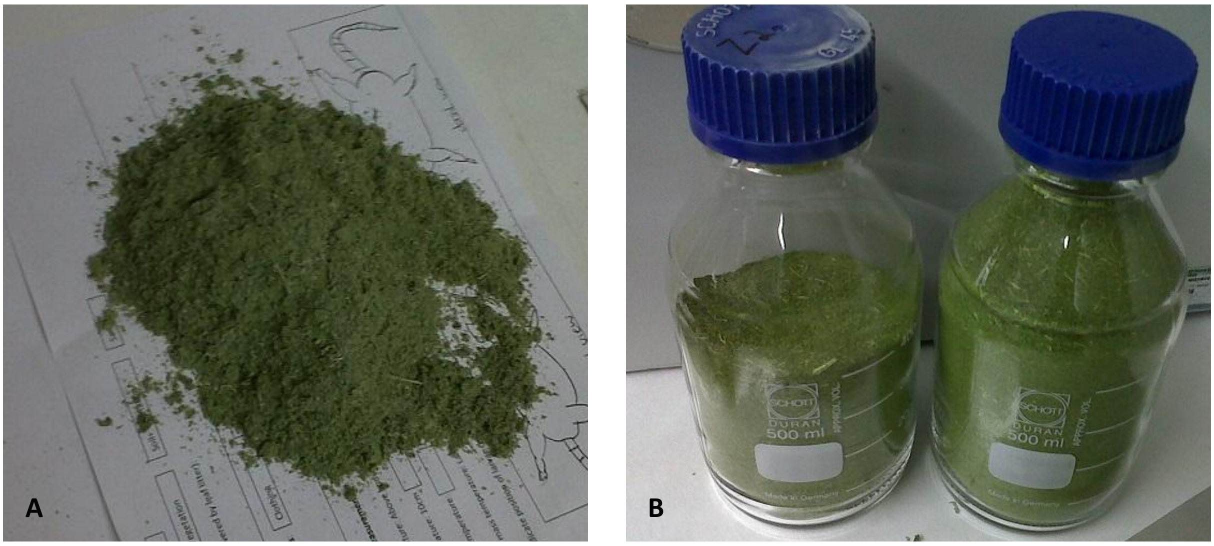

Figure 4.1 Preparation of *Artemisia afra*, (A) powdered dried plant leaves material, and (B) is stored in an air tight glass jar.

4.3. Plant secondary metabolites extraction

Five grams of each dried powered plant materials were dissolved in 150 ml double distilled water, ethanol (95%; v/v) and hexane in a 500 ml flask. The plant mixtures were placed on an electric shaker (U-TER Technology Co., Ltd, China) at 115 rpm revolutions for 24 hours. The extraction mixtures were filtered using Whatman No-1 filter paper (Whatman, UK) to obtain a homogenous solution. The double distilled water filtrates were placed in a water bath at 45°C until totally dried, while ethanol extracts were concentrated on a rotatory evaporator (Laboratory Consumables Pty, RSA) (at 47°C water bath, 6 rpm revolutions) until the solvent totally evaporated. The hexane extracts were exposed to open air for 12 hours until dried in a fume cupboard. The yield percentages of each extract were calculate by dividing dried mass with original plant powered mass.

$$Yield\ (\%) = \frac{Dried\ mass\ (\%)}{Original\ powdered\ mass\ (\%)}\ X\ 100\ (\%)$$

The dried plant extracts were stored in a refrigerator at 4°C until further use. The above stated plant extract preparations were repeated for all tested medicinal plants with the organic solvents respectively. The stored extracts were dissolved in specific volume of double distilled water to prepare a crude solution as calculated by Solis *et al.* 1993:

$$Volume\ (ml)\ = (Beaker\ mass\ after - Beaker\ mass\ before) \times\ (20ml)$$

4.4. Secondary metabolites screening

The qualitative phytochemical screening was done using powdered material and solvent plant extracts according to standard methods described by Edeoga *et al*. (2005).

4.4.1. Test for tannins

The dried powdered (0.5 g: w/w) samples were boiled in double distilled water (20 ml: v/v) and then filtered (using Whatman No-1 filter paper) into another clean test tube. A few drops of ferric chloride (0.1 %: w/v) was added into the solution and observed for brownish green or blue-black colour change for presence of tannins.

4.4.2. Test for phlobatannis

The aqueous (double distilled water) extracts were prepared by boiling dried powdered (0.5 g: w/w) in double distilled water (20 ml: v/v) and filtered (using Whatman No-1 filter paper) into a clean test tube. The aqueous extracts were thereafter boiled with few drops of aqueous hydrochloric acid (1 %: v/v) and observed for the formation of a red precipitate indicating the presence of phlabotannins.

4.4.3. Test for alkaloids

The aqueous (double distilled water) extracts were prepared by boiling dried powdered (0.5 g: w/w) in double distilled water (20 ml: v/v) and filtered (using Whatman No-1 filter paper) into a clean test tube. 3 ml of aqueous extract was stirred with 3 ml of 1% Hydrochloric acid (1%: /v/v) on a steam bath. Mayer’s and Wagner’s reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

4.4.4. Test for saponin

The aqueous extracts were prepared by boiling dried powdered samples (2 g: w/w) inside a water bath and then filtered (using Whatman No-1 filter paper) into a clean test tube. Transferred filtrate (10 ml: v/v) into double distilled water (5 ml: v/v) and shaken vigorously to mix for a stable determined foam. The foaming solutions were further mixed by pipetting with 3 drops of olive oil and then closed test tube while shaken vigorously while observing formation of emulsion for presence of saponin.

4.4.5. Test for flavonoids

A portion of the dried powdered plant samples were mixed with ethyl acetate (10 ml: v/v) and heated over a steam bath for 3 minutes, and filtered using Whatman No-1 filter paper (Whatman, UK). The filtrates (4 ml: v/v) were mixed with ammonia solution (1 ml: v/v) and shaken to observe a yellow colour change indicating presence of flavonoids.

4.4.6. Test for steroids

The ethanolic extracts were prepared by adding ethanol (10 ml: 95 %) into dried plant powder (0.5 g: v/v) and filtered (using Whatman No-1 filter paper). Further added acetic anhydride (2 ml: v/v) into ethanolic extract (5 ml: v/v), then later added sulphuric acid (2 ml: v/v). Then, observed a violet blue or green colour change for the presence of steroids.

4.4.7. Test for terpeniods using the Salkowski test

The solvent extracts (5 ml: v/v) of each plants were mixed with chloroform (2 ml: v/v) into separate test tubes, and later added concentrated sulphuric acid (3 ml: v/v) carefully. The presence of terpenoids is denoted by the formation of two layers and a reddish brown coloured interface layer.

4.4.8. Test for cardiac glycosides using the Keller-Killani test

The solvent extracts (5 ml: v/v) of each plants were treated with glacial acetic (2 m: v/v) acid into separate test tubes containing one drop of ferric chloride solution. Concentrated sulphuric acid (1 ml: v/v) was cautiously added to underlay the solutions. The observed a brownish and green ring colour change indicates the presence of cardiac glycosides.

4.5. Cytotoxicity test: Brine shrimp lethality bioassay

The cytotoxicity tests were conducted using brine shrimp lethality assay as described by Onocha *et al*. (2011) with modifications. The brine shrimp eggs (*Artemia salina*, Joe's Aquarium™, Taiwan) were incubated for about 48 to 72 hours under light to hatch in sea water into brine shrimp nauplii at standard room temperature (24-25°C).

The brine shrimp lethality test was investigated with five plants namely *Artemisia afra*, *Artemisia absinthium*, *Cosmos bipinnatus*, *Tagetes minuta*, and *Foeniculum vulgare*. The stock solution of 1 mg/ml of each extract was used to prepare graded concentrations (0.5, 0.4, 0.3, 0.2, 0.1 mg/ml) respectively in separate vials. Ten brine shrimps nauplii were placed in each vial and incubated under light for 24 h. The control experiment had only 5 ml sea water and 10 nauplii. The shrimp is declared dead when it lies flat at the bottom of the beaker without motion, does not respond to any stimuli, or did not reach the surface of the solution after 20 s of observation. The number of dead individual was counted and recorded for analysis.

4.6. Culicidae mosquito mortality tests

4.6.1 Mosquitoes

The field mosquitoes larvae were collected from Kroonstad in the Free State Province of South Africa (Figure 4.2), at site A near Snake Park location (27°38'27.30" S, and 27°11'20.40" E with latitude 1372 m), and site B near old grave yard (27°39'36.30" S, and 27°10'18.90" E with latitude 1338 m) (Figure 4.3). The collected samples were then sorted into developmental life stages of larval 1st to 4th instar level into labelled white trays. The larvae were identified using larval identification keys as described by Snell (2005) and, later confirmed with morphological characters of adult female mosquitoes using identification keys as described by Harbach (2007) and Snell (2005). The museum voucher specimen was prepared with fourth instar larvae of UFSCulex01.Mod, at the UFS Qwaqwa campus Zoology and Entomology Museum.

According to World Health Organisation (WHO) (1981), the accredited larval developmental level for any insecticidal bioassays and other related treatments is the fourth instar larvae. The other earlier larval developmental levels such as instar 1-3 were held at about temperature of 27 ±2°C, humidity of 70 ±1% and a photoperiod regime of 14:10 hours (light/dark). To maintain the larval colonies, the aquatic larvae were reared in dechlorinated water and fed yeast solution.

Figure 4.2 The map of South Africa showing the collection site for *Culex* mosquito larvae in the Free State Province of South Africa. Panel A illustrates the Free State Province highlighted in red, while B illustrates Kroonstad town highlighted were *Culex* larvae was collected from. (http://www.southafrica-travel.net/samaps/fs_navigator.html).

Figure 4.3 Active collection of field *Culex quinquefasciatus* larvae at site A near Snake Park location (27°38'27.30" S, and 27°11'20.40" E with latitude 1372 m) from standing sewage water leakage at Kroonstad Free State Province.

4.6.2. Larvicidal bioassays

The larvicidal bioassays were evaluated with only four plants namely *Artemisia afra*, *Cosmos bipinnatus*, *Tagetes minuta*, and *Mentha longifolia* due to limited mosquito larvae. The larvicidal activities on all experimental plant extracts were tested on fourth instars larvae as recommended by the WHO (1981), but with some modification. The graded extract concentrations were prepared as 0.1, 0.5, 1, 2, 5, 10 mg/ml into separate McCartney (20 ml) glass vials (Figure 4.3). Ten fourth instar larvae were separately pipetted into each graded concentration tested. The control experiments were conducted using only 5 ml of double distilled water without plant extracts. The steps were repeated for every tested plant species for different extraction type. The experimental treatments and control were kept under the same conditions that were used to maintain *Culex* mosquito larval colonies as described in section 4.6.1. The larval mortalities were counted and recorded at 12 hours and 24 hours for analysis. Larvae was declared dead when it lied flat at bottom of vial without motion, does not respond to any stimuli, or did not reach the surface of the solution.

Figure 4.4 The larvicidal and pupicidal bioassays experimental setup with graded extract concentrations for *Culex* mosquito.

4.6.3. Pupicidal bioassays

The pupicidal bioassays were investigated for only two plants namely *Artemisia absinthium*, and *Foeniculum vulgare* due to limited pupa. The pupicidal activities on all experimental plant extracts were tested on a day old pupae as recommended by the WHO (1981), and with some modification. The graded extract concentrations were prepared as 0.1, 0.5, 1, 2, 5, 10 mg/ml into separate McCartney (20 ml) glass vials. Ten pupa were placed separately into each graded concentration tested solution. The control experiments were conducted using only 5 ml of double distilled water without plant extracts. The steps were repeated for every other plant extracts. The experimental treatments and control were kept under same conditions that are used to maintain *Culex* mosquito larval colonies as described in section 4.6.1. The pupal mortalities were counted and recorded at 12 hours and 24 hours for analysis. Pupa was declared dead when it lies flat at the bottom of vial without motion, does not respond to any stimuli, or did not reach the surface of the solution.

4.6.4. Insecticidal bioassays

The insecticidal bioassay was investigated for *Artemisia afra*, *Artemisia absinthium*, *Cosmos bipinnatus, Tagetes minuta*, *Foeniculum vulgare* and *Mentha longifolia.* The insecticidal bioassays were conducted by preparing graded masses as 0.25, 0.5, 1, 1.5, 2 g of dried powdered leaf material. A Whatman no. 1 filter paper was cut into a circle of 25 mm in diameter, and placed under plastic vials cap to fit. The different masses of the powdered plant were poured onto the filter paper and covered with perforated vial in an inverted position (Figure 4.4). Ten two day old adult *Culex* mosquitoes were incubated inside the closed plastic vial. The steps were repeated for every tested plant species for separate extraction type. The results were recorded at 6, 12, 18 and 24 hours for analysis.

Figure 4.5 The insecticidal bioassay experiment on graded plant powdered material for adult *Culex* mosquito.

4.7. Data analysis

The concentration mortality data for larvicidal, pupicidal and insecticidal bioassays were determined using Probit analysis as described by Finney (1952) to determine the mean lethal concentration (LC_{50}) for tested solutions, mean lethal dose (LD_{50}) for powdered dosage weighed treatments, and mean knock-down (KD_{50}) for knock-down effect of plant powdered material on adult mosquitoes. The Probit analysis calculates the effective mean lethal concentration (solution) or dose of tested insecticidal plant extract, and subjects the values to an insect response curve against various concentrations (relation between extract concentration and insect response). The statistical significant difference of $LC_{50,}$ LD_{50} and KD_{50} values exhibited by plant extracts at different concentration was calculated using repetitive-measure of analysis of variance ANOVA test and Kruskal-Wallis test (Cohen *et al*. 1998; Clewer & Scarisbricks 2001).

CHAPTER 5

RESULTS

5.1. Crude extract percentage yield

The aqueous plant extracts yielded much more dried mass percentage than ethanolic and hexane crude extracts. There was relatively higher extract yield in *Foeniculum vulgare*, *Artemisia afra*, *Artemisia absinthium,* and *Mentha longifolia,* than in *Cosmos bipinnatus* and *Tagetes minuta* (Table 5.1).

Table 5.1 The percentage yield of aqueous, ethanolic and hexane leaf extracts of *A. absinthium, A. afra*, *C. bipinnatus, F. vulgare, M. longifolia* and *T. minuta* from eastern Free State of South Africa.

	% Yield		
Plant Species	**Hexane**	**EtOH**	**Dd H_2O**
Artemisia absinthium	8.2	10.7	18.1
Artemisia afra	6.2	15.4	22.6
*Cosmos bipinnatu*s	1.2	7.0	27.0
Foeniculum vulgare	6.0	11.0	23.1
*Mentha longifoli*a	3.2	7.8	24.2
Tegetes minuta	2.4	7.4	13.6

EtOH- ethanol, Dd H_2O- double distilled water

5.2. Phytochemical screening analysis

Table 5.2 shows the phytochemical constituents detected in the selected medicinal plants under evaluation. Saponins were present in all plants leaf extracts. *A. afra*, *C. bipinnatus* and *T. minuta* revealed presence of terpenoids in all extracts. Alkaloids, flavonoids and steroids were detected mostly in the aqueous and ethanolic extracts than in hexane extracts of the evaluated plants. Cardiac glycosides were not present in all plants while phlobatannis were detected only in the ethanolic extract of *T. minuta* leaves.

Table 5.2 The secondary metabolite constituents in double distilled water, ethanolic and hexane extracts of Basotho medicinal plants collected in Qwaqwa region eastern Free State Province.

Plant Species	**Solvents**	**Alkaloids**	**Tannins**	**Phlobatannins**	**Saponins**	**Flavonoids**	**Steroids**	**Terpenoids**	**Cardiac glycosides**
	ddH_2O	+	-	-	+	+	+	+	-
A. afra	EtOH	-	-	-	+	+	+	+	-
	Hexane	-	-	-	+	-	-	+	-
	ddH_2O	-	+	-	+	+	+	+	-
C. bipinnatus	EtOH	-	-	-	+	+	+	+	-
	Hexane	+	-	-	+	-	-	+	-
	ddH_2O	+	-	-	+	+	-	+	-
T. minuta	EtOH	+	-	+	+	+	+	+	-
	Hexane	+	-	-	+	-	-	+	-
	ddH_2O	+	+	-	+	+	+	+	-
A. absinthium	EtOH	-	-	-	+	-	-	-	-
	Hexane	+	-	-	+	-	-	-	-
	ddH_2O	-	+	-	+	+	+	+	-
F. vulgare	EtOH	+	+	-	+	+	-	-	-
	Hexane	+	-	-	+	-	-	-	-
	ddH_2O	+	+	-	+	+	+	+	-
M. longifolia	EtOH	-	+	-	+	-	+	-	-
	Hexane	-	-	-	+	-	-	-	-

EtOH- ethanol, Dd H_2O- double distilled water; + present; - absent

5.3. Cytotoxicity bioassay analysis

The ethanolic and aqueous extracts had relatively higher cytotoxic effects, whereas the hexane extracts had lower cytotoxic effects on the *Artemia salina* nauplii as shown in Table 5.3. killing all *A. salina* nauplii at all investigated concentration levels. Other ethanolic extracts that showed effects included *Artemisia absinthium* (LC_{50} = 2.89 mg/ml) and *Cosmos bipinnatus* (LC_{50} = 5.66 mg/ml). Most of the aqueous extracts also exhibited cytotoxic effects in *T. minuta* (LC_{50} = 3.16 mg/ml), *Cosmos bipinnatus* (LC_{50} = 4.81 mg/ml), *Artemisia afra* (LC_{50} = 5.39 mg/ml), *Foeniculum vulgare* (LC_{50} = 7.30 mg/ml), and *Artemisia absinthium* (LC_{50} = 7.43 mg/ml). The hexane extracts had cytotoxic effects only for *A. absinthium* (LC_{50} = 15.75 mg/ml) and *Foeniculum vulgare* (LC_{50} = 21.25 mg/ml), but no lethal bioactivity were observed for *A. afra*, *C. bipinnatus* and *T. minuta* towards brine shrimp nauplii.

There was significant difference observed between percentage mortality of tested Basotho plants, with ethanolic extracts ($F_{5,5}$ = 13.69; P < 0.01), hexane extracts ($F_{5,5}$ = 4.02; P < 0.05), while aqueous extracts showed no significant difference in larval killing. Again, the leaf extract concentrations showed significant difference for aqueous extracts ($F_{5,5}$ = 9.80; P < 0.01), ethanolic extract ($F_{5,4}$ = 5.13; P < 0.05) and whereas the hexane extract concentrations had no significant difference.

There was strong positive correlation between extracts concentration and rate of mortality, which included aqueous extracts for *A. afra* (R^2 = 0.80) (Figure 5.1) and ethanolic extracts for *A. absinthium* (R^2 = 0.83) (Figure 5.2), while hexane extracts had a very weak correlation for *A. absintium* (R^2 = 0.16) and *F. vulgare* (R^2 = 0.20) (Figure 5.3).

Table 5.3. The cytotoxic effects of different leaf extracts from Basotho medicinal plants on brine shrimp (*Artemia salina*) nauplii.

		% Mortality											
		A. absinthium			***A. afra***			***C. bipinnatus***			***F. vulgare***		
Conc. [mg/ml]	**Init.nmb.**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**
0.10	10.00	0.00	0.00	20.00	0.00	10.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00
0.20	10.00	10.00	80.00	0.00	0.00	0.00	0.00	10.00	20.00	0.00	10.00	10.00	40.00
0.30	10.00	10.00	80.00	0.00	0.00	0.00	0.00	0.00	20.00	0.00	20.00	0.00	0.00
0.40	10.00	20.00	70.00	20.00	20.00	0.00	0.00	20.00	10.00	0.00	30.00	30.00	0.00
0.50	10.00	40.00	90.00	40.00	30.00	20.00	0.00	30.00	0.00	0.00	30.00	40.00	50.00
1.00	10.00	30.00	100.00	60.00	100.00	100.00	0.00	100.00	100.00	0.00	30.00	80.00	20.00
LC_{50}		7.43	2.89	15.75	5.39	8.15	0.00	4.81	5.67	0.00	7.30	7.01	21.25

conc.-concentration; H_2O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LD_{50}- mean lethal concentration

Table 5.3. The cytotoxic effects of different leaf extracts from Basotho medicinal plants on brine shrimp (*Artemia salina*) nauplii, continued.

		% Mortality					
		M. longifolia			***T. minuta***		
Conc. [mg/ml]	**Init.nmb.**	**ddH$_2$O**	**EtOH**	**Hexane**	**ddH$_2$O**	**EtOH**	**Hexane**
0.10	10.00	0.00	0.00	10.00	20.00	100.00	0.00
0.20	10.00	0.00	10.00	0.00	40.00	100.00	0.00
0.30	10.00	10.00	0.00	0.00	30.00	100.00	0.00
0.40	10.00	0.00	30.00	20.00	20.00	100.00	0.00
0.50	10.00	0.00	20.00	40.00	10.00	100.00	0.00
1.00	10.00	40.00	10.00	40.00	100.00	100.00	0.00
LC$_{50}$		16.55	13.71	18.19	3.16	0.10	0.00

conc.-concentration; H$_2$O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LD$_{50}$- mean lethal concentration

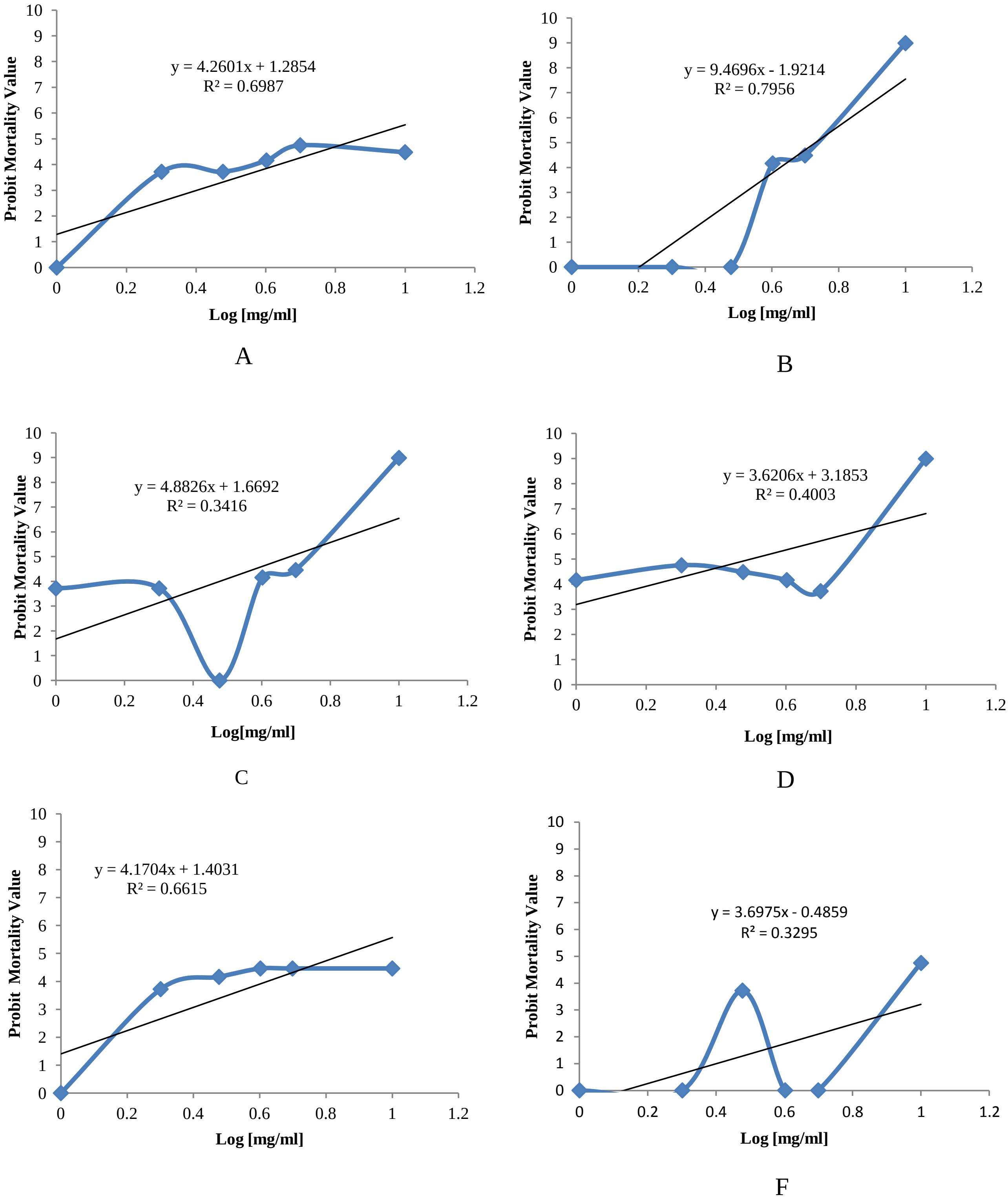

Figure 5.1 The cytotoxicity effects of aqueous extracts from Basotho medicinal plants, panel A) *Artemisia absinthium*, B) *A. afra*, C) *Cosmos bipinnatus*, D) *Tagetes minuta*, E) *Foeniculum vulgare* and F) *M. longifolia* on brine shrimp (*Artemia salina*) nauplii.

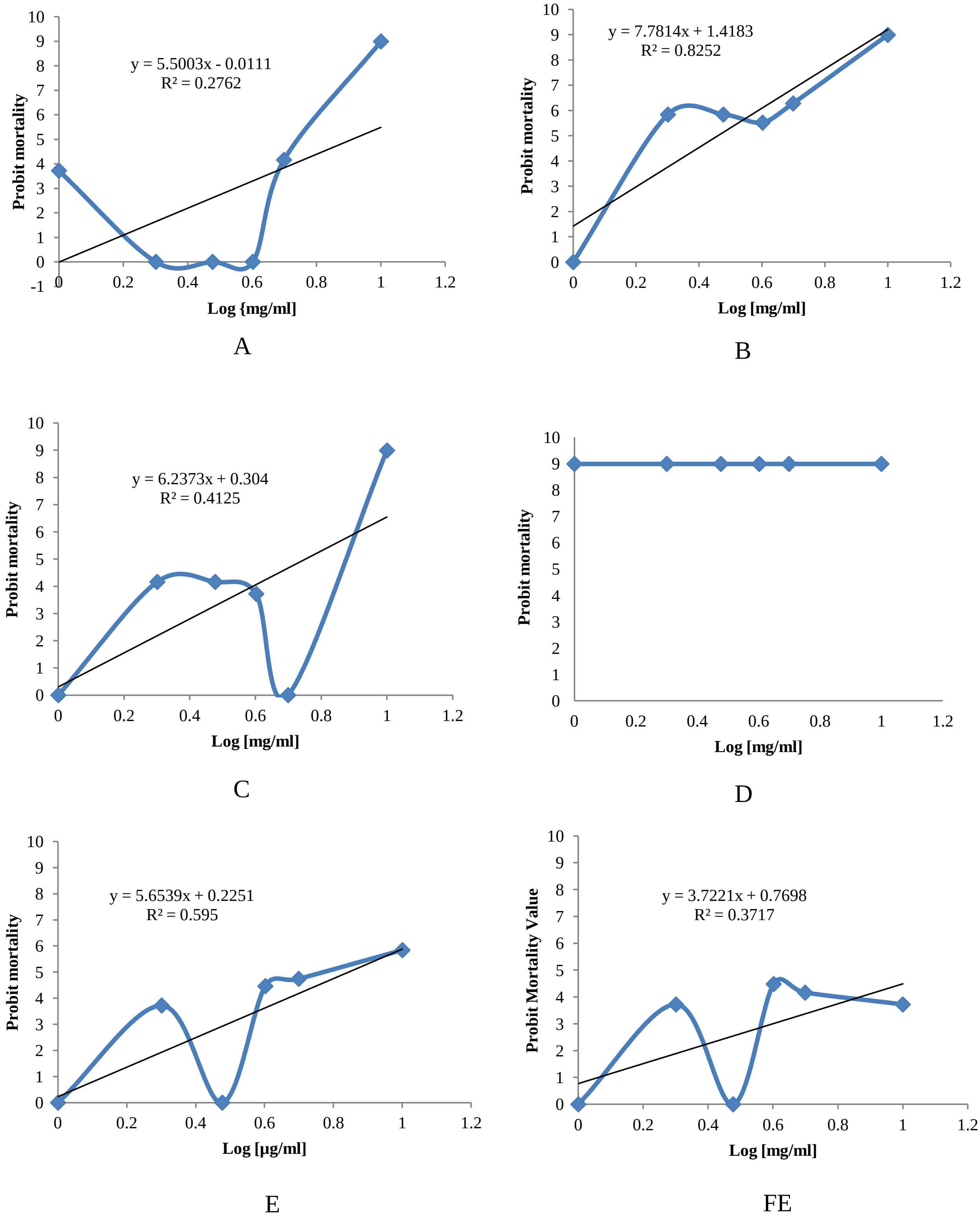

Figure 5.2 The cytotoxicity effects of ethanol extracts from Basotho medicinal plants , panel A) *Artemisia absinthium*, B) *A. afra*, C) *Cosmos bipinnatus*, D) *Tagetes minuta*, E) *Foeniculum vulgare* and F) *M. longifolia* on brine shrimp (*Artemia salina*) nauplii.

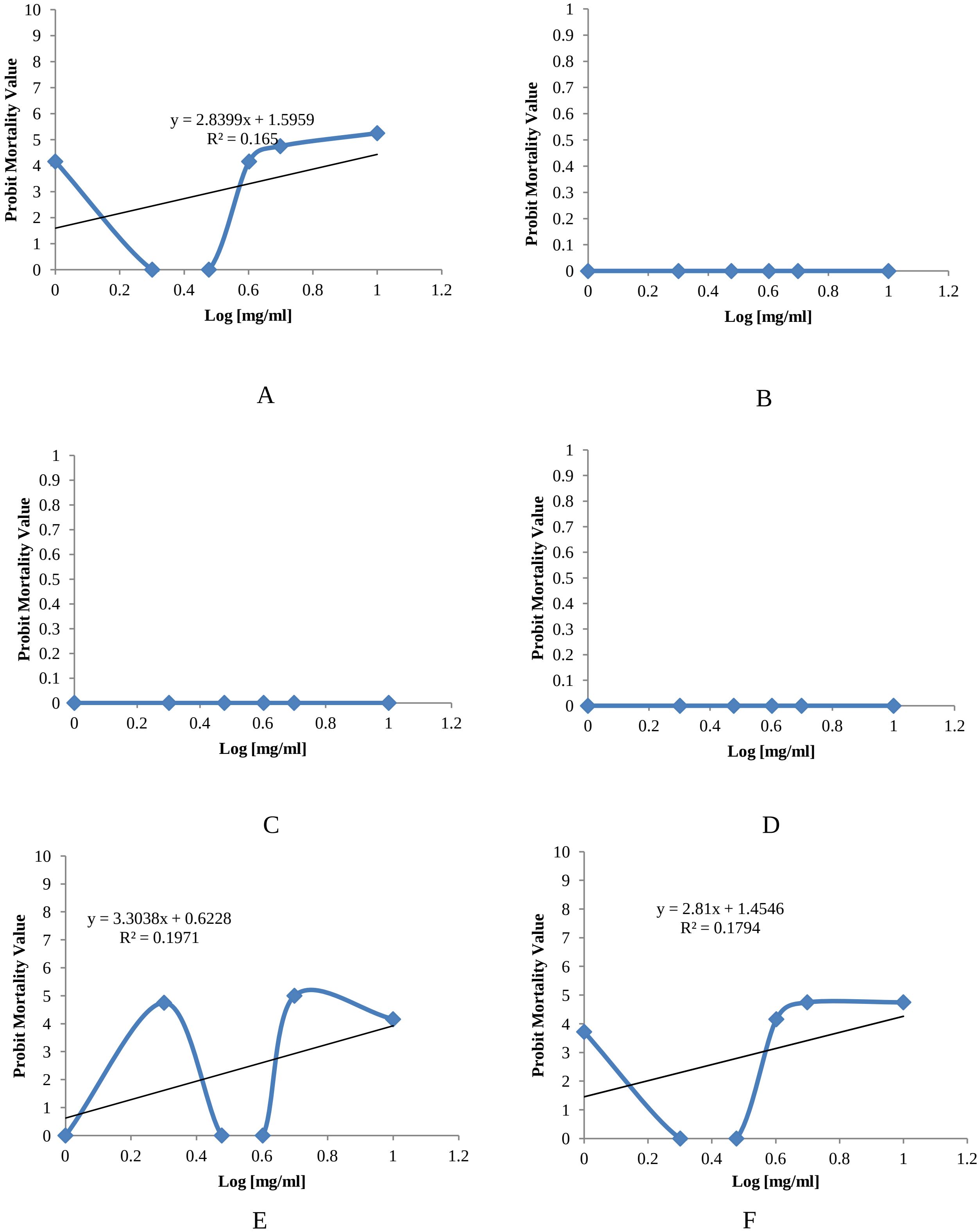

Figure 5.3 The cytotoxicity effects of hexane extracts from Basotho medicinal plants, panel A) *Artemisia absinthium*, B) *A. afra*, C) *Cosmos bipinnatus*, D) *Tagetes minuta*, E) *Foeniculum vulgare* and F) *M. longifolia* on brine shrimp (*Artemia salina*) nauplii.

Mosquito (*Culex quinquefasciatus*) bioassays

5.4. Larvicidal bioassay analysis

The bioactivity of hexane and ethanolic extracts of tested Basotho medicinal plants displayed larvivicidal activity towards *C. quinquefasciatus* larvae after 24 hours of observation (Table 5.4). The *M. longifolia* hexane extracts killed all tested larvae in all evaluated concentration levels (LC_{50} = 0.10 mg/ml), whereas *T. minuta* (LC_{50} = 1.01 mg/ml), *F. vulgare* (LC_{50} = 1.03 mg/ml), *A. afra* (LC_{50} = 1.14 mg/ml), *C. bipinnatus* (LC_{50} = 1.27 mg/ml) and A. absinthium (LC_{50} = 4.93 mg/ml), also displayed larvicidal activity. The ethanolic extract that killed all tested larvae in the evaluated concentration levels was *F.vulgare* with LC_{50} = 0.10 mg/ml, while *A. afra* exhibited larvicidal activity with LC_{50} = 1.02 mg/ml, *M. longifolia* with LC_{50} = 1.05 mg/ml, *T. minuta* with LC_{50} = 1.17 mg/ml, *C. bipinnatus* with LC_{50} = 1.18 mg/ml, and *A. absinthium* with LC_{50} = 1.55 mg/ml respectively. The aqueous extracts of all tested medicinal plants had no fatal effects on the larvae after 24 hours of observation (Figure 5.4).

There was strong positive correlation between concentrations (dose-response) of Basotho plants and mortality rates of mosquito larvae, with ethanolic extracts of *C. bipinnatus* (R^2 = 0.77), *A. afra* (R^2 = 0.77), *T. minuta* (R^2 = 0.75) as well as for *M. longifolia* (R^2 = 0.73) (Figure 5.5), however, hexane extracts of *C. bipinnatus* (R^2 = 0.94), *F. vulgare* (R^2 = 0.84) and *A. afra* (R^2 = 0.81) showed stronger correlation (Figure 5.6).

The graded concentrations and larvae mortality activity showed significant difference between evaluated medicinal plants. The graded concentration had significant difference between Basotho plant ethanolic extracts at $F_{5,5}$ = 9.95 (P < 0. 05), and hexane extracts at $F_{5,5}$ = 4.70 (P < 0.05) . Also, there was observed significant difference among investigated mortality activities but only for hexane extracts at $F_{5,5}$ = 4.38 (P < 0.05) and there was no significant difference between ethanolic extracts of Basotho plants ($F_{5,5}$ = 1.91; P < 0.05).

Table 5.4 The larvicidal effects of different leaf extracts of Basotho medicinal plants against *Culex quinquefasciatus* larvae.

		% Mortality											
		A. absinthium			***A. afra***			***C. bipinnatus***			***F. vulgare***		
Conc. [mg/ml]	**Ini.nmb.**	**ddH$_2$O**	**EtOH**	**Hexane**	**ddH$_2$O**	**EtOH**	**Hexane**	**ddH$_2$O**	**EtOH**	**Hexane**	**ddH$_2$O**	**EtOH**	**Hexane**
0.10	10.00	0.00	90.00	100.00	0.00	20.00	10.00	0.00	0.00	0.00	0.00	100.00	50.00
0.50	10.00	0.00	100.00	90.00	0.00	80.00	10.00	0.00	40.00	10.00	0.00	100.00	80.00
1.00	10.00	0.00	100.00	100.00	0.00	100.00	90.00	0.00	100.00	10.00	0.00	100.00	90.00
2.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	50.00	0.00	100.00	100.00
5.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00
10.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00
LC$_{50}$		0.00	1.55	4.93	0.00	1.02	1.14	0.00	1.18	1.27	0.00	0.10	1.03

conc.-concentration; ddH$_2$O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LC$_{50}$- mean lethal concentration

Table 5.4 The larvicidal effects of different leaf extracts of Basotho medicinal plants against *Culex quinquefasciatus* larvae, continued.

		% Mortality					
		M. longifolia			***T. minuta***		
Conc. [mg/ml]	**Ini. nmb.**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**
0.10	10.00	0.00	20.00	100.00	0.00	0.00	20.00
0.50	10.00	0.00	50.00	100.00	0.00	80.00	100.00
1.00	10.00	0.00	100.00	100.00	0.00	100.00	60.00
2.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00
5.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00
10.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00
LC_{50}		0.00	1.04	0.10	0.00	1.17	1.01

conc.-concentration; ddH_2O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LC_{50}- mean lethal concentration

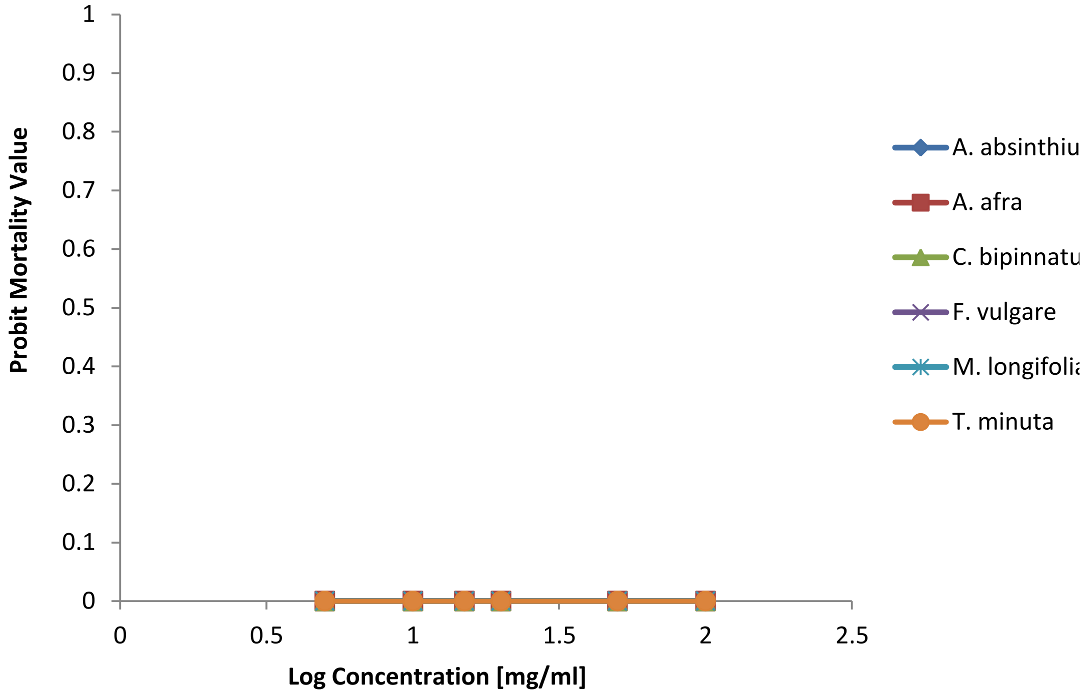

Figure 5.4 The dose-response effects on larvae (*Culex quinquefasciatus*) by aqueous extracts of Basotho medicinal plants.

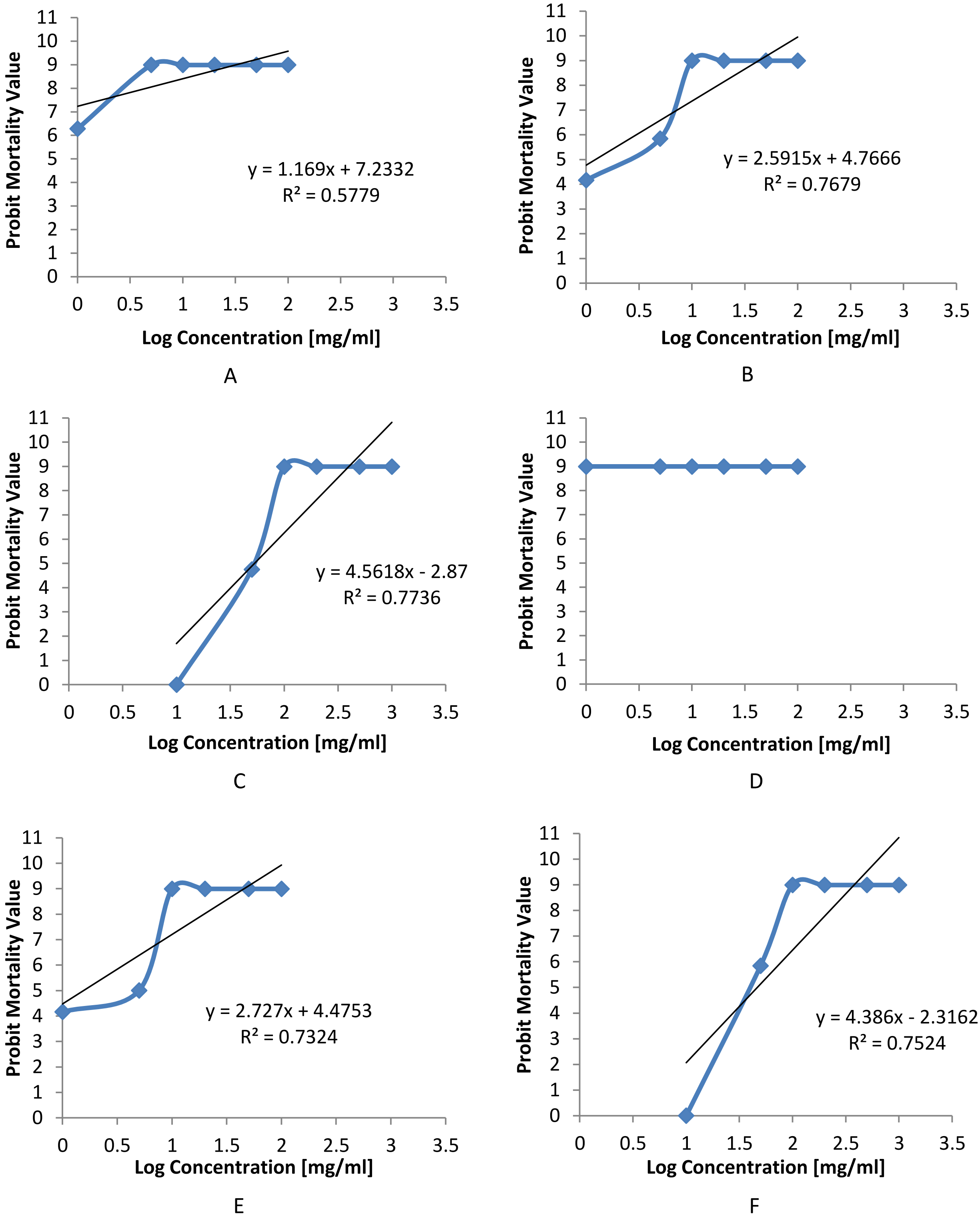

Figure 5.5 The dose-response effects on larvae (*Culex quinquefasciatus*) by ethanolic extracts of Basotho medicinal plants; panel A) *Artemisia absinthium*, B) *Artemisia afra*, C) *Cosmos bipinnatus*, D) *Foeniculum vulgare*, E) *Tagetes minuta* and F) *Mentha longifolia*.

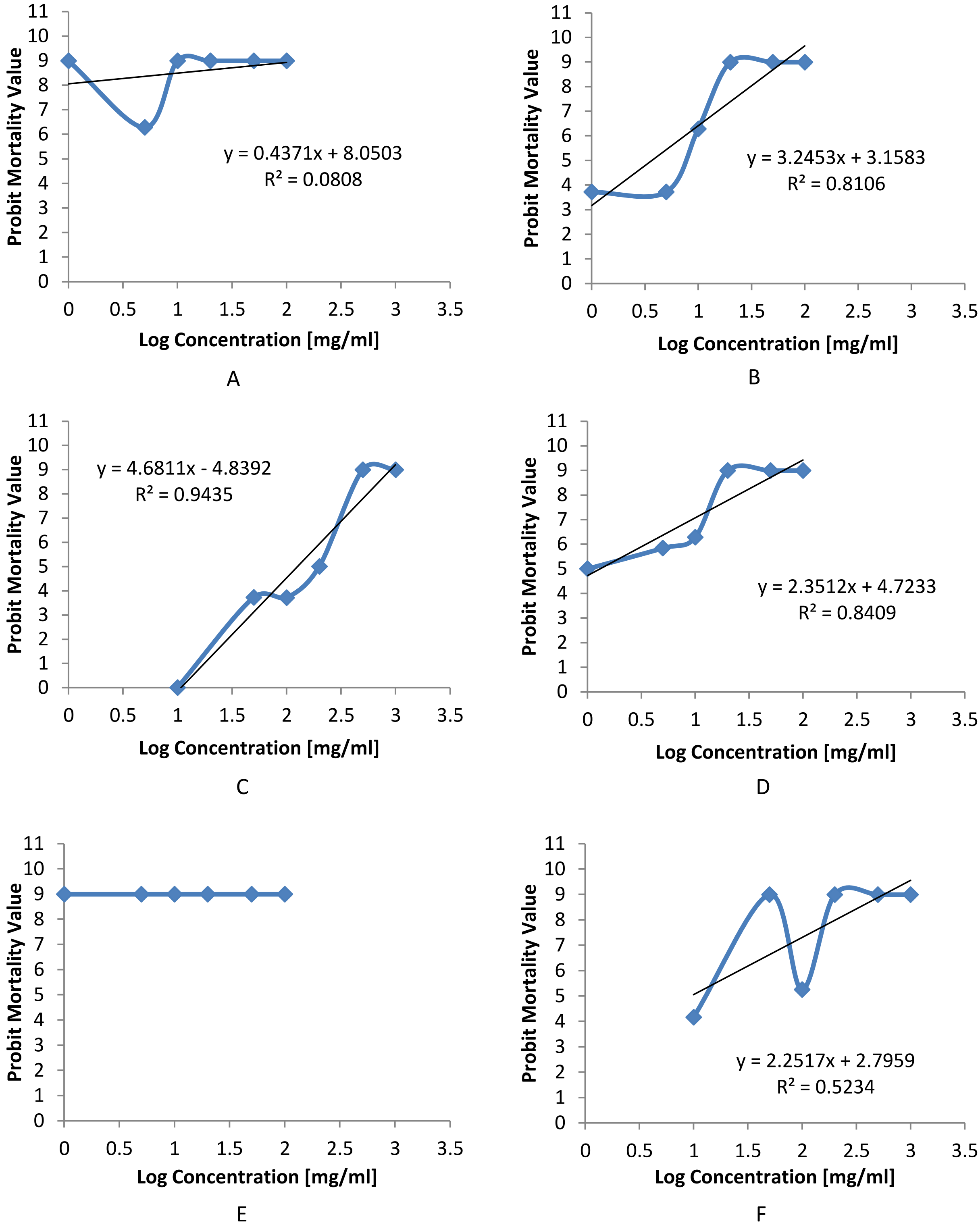

Figure 5.6 The dose-response effects on larvae (*Culex quinquefasciatus*) by hexane extracts of Basotho medicinal plants; panel A) *Artemisia absinthium*, B) *Artemisia afra*, C) *Cosmos bipinnatus*, D) *Foeniculum vulgare*, E) *Tagetes minuta* and F) *Mentha longifolia.*

5.5. Pupicidal bioassay analysis

Table 5.5 indicates pupicidal activity of hexane and ethanolic extracts of tested Basotho medicinal plants against pupa of *C. quinquefasciatus* after 24 hours of observation. The ethanolic extracts displayed pupicidal activity for *A. afra* with LC_{50} = 1.10 mg/ml, *T. minuta* with LC_{50} = 1.11 mg/ml, *M. longifolia* with LC_{50} = 1.13 mg/ml, *C. bipinnatus* with LC_{50} = 1.14 mg/ml, *A. absinthium* with LC_{50} = 1.17 mg/ml, *C. bipinnatus* with LC_{50} = 1.18 mg/ml, and *F. vulgare* with LC_{50} = 1.31 mg/ml respectively. The hexane extracts exhibited pupicidal activity for *A. afra* (LC_{50} = 1.04 mg/ml), *F. vulgare* (LC_{50} = 1.07 mg/ml), *T. minuta* (LC_{50} = 1.12 mg/ml), *C. bipinnatus* (LC_{50} = 1.16 mg/ml), *M. longifolia* (LC_{50} = 1.21 mg/ml) and *A. absinthium* (LC_{50} = 1.29 mg/ml) respectively. The aqueous extracts of all tested medicinal plants had no fatal effects on the larvae after 24 hours of observation (Figure 5.7).

There was strong positive correlation between concentrations (dose-response) of Basotho plants and mortality rates of mosquito larvae, with ethanolic extracts of *M. longifolia* (R^2 = 0.85), *A. absinthium* (R^2 = 0.76), as well as for *A. afra* (R^2 = 0.74) (Figure 5.8). Hexane extracts displayed positive correlation of *A. afra* (R^2 = 0.82), *C. bipinnatus* (R^2 = 0.56) and *F. vulgare* (R^2 = 0.53) (Figure 5.9).

The graded concentration had significant difference between Basotho plant ethanolic extracts at $F_{5,5}$ = 12.28 (P < 0. 01), and hexane extracts at $F_{5,5}$ = 5.90 (P < 0.01). However, there was no significant difference observed among investigated mortality activities of ethanolic extracts ($F_{5,5}$ = 2.59 P < 0. 05) and hexane extracts ($F_{5,5}$ = 1.79; P < 0.05) of Basotho plants.

Table 5.5 The pupicidal (*Culex quinquefasciatus*) effects of different leaf extracts of Basotho medicinal plants from Qwaqwa region in the eastern Free State.

		% Mortality											
		A. absinthium			***A. afra***			***C. bipinnatus***			***F. vulgare***		
Conc. [mg/ml]	**Ini. nmb.**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**	**ddH_2O**	**EtOH**	**Hexane**
0.10	10.00	0.00	0.00	20.00	0.00	40.00	40.00	0.00	20.00	50.00	0.00	70.00	40.00
0.50	10.00	0.00	70.00	0.00	0.00	20.00	90.00	0.00	20.00	10.00	0.00	100.00	100.00
1.00	10.00	0.00	100.00	40.00	0.00	80.00	80.00	0.00	30.00	60.00	0.00	100.00	80.00
2.00	10.00	0.00	100.00	90.00	0.00	100.00	100.00	0.00	100.00	90.00	0.00	100.00	100.00
5.00	10.00	0.00	100.00	90.00	0.00	100.00	100.00	0.00	100.00	80.00	0.00	100.00	100.00
10.00	10.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00
LC_{50}		0.00	1.17	1.29	0.00	1.10	1.04	0.00	1.14	1.16	0.00	1.31	1.07

conc.-concentration; ddH_2O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LC_{50}- mean lethal concentration

Table 5.5 The pupicidal (*Culex quinquefasciatus*) effects of different leaf extracts of Basotho medicinal plants from Qwaqwa region in the eastern Free State, continued.

		% Mortality					
		M. longifolia			***T. minuta***		
Conc.[mg/ml]	**Ini.nmb.**	**ddH$_2$O**	**EtOH**	**Hexane**	**ddH$_2$O**	**EtOH**	**Hexane**
0.10	10.00	0.00	30.00	80.00	0.00	50.00	60.00
0.50	10.00	0.00	40.00	50.00	0.00	100.00	50.00
1.00	10.00	0.00	70.00	0.00	0.00	90.00	90.00
2.00	10.00	0.00	90.00	50.00	0.00	100.00	100.00
5.00	10.00	0.00	100.00	100.00	0.00	100.00	90.00
10.00	10.00	0.00	100.00	100.00	0.00	100.00	90.00
LC$_{50}$		0.00	1.13	1.21	0.00	1.11	1.12

conc.-concentration; ddH$_2$O- double distilled water; ini.numb.-initial number; EtOH- ethanol; LC$_{50}$- mean lethal concentration

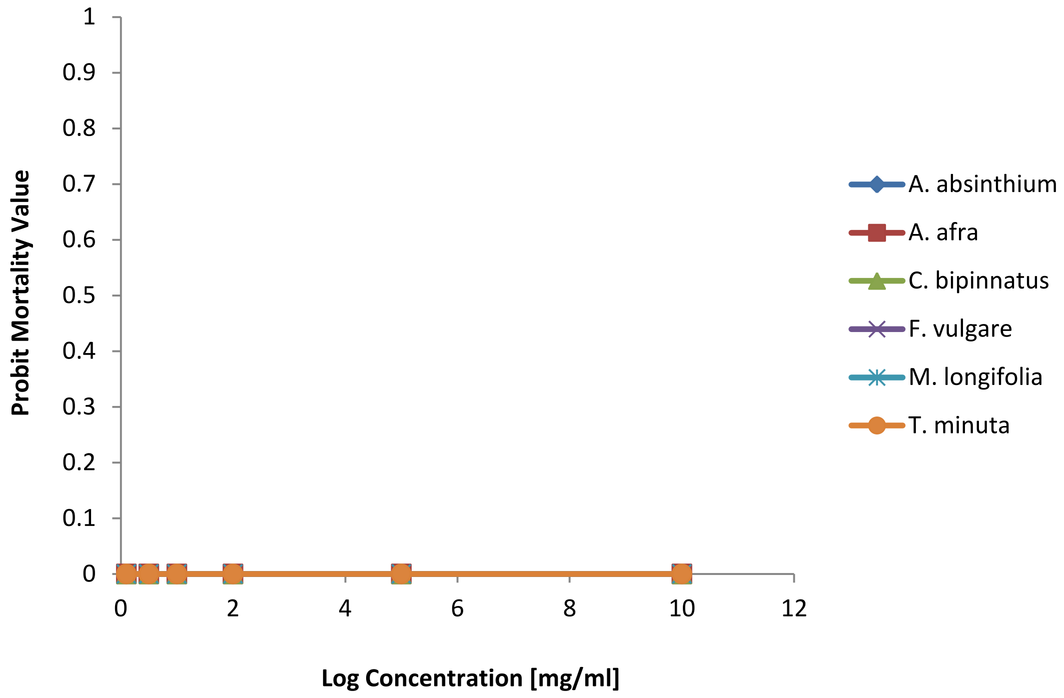

Figure 5.7 The bioactivity effects of aqueous extracts of Basotho medicinal plants towards pupa (*Culex quinquefasciatus*).

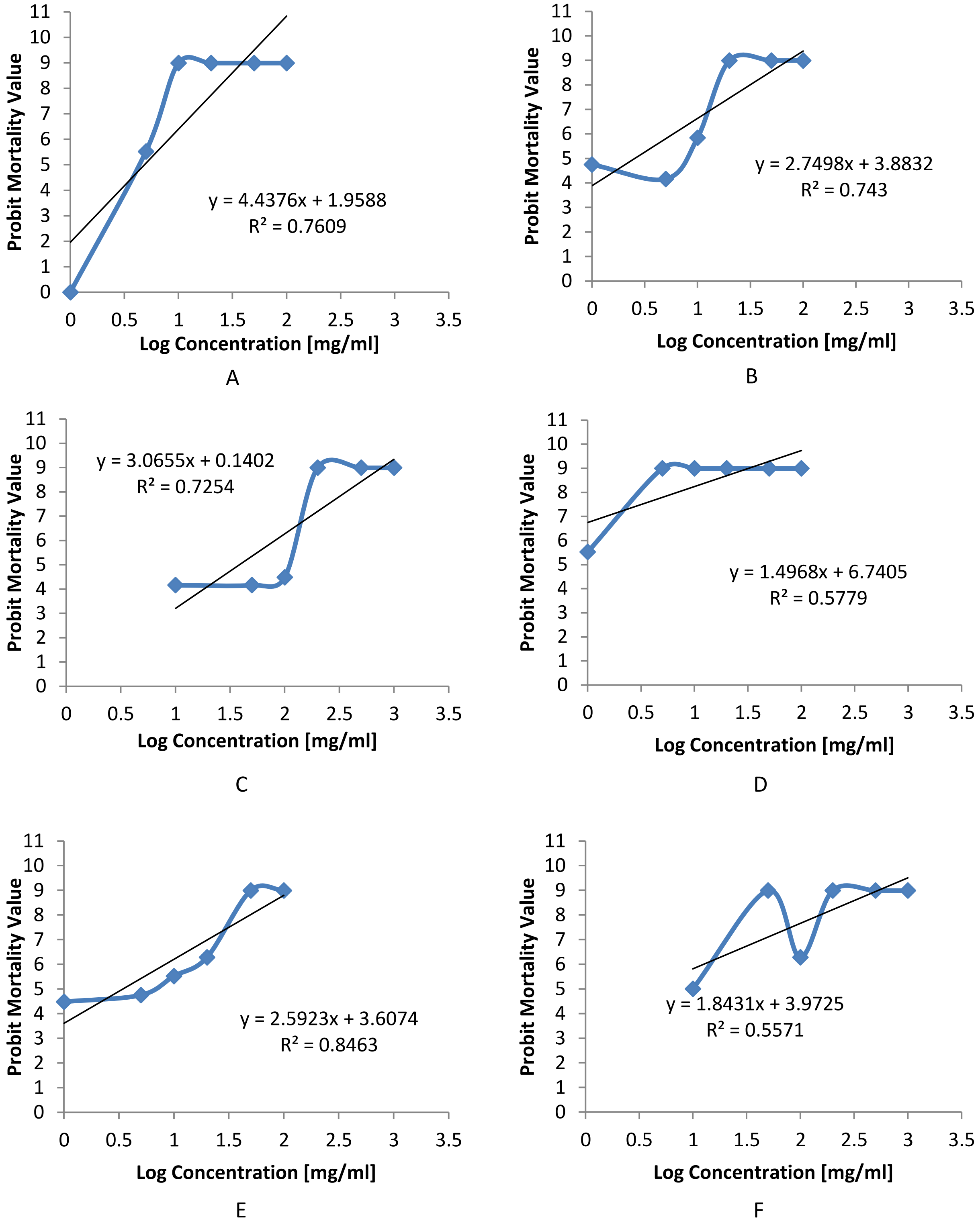

Figure 5.8 The dose-response effects on pupa (*Culex quinquefasciatus*) by ethanolic extracts of Basotho medicinal plants, panel A) *Artemisia absinthium*, B) *Artemisia afra*, C) *Cosmos bipinnatus*, D) *Foeniculum vulgare*, E) *Tagetes minuta* and F) *Mentha longifolia*.

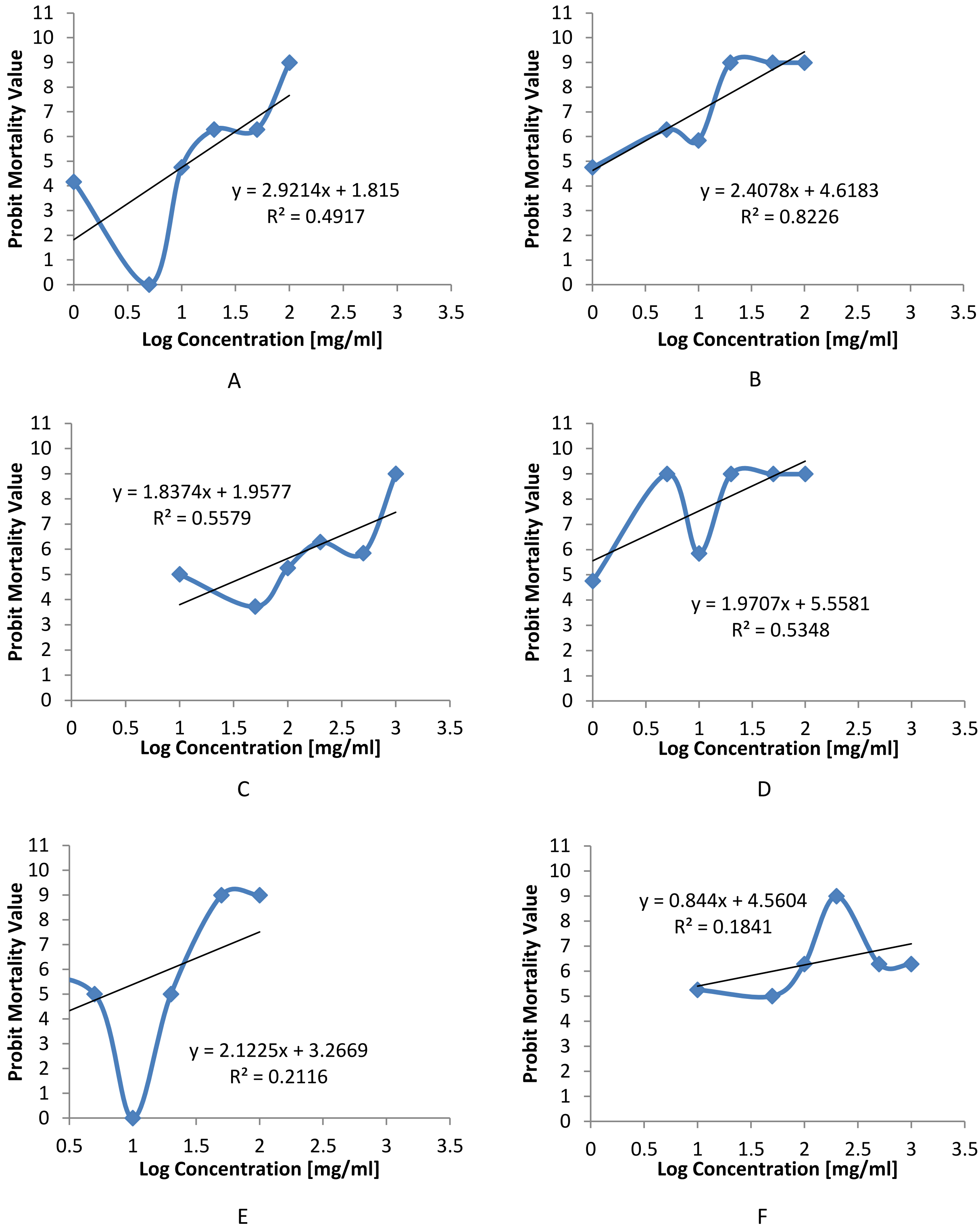

Figure 5.9 The dose-response effects on pupa (*Culex quinquefasciatus*) by hexane extracts of; Basotho medicinal plants, panel A) *Artemisia absinthium*, B) *Artemisia afra*, C) *Cosmos bipinnatus*, D) *Foeniculum vulgare*, E) *Tagetes minuta* and F) *Mentha longifolia*.

5.6. Insecticidal bioassay analysis

The most effective average rate of knock-down was observed in *M. longifolia* (KD_{50} = 4.91 min^{-1}), followed by *F. vulgare* (KD_{50} = 9.87 min^{-1}), *T. minuta* (KD_{50} = 12.39 min^{-1}), and *A. afra* (KD_{50} = 19.02 min^{-1}) after a 6 hour period, were all tested adult mosquitoes showed characters such as difficulty in walking, uncoordinated flying, convulsions, twitching legs and muscle contractions (Figure 5.10). There was no knock-down effect induced by *A. absinthium* and *C. bipinnatus* after the 6 hour period on *C. quinquefasciatus* adult mosquitoes.

The average range of knock-down and percentage mortality in all doses of tested leaf powder was greater in *M. longifolia* (95 - 70 %), *F. vulgare* (75 – 67 %), *T. minuta* (55 – 43 %) and *A. afra* (45 – 25 %) after 12 hours of evaluation (Figure 5.11). Furthermore, there was still no knock-down effect induced by *A. absinthium* and *C. bipinnatus* even after the 12 hour period on *C. quinquefasciatus* adult mosquitoes.

The plants which exhibited insecticidal activities are *M. longifolia* (LD_{99} = 0.25 g), *F. vulgare* (LD_{99} = 0.25 g), *T. minuta* (LD_{99} = 0.25 g) and *A. afra* (LD_{99} = 0.25 g), that killed all tested *C. quinquefasciatus* adult mosquitoes in all evaluated graded doses after the 24 hour period. There was no fatal bioactivity observed in both *A. absinthium* and *C. bipinnatus* towards adults of *C. quinquefasciatus* mosquitoes for all evaluated graded doses after the 24 hour period. The mortality ratio between the evaluated Basotho plants provided significant difference ($F_{5,4}$ = 283.11; $P < 0.01$), whereas there was no significant difference between graded doses for all tested Basotho plants.

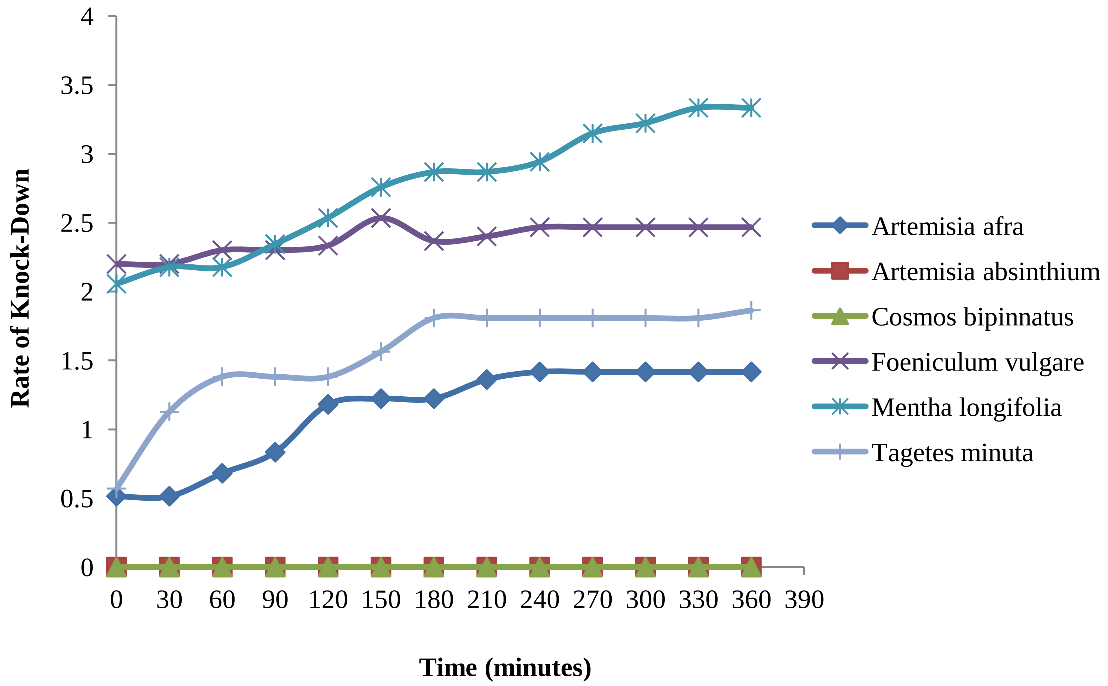

Figure 5.10 The average rate of knock-down effect on mosquitoes (*Culex quinquefasciatus*) by the powdered Basotho medicinal plants after 6 h of exposure.

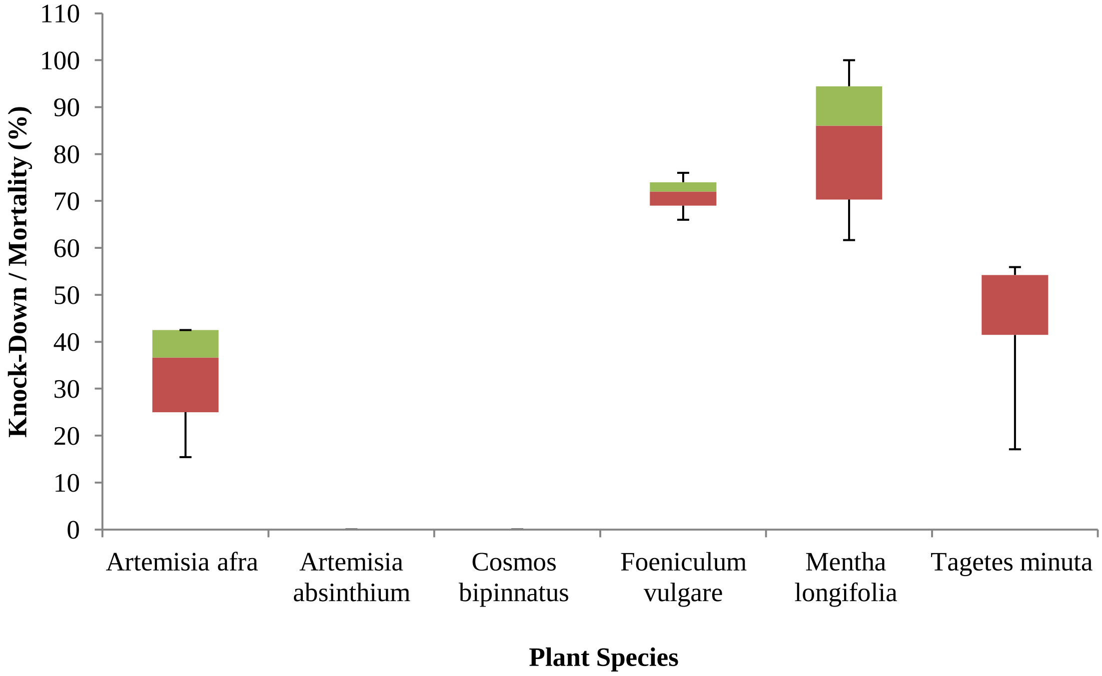

Figure 5.11 The average range of knock-down and mortality effects on mosquitoes (*Culex quinquefasciatus*) by the powdered Basotho medicinal plants after 12 h of exposure.

Table 5.6 The insecticidal effects of Basotho medicinal plants on *Culex quinquefasciatus* mosquitoes after 24 h of exposure.

	% Mortality					
Plant Species	**0.25 g**	**0.5 g**	**1.0 g**	**1.5 g**	**2.0 g**	**LD_{99}**
Artemisia afra	100.0	100.0	100.0	100.0	100.0	0.25
Artemisia absinthium	0.0	0.0	0.0	0.0	0.0	0.0
Cosmos bipinnatus	0.0	0.0	0.0	0.0	0.0	0.0
Tagetes minuta	100.0	100.0	100.0	100.0	100.0	0.25
Foeniculum vulgare	100.0	100.0	100.0	100.0	100.0	0.25
Mentha longifolia	100.0	100.0	100.0	100.0	100.0	0.25
Control	0.0	0.0	0.0	0.0	0.0	0.0

CHAPTER 6

DISCUSSION

The presence of secondary metabolites contributes to the medicinal value of a plant and plays a role of inducing physiological or physiochemical actions towards other organisms (Edeoga *et al*. 2005). Secondary metabolites such as saponins are among common compounds found in medicinal plants and usually seen to co-occur along with other secondary compounds to form complex molecules such as sapogenins (Hopkins & Hüner 2009). The presence of saponins in all the tested Basotho medicinal plants possibly indicates their role in defence against plant invading organisms in most plants. The natural or secondary compounds are sparsely or unevenly distributed, while others are restricted to only some plant parts (fruits, leaves or stem), by age or species specific (Salisbury & Ross 1992). Secondary metabolites that were detected in *Artemisia afra* and *A. absinthium* are said to be common among the *Artemisia* genus, namely, terpenoids, flavonoids, saponins and steroids, but not for tannins (Table 5.1) (Tan *et al*. 1998; Devmurari *et al*. 2010). The presence of terpenoids, flavonoids, saponins, steroids and tannins in leaves of *Cosmos bipinnatus* that are among compounds that play a role in plant defence might bring interests for further studies, since most studies have been reporting the bioactivities of *C. bipinnatus* floral parts (Jang *et al*. 2008). The genus of *Tagetes* was claimed to be ~~a~~ multipurpose due to their variety of bioactivities performed by members of the genus (Vasudevan *et al*. 1997). The phytochemical metabolites detected in *Tagetes minuta* were also reported in another study, and these included major compound derivatives of terpenoids, flavonoids, phenols, steroids while alkaloids were in small quantities (Li-wei *et al*. 2012). The secondary metabolites found in *Foeniculum vulgare* and *Mentha longifolia* add up to major compounds of alkaloids and tannins that were previously not reported to be in significant quantities, furthermore they confirm presence of steroids, and flavonoids in the ethanolic extracts of *F. vulgare* leaves (Sumbul *et al*. 2011).

The plant specificity and restriction of important bioactive secondary metabolites like cardiac glycosides and phlobatannis might be responsible for their absence in the evaluated Basotho medicinal plants. However, other factors that affect presence of a secondary metabolites in the extracts include polarity of extracting solvent, method of extractions, how was the plant stored, solubility and mobility of targeted extract, and geographical location (van Wyk *et al*. 1997).

Most of the detected major secondary metabolites are known for their biological activities such as for plant defence and protection (Hopkins & Hüner 2009). The aqueous and ethanolic extracts of *A. absinthium*, *A. afra*, *C. bipinnatus* and *T. minuta* showed significant cytotoxic effects against brine shrimp nauplii (*Artemia salina*) with mean lethal concentrations (LD_{50} value) performing below 100 µg/ml are considered to be toxic (Table 5.3), and a similar cytotoxic effect trend was observed in study (Ashafa 2013). The brine shrimp are sensitive and vulnerable to toxins exposure (Meyer *et al*. 1992; Cronin *et al*. 2001), therefore aqueous and ethanolic extracts might have contained bioactive ingredients that are toxic towards brine shrimp nauplii. The hexane extracts are suitable for extracting lipids and oils that form an immiscible mixture with aqueous solution, forms a fatty layer on the surface, and so did not come in direct contact with nauplii, whereas the hexane extracts had no effect on brine shrimp nuaplii in the water medium (Table 5.3). The outcome of the cytotoxicity bioassay can be used to predict possible mortality effect of other species or cancer cells that is exposed to similar toxins (Calleja *et al*. 1993; Cronin *et al*. 2001).

The compared dose-response effect between species *A. salina* nauplii and *Culex quinquefasciatus* larvae as well as pupa stages, indicated to differ greatly since the hexane extracts were active against immature aquatic stages of *C. quinquefasciatus* mosquito and not for brine shrimp nauplii. The evaluated larvae and pupa were collected from the field, and their natural environment exposure might play a role in toxin tolerance than *A. salina* nauplii. Also, the two species differ in physiological, metabolic and behavioural aspects hence they responded differently to the investigated dosages (Calleja *et al*. 1993; Cronin *et al*. 2001).

The larvicidal and pupicidal effects exhibited might be due to the availability of bioactive compounds present in plant extracts. Each Basotho plant extract displayed larvicidal and pupicidal activity uniquely indicating difference in plant extracts action towards *Culex quinquefasciatus* immature stages. *A. afra* foliage parts are known to contain several sesquiterpene lactones such as guaianolides and glaucolides, that might have caused muscle convulsions making swimming difficult for *C. quiquefasciatus* larvae to end up drowning (Jakupovic *et aI*. 1988; MacÍas *et al*. 2000). Other isolated non-volatile compounds from *A. afra* included triterpenes (α-amyrin, β-amyrin and friedelin) and alkanes (ceryl cerotinate and N-nonacosane), as well as surface flavonoids (methyl ethers of luteolin), that are considered toxic to arthropods in significant concentration (Brendler *et al*. 2010; Patil *et al*. 2011).

The presence of a variety of compounds in *T. minuta* such as dihydrotagetone, tagetones, ocimenones and piperitone previously isolated (Nchu *et al*. 2011), may cause increased bioactivity compared to individual compounds because of synergistic effects. Perich *et al.* (1994) reported that *T. minuta* extracts (soxhlet) had greater larvicidal activity against larvae of *Aedes aegypti* and *Anopheles stephensi* mosquitoes, therefore comparatively, *T. minuta* ethanolic (Figure 5.5) and hexane (Figure 5.6) extracts in this study also showed larvicidal and pupicidal bioactivities towards *C. quiquefasciatus* mosquitoes.

The dose-response action of ethanolic and hexane showed better larvicidal effects of the toxins from *M. longifolia* and *C. bipinnatus* leaf extracts, whereas the ethanolic and hexane extracts had fewer secondary metabolites present. There might be false extract effects in that the solvent might have influenced the dose response for total larvae mortalities observed against *C. quniquefasciatus* mosquito. Furthermore, Ansari *et al*. (1999) showed that essential oil form *Mentha piperita* has 100% mortality on larvae of *C. quniquefasciatus* mosquito within 24 h, so the response from *M. longifolia* in this study comparatively showed similar bioactivities.

The plants pupicidal effects seemed to differ greatly between evaluated Basotho plants (Table 5.5). The study by Martin *et al*. (2012) showed that the ethanolic extracts of *A. absinthium* significantly improved the yield of some bioactive monoterpenoids as well as sesquiterpenoids against insect pests. Bailen *et al*. (2013) isolated and identified two major compound of *A. absinthium* to be sesquiterpenoids lactone hydroxypelenolide that is known to disrupt cell wall of fungal and invasive bacteria (Chadwick *et al*. 2013), and again to affect insect metabolism as well as central nervous system (Ikemoto *et al*. 1995; Koul 2008), and casticin which act as an insect antifeedant in significant concentrations. The *C. quinquefasciatus* pupa in this study might have drowned in solution due to paralyses which was led by the disruption of the nervous system as a consequence of the bioactive compounds present in the extract.

Previous studies on *F. vulgare* using essential oils against mosquito aquatic larvae, revealed that seed oils were effective on both 2^{nd} and 4^{th} larval instar of *Culex pipiens* (Zoubiri *et al.* 2011), while the chemical isolates from foliage extracts were found toxic against *Culex pipiens* larvae, with terpineol and 1,8-cineole being the most effective components against *Anopheles dirus* and *Aedes aegypti* larvae (He & Huang 2011). Usually, most monoterepnoid such as terpineol and 1,8-cineole are neurotoxic in many insect and or arthropods, and might have caused paralyses as well as significantly disrupting the nervous system enough to cause mosquito *C. quinquefasciatus* pupa deaths. The increase in graded concentrations of Basotho medicinal plants extracts had a direct relation to influence the increase in mortality rates of *C. quinquefasciatus* larvae and pupa.

The mosquito wild larvae and pupa of *C. quinquefasciatus* prefer to inhabit polluted waters and have become tolerant to toxins present in water bodies over time (Service 1993). The aqueous extracts did not have any fatal larvicidal or pupicidal effects. However, aqueous solvents have lower selectivity in extracting biologically active secondary (phytochemical) constituents from plant material (Johnson & Lussas 1983; Ferreira-Dias *et al*. 2003). Also, the larvae and pupae of *C. quinquefasciatus* mosquitoes might have some tolerance mechanism towards aqueous extract since they prefer to dwell in relatively polluted water pools that are situated near human settlements (Service 1993). The larvae and pupa might have being previously exposed to chemical pollutants (toxins) that are similar or show to induce similar effects, on which most Basotho plants possess, and have built some metabolic resistance against them over longer period of time in their natural habitat.

Only leaves of *M. longifolia, F. vulgare, T. minuta* and *A. afra* showed to contain insecticidal bioactive effects. The rate and range of mosquito knock-down effect exhibited by *M. longifolia, F. vulgare, T. minuta* and *A. afra* shows to possess rapid fatal effects, and an average knock-down effect was observed to be under 20 minutes during the evaluations and plants are considered to possess potential insecticidal activity (Figure 5.10). A study carried out on three Culicidae mosquitoes of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* by Ansari *et al*. (1999) revealed that another mint species of *M. piperita* L. leave essential oils have repellent activity towards adults of *C. quniquefasciatus* for a period of 7 h of exposure. The intense odour of *M. longifolia* leaves mainly consists of extremely volatile compounds of monoterpenes and sesquiterpenes such as cis-piperitone epoxide, piperitenone oxide, carvone, pulegone, menthone, thymol, β-thujone (Sharopov *et al*. 2012), however their quantities tend to differ due to their geographical distribution but the presence of essential chemical compounds do not correlate with the apparent geographical variation (Viljoen *et a*l. 2006). The adult mosquitoes were all dead within 12 h and (Figure 5.11), that might be because most volatile compounds affect nervous system and might have been active against adult *C. quiquefasciatus*. The compounds can again have fumigant effect enabling them to penetrate through to disrupt vital metabolic reaction that might deprive adult mosquitoes from getting enough oxygen through and death result from suffocation.

A study by Perich *et al.* (1995) on *T. minuta* floral parts reported repellence and fatal properties against adult *Aedes aegypti* and *Anopheles stephensi* mosquitoes, which are of medical importance as vector of deadly disease such as malaria and yellow fever, the compounds isolated by hydrogenate fraction produced 4 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. These compounds in *T. minuta* were highly suspected to be responsible for the toxicity exhibited against the tested *C. quiquefasciatus* adult mosquitoes (Perich *et al.* 1995; Nchu *et al*. 2011; Sadia *et al*. 2013). The volatile leaf compounds might be biologically active to have significantly killed all adult *C. quinquefasciatus* in less than the targeted time, and compounds include cis-ocimene, beta-ocimene and 3-methyl-2-(2-methyl-2-butenyl)-furan (Alok *et al*. 2005; Nchu *et al*. 2011), with fumigant insecticidal effects on adult mosquitoes showed neurotoxic symptoms.

A. afra leaves mainly consists of volatile compounds of monoterpenes and sesquiterpenes e.g. thujone, campor, 1,8-cineole and borneol that affect nervous system and might have been active against adult *C. quiquefasciatus*. The volatile monoterpenes are among major essential oils components of *A. afra*. contributing to the plant aromatic (odour) properties (Lambers *et al*. 1998; Hopkins & & Hüner 2009), and usually act as fumigants of insecticides that can penetrate through membranes and mediums to cause metabolic disturbance (Yu 2008; Zahran & Abdelgaleil 2010). The adult *C. quinquefasciatus* in this study might have been killed by metabolic disturbance such as nerve shock or difficulty in respiration from suffocation that lead to death in higher concentrations after longer exposure periods.

CHAPTER 7 ~~6~~

CONCLUSION

General conclusion

The availability of bioactive ingredients affected the effectiveness of the extract and mosquito response to exposed compounds. The ethanolic and aqueous extracts of Basotho plants showed significant toxic effects against brine shrimp nauplii than hexane extracts and are considered to contain cytotoxic activities. Furthermore, the evaluated Basotho medicinal plants namely *Tagetes minuta, Cosmos bipinnatus, Artemisia absinthium, Foeniculum vulgare* and *Artemisia afra* can be a source of antitumor bioactive ingredients and recommended for further cancer research. The outcome of this study indicated that the *Artemia salina* nauplii response to exposed dosage of tested toxin cannot be related or used to predict dose responses of other species (mosquitoes), since the compared responses varied towards different extracts and concentration level. Although *A. salina* nauplii are sensitive to applied evaluated toxins and the mode of action of similar or related phytochemical biologically active compounds exhibit in similar action, the fact is that the interspecies (and intraspecies) association to preliminarily assume dose responses of toxins not encouraged.

The ethanolic and hexane extracts of the investigated Basotho medicinal plants contain larvicidal and pupicidal properties. The larvicidal activity of ethanolic extracts was greater for *F. vulgare*, *A. afra*, *M. longifolia, T. minuta, C. bipinnatus* and *A. absinthium* respectively. Whereas, the hexane extract toxicity differ slightly from ethanolic extracts, and was higher in *M. longifolia, T. minuta, F. vulgare, A. afra, C. bipinnatus* as well as *A. absinthium* respectively. The Basotho plants contain pupicidal properties with greater activity observed for *A. afra, T. minuta, M. longifolia, C. bipinnatus* and *A. absinthium* for both ethanolic and hexane extracts respectively.

Most previous studies have analysed and quantified some of the biologically active chemical compounds from the selected Basotho medicinal plants, and the major compounds have indicated possible larvicidal and pupicidal active ingredients, *A. absinthium* with (sesquiterpenoids lactone hydroxypelenolideand casticin), *A. afra* (β-α-amyrin), *C. bipinnatus* (phenolic compounds), *T. minuta* (tagetones and ocimenones), *F. vulgare* (terpineol and 1,8-cineol), and *M. longifolia* (piperitenone oxide and carvone), are considerd water soluble compounds and could have been active in killing the mosquito larvae and pupa. The toxic effects on larvae and pupa might have been rendered by bioactivities of one or a combined action of few phytochemical constituents working in synergy. Other follow up studies should look more into the mode of action at a molecular level, so as to have a clearer picture of how does the Basotho medicinal plant extracts act to pose response from pupa and larvae.

The investigated Basotho medicinal plants had insecticidal activities except for *C. bipinnatus*. The presence of highly volatile chemical compounds must have played a role in higher potency of these plants leaves at significant toxin concentrations. The plants that contain highly volatile compounds include *M. longifolia* (carvone, pulegone, and menthone), *T. minuta* (dihydrotagetone and ocimenones), *A. afra* (campor, 1,8-cineole and borneol), *F. vulgare* (terpineol and 1,8-cineol), and *A. absinthium* with (α-β thujone and casticin). The mode of actions that might have been exhibited by these Basotho plants includes functions like disrupting nervous system, tempering with structure, growth and development. Further research still needs to be done on the plant's extractmolecular actions and mosquito responses (physiological, behavioural and structural) before any recommendation for local communities to continue or modify their current strategies using these Basotho plants.

The practice of utilising natural botanical insecticides is considered safer than synthetic insecticides and the plants are easily accessible. The botanical insecticides extracts are biodegradable and uses many modes of action in controlling mosquitoes. Despite the necessity to explore plant based insecticides for mosquito control agents, there is still a future need to improve on the longevity of effective of most developed insecticides. The improved botanical insecticides must deprive mosquitoes from developing resistance against them. The majority of mosquitoes that are considered to be of medically and economic importance by World Health Organisation have been documented to have developed resistance to most synthetic insecticides. The Basotho medicinal plants would offer a wide range of biologically active compounds that are involved in controlling mosquito populations

The key focal point in mosquito control is reducing mosquito populations to manageable densities or reducing female biting animal and humans. The stumbling feature of mosquitoes is that the complete their life stages in water as immature pupae and larvae, and to further mature into arboreal adults. The tested Basotho medicinal plants possessed larvicidal, pupicidal and insecticidal activities against mosquitoes of *C. quinquefasciatus*. The outcome from this study validates the traditional used of Basotho medicinal plants namely, *M. longifolia, T. minuta, A. afra, F. vulgare, A. absinthium* and *Cosmos bipinnatus*, as insecticides in the Free State Province. The plants or methodologies can be employed for control of other socioeconomic important mosquitoes like *Aedes* or *Anopheles* species. In South Africa, malaria is currently confined to the lower altitude regions such as Limpopo, Mpumalanga and north eastern KwaZulu-Natal provinces, up to areas along the border with Mozambique and Swaziland. These plants could be cultivated in the above mentioned areas in order to reduce mosquito proliferations.

Native people in most developing countries including South Africa still rely on traditional medicinal for pest control. The outcome from this study reveal the use of botanicals natural products as pest control and further documents the traditional use of Basotho plants as pesticides in eastern Free State province of South Africa.

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