BIOACTIVITY AND CHANGES IN GENE EXPRESSION PROFILE MEDIATED BY TEA (*CAMELLIA SINENSIS*) EXTRACT ON *ANOPHELES GAMBIAE* SENSU STRICTO LARVAE

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Bioactivity and Changes in Gene Expression Profile Mediated by Tea (*Camellia sinensis*) extract on *Anopheles gambiae* sensu stricto Larvae

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DECLARATION

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

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DEDICATION

I wish to dedicate this thesis to all who financially contributed towards my MSc studies, understood and invested in my potential. Thanks and may God bless you abundantly.

"Well, I believe that the depth of your struggle can determine the height of your success. I was inspired to come out of everything I've been through and end up in a place where I never thought that I would be." *R. Kelly*

"The harder the struggle, the more glorious the triumph, self-realization demands very great struggle". Swani Sivananda

"The struggle is always worthwhile, if the end be worthwhile and the means honorable; foreknowledge of defeat is not sufficient reason to withdraw from the contest." Steven Brust

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LIST OF ABBREVIATIONS

20E	:	20-hydroxyecdysone
AgILP1	:	*Anopheles gambiae* Insulin-like peptide gene 1
AgJHAMT	:	*Anopheles gambiae* Juvenile Hormone Acid Methyl Transferase
AgSRPN6	:	*Anopheles gambiae* Immune-Responsive Serpin Protein 6
AKH	:	Adipokinetic Hormone
ANOVA	:	Analysis of Variance
ARQU	:	Arthropod Rearing and Quarantine Unit
cAMP	:	cyclic Adenosine Monophosphate
cDNA	:	Complementary Deoxyribonucleic Acid
CSP	:	Circumsporozoite Protein
CYP6M2	:	Cytochrome P450 family 6 subfamily M polypeptide 2
DEET	:	N, N-diethyl-3-toluamide
dFOXO	:	Forkhead box class O transcription factor
DNA	:	Deoxyribonucleic Acid
EGCG	:	(-)-epigallocatechin-3-gallate
Hsp70	:	Heat shock protein 70kDa
icipe	:	International Centre of Insect Physiology and Ecology
IGRs	:	Insect Growth Regulators

IIS	:	Insulin/Insulin-like signaling
IMM	:	Integrated Malaria Management
IRS	:	Indoor Residual Spraying
ITNs	:	Insecticide Treated Nets
IVM	:	Integrated Vector Management
JH	:	Juvenile Hormone
Kdr	:	Knockdown resistance gene
LC/ESI-Qtof/MS	:	Liquid Chromatography Electron Spray Ionization Quadruple time of flight coupled with Mass Spectrometry
LC_{50}	:	Lethal Concentration that killed 50% of test population
LLINs	:	Long-Lasting Insecticide treated Nets
LRIM1	:	Leucine-Rich Immune Protein 1
LSM	:	Larval Source Management
MAEBL	:	Apical membrane Protein/Erythrocyte Binding like Protein
mRNA	:	messenger Ribonucleic acid
NCBI	:	National Centre for Biotechnology Information
ppm	:	Parts per million
RNA	:	Ribonucleic Acid
RT-*q*PCR	:	Reverse Transcriptase quantative real time Polymerase Chain Reaction

SIT	:	Sterile Insect Technology
TEP1	:	Thioester-containing Protein 1
TLC	:	Thin Layer Chromatography
TOR	:	Target of Rapamycin
TRAP	:	Thrombospondin-related Anonymous Protein
UCPs	:	Uncoupling Proteins
UOS3	:	Up-regulated-in-Oocysts Sporozoite Protein 3
VGSC	:	Voltage gated sodium channels
WHO	:	World Health Organization

ABSTRACT

Malaria, a deadly disease caused by *Plasmodium falciparum*, is vectored to humans by infected female *Anopheline* mosquitoes. Malaria intervention tools and specifically those regulating mosquito populations have reduced malaria incidences by 42% in Africa. However, residual transmission accounts for 214 million new cases, > 80% fatalities and 269 million people at risk of contracting the disease. The inexhaustive coverage of these vector controls, widespread insecticide resistance, and shifts in feeding habits of mosquito vectors prompts the need for novel interventions to reduce residual malaria transmission. The targeting of immature mosquito stages affects both vectorial capacity and vector competence. In this study, the effects of tea (*Camellia sinensis*) leaf extract and its bioactive constituents on *An. gambiae s.s* larvae physiology were investigated. Late third/early fourth instar larvae were challenged with increasing doses of *C. sinensis* extract and survival rates recorded every 24 h for 72 h continuously. Following bioassay-guided fractionation, the bioactive ingredients were analyzed using liquid chromatography electron spray ionization quadruple time of flight coupled with mass spectrometry (LC/ESI-Qtof/MS). Hormetic responses to the sublethal doses were transcriptionally assessed by quantifying gene expression levels of *AgamJHAMT*, *AgamILP1*, *Hsp70* and *CYP6M2* with RT-*q*PCR. Generally, the extract exhibited toxicity to mosquito larvae in a dose-dependent relationship. At 250 and 500 ppm, the extract elicited > 90% mortality after 24 h post exposure. Although only moderate larval mortality was observed with 100 ppm of the crude extract, this exposure induced growth disruption effects on mosquito larvae. No effects were observed at 25 ppm and 50 ppm. The bioactive polar fraction elicited 100% larval mortality at 25 ppm. The LC_{50} of the crude extract at 24 h was 117.15 ppm (95% CI 112.86-127.04) while active fraction was 5.52 ppm (95% CI 2.68-9.65). UPLC/ESI-Qtof/MS analysis revealed that proanthocyanidins were the toxicity-mediating compounds in the bioactive fraction. Gene expression analysis showed that, relative to controls, *Hsp70* (*AGAP004581*) was significantly up-regulated by 159.7863 fold while the other three larval genes

AGAP005256 (*AgamJHAMT*), *AGAP010605* (*AgamILP1*) and *AGAP008212* (*CYP6M2*) were down-regulated by 265.0278, 9.7136 and 621.6678 fold respectively (t-test, $P < 0.001$). The study showed that, tea leaf extract and its constituents have potential of controlling *An. gambiae* s.s larvae and inspires new larviciding chemistry for control of malaria mosquitoes.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria remains a scourge to human life with an estimated 438,000 deaths reported globally in 2015 and 3.3 billion people at risk of contracting the disease (WHO, 2015). Africa bears the heaviest burden as more than 80% of reported cases occur in sub-Saharan Africa (WHO, 2015). Effective malaria control in Africa is devoid of transmission blocking vaccines (Crompton *et al*., 2010; Arama & Troye-Blomberg, 2014), inexhaustive understanding of vector ecology (Ferguson *et al.*, 2010), and widespread resistance to conventional antimalarial drugs and insecticides by *Plasmodium* and *Anopheles* mosquitoes, respectively (Alonso *et al.*, 2011; Ranson & Lissenden, 2016). Additionally, shifts in feeding habits by *Anopheline* mosquito vectors renders the existing vector control strategies less effective and humans become more vulnerable to *Plasmodium* infection (Sokhna *et al.*, 2013; Killeen, 2014; Ranson & Lissenden, 2016). Thus, novelty in targeting the residual transmission is urgently required to reduce the basic malaria reproduction rate to at least less than 1 (Govella & Ferguson, 2012; Russell *et al.*, 2013).

One feasible approach with forgotten success is larviciding, a component of integrated vector management (IVM) and larval source management (LSM), that eradicated malaria in Brazil and Egypt (Killeen *et al.*, 2002a; Walker & Lynch, 2007; Tusting *et al.*, 2013). The approach suppresses both indoor and outdoor feeding mosquito populations. Larviciding approach is favored by the fact that the immature stages are relatively confined, immobile, chemically susceptible, and meager chances of developing resistance (Killeen *et al.*, 2002b). Vector competence is a genetic trait of mosquitoes (Beerntsen *et al*., 2000; Cohuet *et al.*, 2010) that is greatly influenced by the quality of environment the aquatic juveniles encounter in the course of their development (Okech *et al*., 2007; Moller-Jacobs *et al*., 2014). Manipulation and/or

modification of the bio-physicochemical parameters of larval ecology reduces the physiological fitness of the resultant adult or induce delayed maturity, development perturbation and death (Fillinger & Lindsay, 2011). Therefore, larval source reduction using bioactive compounds appears promising vector control strategy (Fillinger & Lindsay, 2006; Tusting *et al.*, 2013).

Mosquitoes develop in diverse habitats that might influence their bionomics and life history traits (Mwangangi *et al*., 2007). Mosquito larvae under optimal conditions take 7-14 days to develop into adults, but the length of this period can vary depending on the quality of larval environment (Olayemi & Ande, 2009). Juvenile stages are highly sensitive to perturbed physiological environments (Moller-Jacobs *et al*., 2014). Suboptimal environments are perceived by conserved sensory systems such as insulin/insulin-like signaling (IIS), steroid hormone, transformed growth factor-beta-like (TGF-β) and guanylate cyclase pathways altering the physiology in response to the surrounding (Cornils *et al.*, 2011; Davies *et al.*, 2014). This prompts for adaptive responses leading to developmental arrest (diapause / "status quo") (Vinogradova, 2007), life history trade-offs (Zera & Harshman, 2001) and reduced behavioral activities or performance (Ricklefs & Wikelski, 2002). The sensory inputs from these diverse breeding habitats inductively shape the transcriptomic expression and regulation of genes vital for development, reproduction, stress response, heat shock proteins, environmental signaling and xenobiotic detoxification (Feder & Hofmann, 1999; de Nadal *et al.*, 2011; King & MacRae, 2015).

Mosquito larval control has for millennia relied on the use of chemicals such as malathion, fenthion, temephos, endosulfan, deltamethrin, pirimiphos-methyl, among others (Khater, 2012). Significant reduction of mosquito population has been achieved through persistent application (Walker, 2002). However, resurgence of resistant strains, inevitable environmental concerns due to chemical pollution, and toxicity to non-targets limited continued use of these insecticides (Khater, 2012). This has made it imperative to search for ecofriendly alternatives as effective as the aforementioned chemicals

(Khater, 2012). Plants constitutively and/or inductively synthesize structurally diverse noxious allelochemicals to mediate defense against biotic and abiotic stresses (Mithöfer & Boland, 2012). These include N-containing alkaloids, glucosinolates, phenolics, flavonoids, terpenoids, cyanogenic glycosides and many others. Many of these allelochemicals have shown efficacy in controlling insect pests and disease transmitting vectors (Isman, 2006; Rattan, 2010). The chances of resistance development to these allelochemicals are minimal because the chemicals target multiple insect physiological processes (Isman & Akhtar, 2007). These compounds interact with insect cellular components disturbing digestion, hormonal balance, membrane potential, modulation of ion channels, neuronal coordination and signaling pathways resulting in either death or impaired development and fitness (Mithöfer & Boland, 2012).

Tea (*Camellia sinensis*) leaves are used for preparation of a non-alcoholic drink consumed worldwide for its psycho-activity and health benefits (Khan & Mukhtar, 2013). The immature tea leaves are rich in methylxanthines (caffeine, theobromine, theophylline), catechins (catechin, gallocatechin, catechin gallate (CG), gallocatechin gallate, epicatechin, (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin-3-gallate (EGCG)), flavonoids, proteins, vitamins, glycosides (kaempferol, quercetin, myricetin) (Graham, 1992). The catechins have antimalarial, antiviral, antibacterial, anti-carcinogenic, anti-oxidant, anti-inflammatory, anti-arthritis, anti-angiogenic, neuroprotective, cardioprotective and cholesterol-reducing activities (Sannella *et al.*, 2007; Chacko *et al.*, 2010; Afzal *et al.*, 2015; Fujiki *et al.*, 2015). In nature, the catechins undergo auto-oxidation to form tannins, which are water soluble compounds with strong antioxidant activities. Tannins can occur as either condensed tannins (CT) or hydrolyzable tannins (HT) depending on the degree of the heterocyclic ring polymerization (Barbehenn & Peter Constabel, 2011). Tannins have both beneficial and detrimental biological effects in that they are strong antioxidants as well as astringent compounds (Chung *et al.*, 1998; Amarowicz, 2007). CTs are the most abundant polyphenols and occur as either oligomeric or polymeric flavonoids (proanthocyanidins)

(Dixon *et al.*, 2005). The CTs have been reported to have feeding deterrence and insect growth reduction effects (Barbehenn & Peter Constabel, 2011).

Previous studies reported the effects of tea extracts in causing intoxication of *Drosophila melanogaster* and *Aedes*, *Culex* mosquito larvae (Ranaweera, 1996; Žabar *et al.*, 2013). Further, recent studies by Lopez *et al.* (2015) reported that tea polyphenols negatively impacted *D. melanogaster* development and reproduction viability. Based on these reports, this study was designed to evaluate the effects of tea phytochemicals on survival, development, and gene expression of *An. gambiae* s.s larvae. Here, for the first time- tea leaf extract is reported to have interfered with survival and development of *An. gambiae* s.s mosquitoes. This could be partly due to proanthocyanidins and other bioactive compounds present in tea extract which could be acting by affecting expression of regulatory genes required for mosquito development and survival. Therefore, our study demostrates tea as a potential bio-insecticide candidate and inspires new chemistries for larval control of mosquitoes.

1.2 Statement of the problem

Malaria presents unacceptable public health burden. Although the malaria vaccine RTS,S under clinical trials could reduce *Plasmodium* infections, mosquito population reduction remains the mainstay to effective malaria control. Despite the widespread deployment of various vector control interventions over the last decade, residual malaria transmission still accounts for more than 80% of fatalities reported globally in 2015 from Africa and 269 million people estimated at risk of contracting malaria. The incomplete coverage of the existing vector controls, unsustainability due to widespread insecticide resistance, and behavior plasticity of mosquito vectors prompts novel interventions aimed at reducing residual malaria transmission. Thus, targeting juvenile aquatic stages require reconsideration with cheaply accessible and ecofriendly arsenals aimed to reduce malaria-transmitting vector populations. Additionally, many studies have paucity of information regarding the bioactivity targets of the plant extracts

under study. The current study investigated the bioactivity of tea leaf extracts against *An. gambiae s.s* larvae to isolate potent larvicidal compounds and unravel their probable molecular targets.

1.3 Justification of the study

The high degree of ecological and behavioral plasticity exhibited by members of the *An. gambiae* complex challenges malaria control efforts (Sokhna *et al.,* 2013). Additionally, their capacity to tolerate high doses of insecticides enables them bypass the current vector control strategies (Ranson & Lissenden, 2016). Thus, mosquito larval control using bio-larvicides of plant origin has potential of suppressing mosquito vector populations and disease incidences. Over reliance on chemical insecticides in pest control has negatively impacted environmental safety warranting for new ecofriendly chemistries. In this regard, prospection for new plant compounds with ability to cause growth disruption and delayed maturity in harmful arthropods is worthy for suppression of malaria-transmitting vectors. In this study, *C. sinensis* leaf extract was selected for evaluation based on previous reports that its phytochemicals elicited deleterious effects on insect growth (Lopez *et al*., 2015). No scientific assessments have been carried out to demonstrate its potential to control larval stages of *An. gambiae s.s* mosquito. The findings of this study justify recommendation of tea extract or its constituents for development of potent bio-larvicide to control mosquitoes.

1.4 Objectives

1.4.1 General objective

To determine bioactivity of *C. sinensis* extract against the larval stages of *An. gambiae s.s* and quantify differentially expressed genes in larvae treated with sublethal doses of extract.

1.4.2 Specific objectives

(i) To determine the larvicidal activity of *C. sinensis* crude and solvent fraction extracts

(ii) To fractionate and identify the bioactive compounds present in *C. sinensis* extract.

(iii) To determine expression of larval genes *CYP6M2*, *Hsp70*, Insulin-like peptide gene 1 and Juvenile hormone acid methyltransferase following exposure to *C. sinensis* extract

1.5 Null hypotheses

(i) *C. sinensis* leaf extract does not evoke larvicidal activity

(ii) Exposure of *An. gambiae s.s* larvae to tea extracts does not exert adaptive physiological and/or genetic changes

CHAPTER TWO

LITERATURE REVIEW

2.1 *Anopheles gambiae* complex as vectors of *Plasmodium falciparum*

Mosquitoes of *Anopheles gambiae* Giles complex form the most variable biological species distributed all over Africa owing to the favorable ecological conditions prevailing across the tropical countries (Sinka *et al*., 2010). The mosquito complex comprises of closely related species including *An. gambiae sensu stricto, An. listeri, An. quadriannulatus, An. funestus, An. bwambae, An. arabiensis, An. merus* and *An. melas* that vary in behavior and geographical locations (Sinka *et al*., 2010). Among the eight known species, only three are competent vectors of malaria across Africa *viz An. gambiae s.s, An. funestus* and *An. arabiensis* (Sinka *et al*., 2010). Vector complexity variation among these three mosquito species demonstrates spatio-temporal distribution that account for their differences in survival, adaptability and ecological dispersal in restricted environments, and plasticity in vector bionomics (Sinka, 2013). *An. gambiae s.s* is the most speciated vector demonstrating distinct behavioral traits including anthropophilicity, a characteristic designated to its competence in malaria transmission (Sinka, 2013).

The discovery that mosquitoes transmit malaria parasites was milestoned by Ronald Ross in 1897 (Ross, 1897; Cox, 2010). Female *Anopheline* mosquitoes support the sporogonic development of *Plasmodium* parasites soon after ingestion of malaria-infected blood (Smith *et al.*, 2014). Within the mosquito midgut lumen, maturation of male and female gametes is followed by fertilization to form a flagellated motile zygote (ookinete) (Aly *et al*., 2009). Mosquito-derived molecules such as xanthurenic acid coupled with temperature and pH shifts facilitate this process (Billker *et al.*, 1997; Billker *et al*., 1998). The ookinete traverses the midgut epithelia into the hemocoel and hemolyph differentiating into an oocyst. Within a span of 10-14 days, the oocyst undergo sporogony phase resulting in the formation of sporozoites that invade and lodge

within the salivary glands (Smith *et al.*, 2014). During blood feeding on a new host, the infective sporozoites are injected together with saliva into host bloodstream, closing transmission cycle.

The developmental events of *Plasmodium* within the vector mosquito involve complex compendium of interacting molecules (Sreenivasamurthy *et al*., 2013). These molecules facilitate or inhibit *Plasmodium* parasites development during the co-evolution relationship within the mosquito midgut, hemolymph and salivary glands (Figure 2.1). Ookinete invasion of mosquito midgut epithelium act as a bridge-to-cross for successful colonization in the vector and faces counteraction responses from the vector. The ookinete invasion process has been (and continues to be) a debated topic with numerous theories put forth to explain the phenomenon (Baton & Ranford-Cartwright, 2005). However, midgut proteins epithelial serine protease (ESP) and cactus have been reported to facilitate *Plasmodium* ookinete entry into midgut lamina (Rodrigues *et al.*, 2012). The vector resists colonization of *Plasmodium* parasites by producing serine protease inhibitor immunoresponsive protein *Ag*SRPN6, leucine-rich repeat immune protein 1 (LRIM1), prophenoloxidase components and thioester-containing protein 1 (TEP1) (Abraham *et al.*, 2005; Whitten *et al.*, 2006).

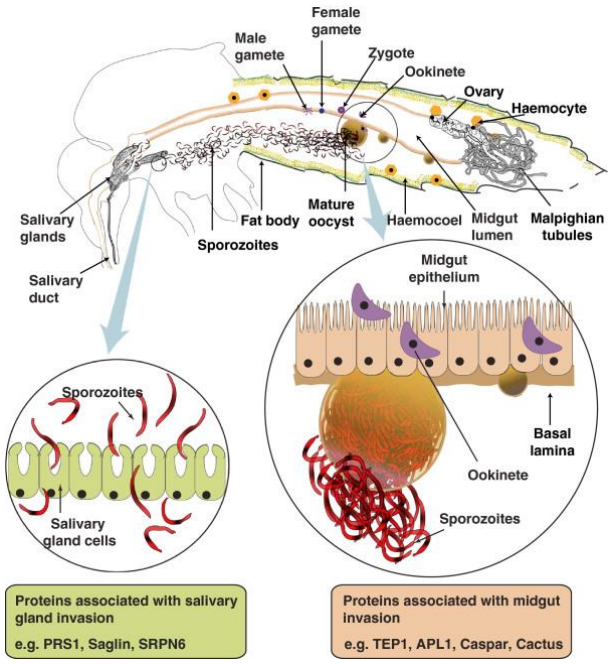


Figure 2.1: Mosquito-*Plasmodium* crosstalk interacting proteins.

Transversing through the midgut epithelia by ookinetes is aided by interaction of TEP1, APL1, Caspar and Cactus proteins. Salivary gland invasion by *Plasmodium* sporozoites involves interaction of AgSRPN6, Saglin and PRS1. Adapted from Sreenivasamurthy *et al.*, 2013.

A number of surface-specific proteins mediate the binding and invasion process of mosquito salivary glands by sporozoites. These are microneme-secreted proteins such as circumsporozoite protein (CSP), apical membrane protein/erythrocyte-binding like protein (MAEBL), thrombospondin-related anonymous protein (TRAP) and up-regulated-in-oocysts sporozoite protein 3 (UOS3) (Mikolajczak *et al.*, 2008). The interaction between *Anopheles* Saglin proteins with *Plasmodium* TRAP aid in internalization of sporozoites into salivary glands of mosquitoes (Ghosh *et al.*, 2009).

2.2 Vector control as a component of integrated malaria management (IMM)

2.2.1 Insecticides for controlling adult mosquitoes

World Health Organization (WHO, 2015) recommends the use of insecticides as the first line protection against mosquito bites. This is true because the adult female mosquitoes are the main carriers of malaria parasites. Currently, the commonly used neurotoxic insecticides are organophosphates, carbamates and pyrethroids (Nkya *et al*., 2013). Organophosphates and carbamates inhibit mosquito acetyl cholinesterase (*AChE*), while pyrethroids block voltage-gated sodium channels (VGSC). The high neural toxicity of these chemicals to mosquitoes and relative safety to humans have won them recommendation for indoor residual spraying (IRS) and impregnating bed nets (Zaim *et al.*, 2000; Lengeler, 2004). Widespread coverage of long-lasting insecticide-treated nets (LLINs) coupled with IRS and case management has considerably reduced malaria transmission and deaths (Pluess *et al.*, 2010; WHO, 2015). On the other hand, the operational scale up of these interventions which rely on chemical insecticides have forced mosquitoes to evolve from insecticide-susceptible into resistant organisms thus limiting vector control efforts all over Africa (Mutunga *et al.*, 2015; Ranson & Lissenden, 2016). Reduced efficacy of LLINs and IRS has been reported in many malaria endemic African countries (N'Guessan *et al.*, 2007; John *et al.*, 2008; Glunt *et al.*, 2015). Two possible reasons have been attributed:- (i) limited tool box for malaria control by over relying on few insecticides particularly pyrethroids and (ii) highly evolvable genomes accounting for physiological plasticity among members of *An. gambiae* complex (Neafsey *et al.*, 2015).

Various mechanisms of insecticide resistance have been discussed in lengthy in several studies (Nkya *et al*., 2013). Physiological, genetic and/or behavioral changes by mosquitoes result in remarkable insecticide avoidance, delayed/slowed penetration, target site modifications through mutations or rapid detoxification (Figure 2.2) (Nkya *et al*., 2013; Liu, 2015). Target site insensitivity and elevated insecticide detoxification are

the main contributors of insecticide resistance in Africa (Hemingway *et al.*, 2004; David *et al*., 2013; Liu, 2015). For instance, over 100 genes encoding cytochrome P450 enzymes have been identified and associated with rapid detoxification of insecticides through hydroxylation and oxidation (David *et al*., 2013). Point mutations of target proteins and frequent changes in allelic regions conferring insecticide resistance have been studied extensively. For instance, Nauen (2007) reported that *AChE* target gene mutations (G119S *Ace-1R*) are associated with organophosphate and carbamate resistance among *An. gambiae s.s* mosquitoes. Similarly, *Ace-1R* and knock down resistance (*Kdr*) allelic mutations (L1014F and L1014S) have been reported to confer resistance to organophosphates, carbamates and pyrethroids in *An. gambiae s.s* and *An. arabiensis* (Chen *et al.,* 2008; Reddy *et al.*, 2013). Additionally, *Vgsc-1014F* was recently reported to confer resistance to pyrethroids in *An. gambiae s.s* and *An. arabiensis* but occur in low frequencies (Ochomo *et al.*, 2015). Cross-induction and selection of insecticide resistance alleles among mosquito populations have been reported to elicit strong resistance to multiple insecticides (Edi *et al*., 2012). Successful implementation of vector control has been greatly hindered by this variable genetic trait among malaria vectors rendering reliability of insecticides untrustworthy (David *et al*., 2013; Gatton *et al.*, 2013). Although efforts to generate resistance-resilient chemistries are underway (Mutunga et al., 2016), there is need for complementary approaches in vector control.

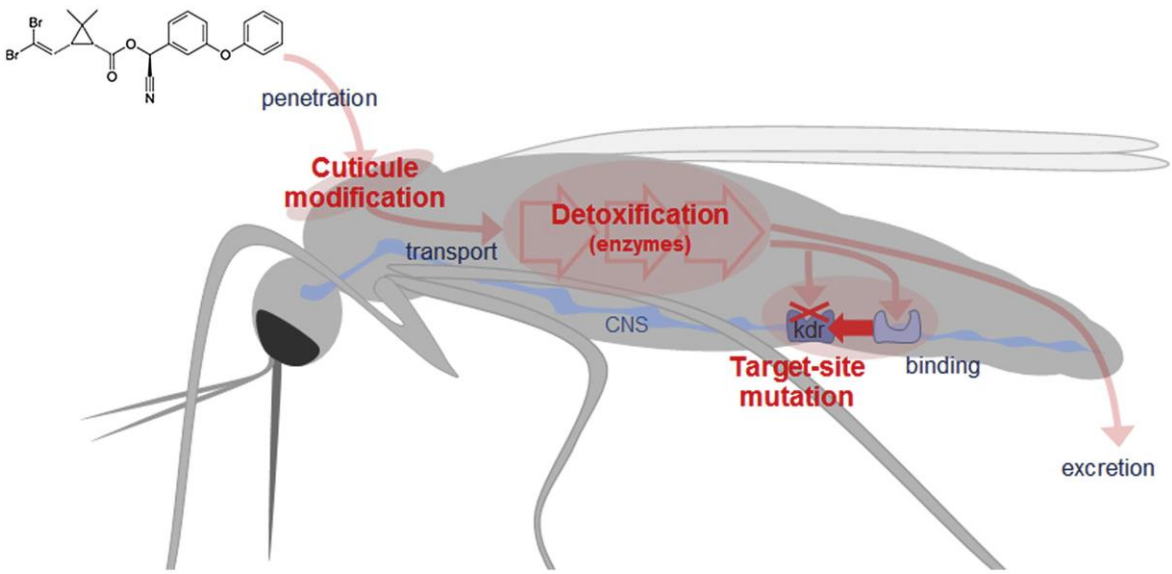


Figure 2.2: Toxicity steps of pyrethroid insecticides and associated resistance mechanisms in mosquitoes.

Following uptake, neurotoxic-targeting pyrethroids bind to chemoreceptors at knock-down resistant site and acetylcholine receptors mediating toxic effects. Mosquitoes have developed resistance mechanisms by modifying the cuticle, upregulating detoxification enzymes and mutations of target proteins to accelerate removal of insecticides (Nkya *et al.*, 2013**)**.

2.2.2 Sterile insect technology (SIT) and paratransgenesis

The increase in insecticide resistance by mosquitoes and advancement of technology has prompted scientists to look for ways of eliminating or suppressing malaria transmitting mosquito populations. Sterile insect technology (SIT), which relies on mass production and release of UV-irradiated sterile male mosquitoes, can potentially suppress mosquito populations over time by inducing sterility to wild females (Alphey *et al.*, 2010; Lees *et al.*, 2015). Mating the wild females reduces reproduction potential as the treatment generates chromosomal aberrations and dominant lethal mutations in sperms. The technique is species-specific and environmentally considerate. A biological technique utilized in vector control is the use of *wolbachia*-induced cytoplasmic incompatibility (incompatible insect technology–IIT) whereby sperms from *wolbachia*-

infected males induce declined fertility of uninfected females (Wilke & Marrelli, 2015). Further, the completion of sequencing, annotation and publishing of *An. gambiae s.s* genome (Holt *et al.,* 2002) has facilitated comprehensive mining of genetic elements for management of mosquitoes (Klassen, 2004; Wilke & Marrelli, 2012). Genetic engineering of male mosquitoes with genes encoding for embryonic lethality has been applaused as a promising technology to control mosquito-borne diseases (Gabrieli *et al.*, 2014). The released transgenic mosquitoes compete with fertile males for mates diminishing the next generation population as the embryo survival is not favored (Benedict & Robinson, 2003). In addition to the high cost involved in this technique, low public acceptance of genetically modified organisms limits its implementation in Africa (Prakash *et al.,* 2011).

2.2.3 Personal protection with repellents

Besides insecticide treated nets (ITNs) and IRS, repellents have been used to keep mosquitoes at bay (Maia & Moore, 2011). Application of N,N-diethyl-3-toluamide (DEET) on attire and human skin repel mosquitoes by interfering with mosquito olfaction sensory system (Ditzen *et al.,* 2008). Binding of active molecules onto sensory chemoreceptors block the signal transduction cascade causing the mosquito to be repelled away from treated surface (Bohbot & Dickens, 2010). In other studies, it was claimed that DEET mask human kairomones required to attract mosquitoes to humans offering up to 98% protection efficacy (Dogan *et al.,* 1999; Bernier *et al.,* 2006). However, its toxicity claims and cost ineffectiveness (Briassoulis *et al*., 2001) has prompted for plant-based repellents (Maia & Moore, 2011; Deletre *et al.*, 2013).

2.2.4 Larval control

Larval Source Management (LSM) complements the current mosquito control strategies by suppressing both indoor and outdoor feeding mosquitoes (Walker & Lynch, 2007). Being a component of IVM, it involves destroying the larvae breeding habitats through chemical larviciding, biological control agents and physical filling of breeding

grounds (Tusting *et al.,* 2013). The approach has been less-practiced though successful elimination of malaria has been achieved through LSM in Brazil (Shousha, 1948) and Egypt (Soper & Wilson, 1943). The first larval control backdates to 1899 when Ronald Ross used kerosene to kill mosquito larvae in Sierra Leone (Bockarie *et al.,* 1999). Today, the oils used for larviciding are biodegradable and mostly obtained from plants (Okumu *et al*., 2007; Kweka *et al*., 2011; Mdoe *et al*., 2014). Essential oils act through suffocation mechanism and inducing toxicity to mosquitoes with their chemical constituents especially the terpenes (Pitasawat *et al.,* 2007; George *et al*., 2014). The lipophilic nature of the oils interferes with biochemical, metabolic, physiological, and behavioral processes of mosquitoes culminating to death or impaired development (Marimuthu *et al.*, 1997).

Chemical larvicides such as malathion, fenthion, temephos, endosulfan, deltamethrin, pirimiphos-methyl among others significantly reduced larvae population upon application (Khater, 2012). However, environmental safety concerns, resurgence of resistant mosquitoes, killing non-targets warrantied for ecosmart bio-insecticides (Imbahale *et al*., 2011; Khater, 2012). Plant compounds have shown potency in controlling developmental stages of mosquitoes (Ghosh *et al.,* 2012; Imbahale & Mukabana, 2015). As a hormonal agonist, neem azadirachtin disrupt development of mosquitoes by mimicking developmental hormones, inducing apoptosis signals and causing cell-cycle arrest (Salehzadeh *et al*., 2003). Others damage larvae midguts or interfere with digestive enzymes causing death (Procópio *et al.,* 2015). Monomolecular surface films are being tried for effective control of aquatic stages of mosquitoes (Mbare *et al.*, 2014b). For instance, Aquatin® mosquito formulation has been reported to inhibit adult emergence by 85% in *An. gambiae s.s* and *An. arabiensis* (Mbare *et al.*, 2014b). Non-chemical strategies have also been included in efforts to fight against mosquitoes. For instance, *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* toxins have been reported effective against mosquito larvae in Africa (Majambere *et al.*, 2007; Imbahale *et al.*, 2012; Nartey *et al.*, 2013). Activation of these toxins by insect midgut alkaline environment and enzymes interfere with larvae digestion resulting in paralysis

and death (Schnepf *et al*., 1998). However, resistance to these toxin crystals has been reported in mosquitoes compromising their effectiveness (Wirth, 2010).

A novel strategy has been proposed to auto-disseminate larvicides to target larval habitats by employing the blood-fed adult female mosquitoes (Sihuincha *et al*., 2005; Devine & Killeen, 2010; Gaugler *et al.*, 2012). Field trials of this approach on *Aedes aegypti* with pyriproxyfen resulted in > 95% habitat coverage (Devine & Killeen, 2010). This appears to be a promising approach to suppress mosquito population by rendering larval habitats unproductive for prolonged periods.

2.3 Insect Growth Regulators (IGRs)

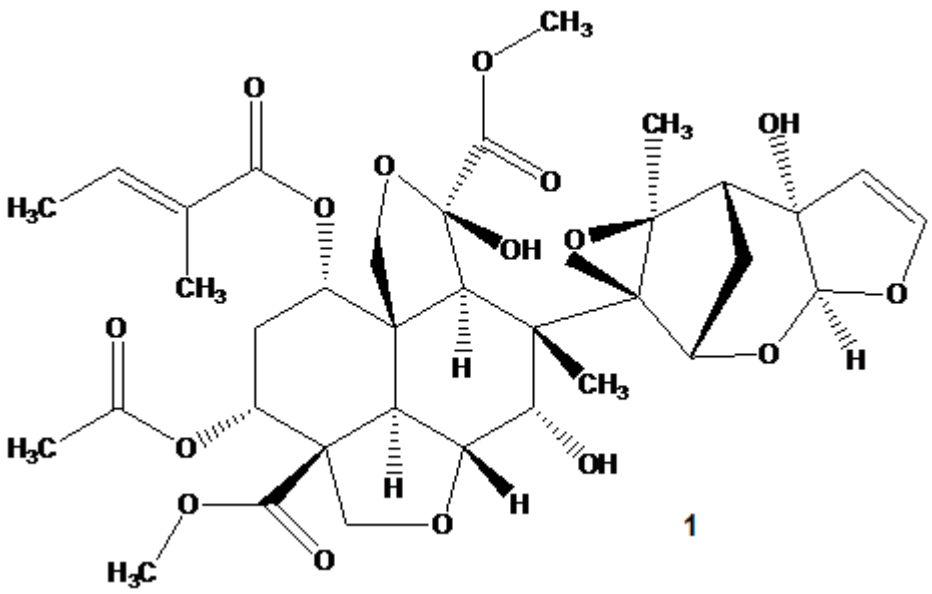
Chemicals such as pyriproxyfen, fenoxycarb, buprofezin, halofenzoide, methoprene and diflubenzuron interfere with the metamorphosis of the insect and prevent adult emergence from the pupal stages (Tunaz & Uygun, 2004; Mbare *et al.*, 2014a; Bensebaa *et al.*, 2015). They have a proven history of selective insect toxicity and low non-target impacts (Mbare *et al.*, 2013). Juvenile hormone analogs such as pyriproxyfen, fenoxycarb and methoprene suppress mosquito embryogenesis and metamorphosis (Ohba *et al.*, 2013). Application of these JH analogs to larval habitats results in abnormal morphogenesis and completely inhibits adult emergence even at low concentrations (Staal, 1975; Hirano *et al.*, 1998). Additionally, topical applications on gravid females, as well as supplementation of adult diet with pyriproxyfen and pyriproxyfen-treated nets have been reported to interfere with mosquito reproduction and egg hatching viabilities (Ohashi *et al.*, 2012; Ohba *et al.*, 2013). A sterilization phenomenon has been reported on the employment of these juvenile hormone analogs to control pests and insect vectors (Tunaz & Uygun, 2004). Diflubenzuron effectively inhibits chitin synthesis by blocking incorporation of sugars into the growing chitin chain (polymerization step) (Cohen, 1987). Buprofezin blocks assembly of chitin precursors and deposition (Muthukrishnan *et al.*, 2012). Damage to newly formed cuticle cannot withstand hemolymph pressure and muscle tractions. These compounds

are relatively as effective as larvicidal organophosphates though inaccessible to low income countries due to their high cost. This makes it imperative to search for cheap and locally available plant-based insecticides.

2.4 Plant-based IGRs

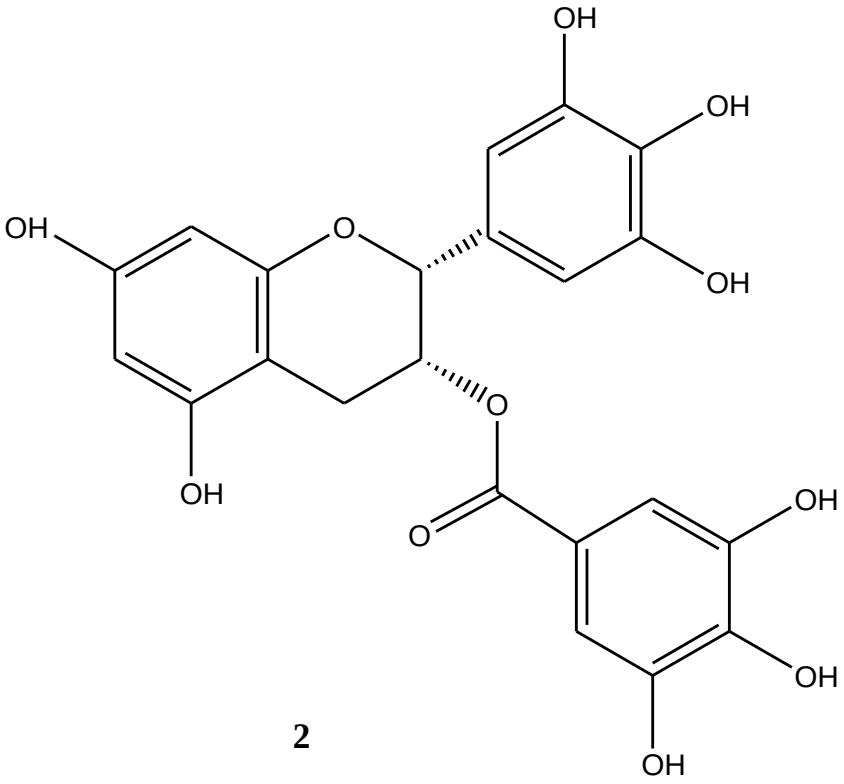
Uncontrollable application of chemicals geared towards elimination of mosquito-borne diseases has negatively impacted environment and biodiversity. Resurgence of insecticide-resistant strains of mosquitoes has also emerged as a result (Nkya *et al*., 2014). To circumvent these drawbacks, researchers have turned to nature for ecofriendly alternatives (Isman, 2006).

Constitutively and/or inductively, plants biosynthesize allelochemicals with insect growth regulatory effects (Mithöfer & Boland, 2012). Naturally occurring IGRs have been isolated from plants of *Meliaceae, Lamiaceae, Asteraceae, Verbenaceae, Rutaceae, Fabaceae* and *Euphorbiceae* families. Well characterized noxious plant-based IGRs are limonoids (Roy & Saraf, 2006) predominantly from *Azadirachta indica* (Mordue(Luntz) & Nisbet, 2000; Mordue (Luntz) *et al*., 2005), *Melia azedarach* (Nathan *et al.*, 2006), *Melia volkesinii* (Al-Sharook *et al.*, 1991), *Turraea mombassana, T. wakefeldii, T. abyssinica, Trichilia roka* (Ndung'u *et al.*, 2004) and *Citrus reticulata* (Jayaprakasha *et al.*, 1997; Khalil *et al.*, 2003; Bilal *et al.*, 2012); phytoecdysteroids such as stigmasterol, 22-monacetomide, 20-hydroxyecdysone (20E) and γ-sitosterol (Aly *et al*., 2011; Nyamoita, 2013) and antijuvenile hormones such as precocenes (Saxena & Saxena, 1992). Structural-bioactivity relationship of these compounds has been reported. For instance, steroidal terpenoid ring structures such as of Azadirachtin (**1**) strongly interferes with molting and reproduction fitness by mimicking and/or inhibiting synthesis of insect developmental hormones (Wang *et al.*, 2014). Other compounds inhibit key enzymes involved in insect chitin and cuticle synthesis, modification and degradation causing abortive molting.



2.5 Tea as a candidate bio-larvicide

Emerging evidence showing that tea phytochemicals negatively affect the development and reproduction viability of insect model *D. melanogaster* suggests their potential as candidate bio-insecticides (Lopez *et al.*, 2015). Additionally, Žabar *et al* (2013) and Ranaweera (1996) demonstrated potent larvicidal activity of tea phytochemicals. Polyphenols especially (-)-epigallocatechin-3-gallate (EGCG) (**2**) were reported as inducer of the observed effects. Other bioactive compounds are condensed tannins (proanthocyanidins) (**3**), oligomer or polymers of catechins, which have been reported to induce feeding deterrence and insect growth reducing effects (Barbehenn & Peter Constabel, 2011).



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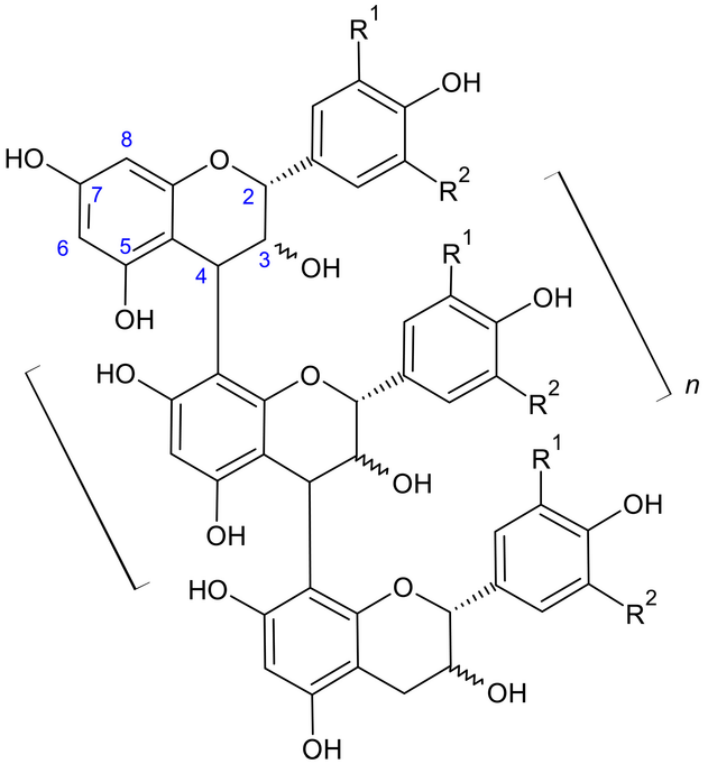


Propelargonidin	$R^1 = H$
	$R^2 = H$
Procyanidin	$R^1 = OH$
	$R^2 = H$
Prodelphinidin	$R^1 = OH$
	$R^2 = OH$

3

2.6 Mosquito development as vector control target

Mosquitoes being holometabolous insects possess a four-stage lifecycle: egg, four larval instars, pupa and adults (Figure 2.3). The lifecycle is indeed complex revolving between aquatic and terrestrial phases for immature stages and adult stage, respectively (Becker *et al.*, 2010). The larval stages feed on organic matter and algae found in water and thus colonize an array of aquatic environments, but majority are restricted to fresh water masses (Harbach, 2007). The evolutionary morphogenetic and phenotypic characteristics acquired on colonization of these larval ecological niches influence mosquito vector competence trait. Larval control is less costly and suppression of large population is possible (Maheu-Giroux & Castro, 2014).

Targeting the immature aquatic stages proves an ideal approach of eliminating malaria (Killeen *et al.*, 2002a; Walker & Lynch, 2007). This has been favored by immobility of larval stages, confinement to water bodies, chemical susceptibility and meager chances of developing resistance (Killeen *et al.*, 2002b). Additionally, perturbation of larval habitat bio-physicochemical characteristics reduces vector competence and vectorial capacity of resultant mosquitoes (Okech *et al*., 2007; Moller-Jacobs *et al*., 2014). Successful stories have been reported in Brazil, Egypt and Zambia (Fillinger & Lindsay, 2011) with increasing efforts of larval source management (LSM) being currently revived in Kenya (Fillinger & Lindsay, 2006), Gambia (Majambere *et al.*, 2007), Tanzania (Chaki *et al.*, 2014; Maheu-Giroux & Castro, 2014), and Burkina Faso (Diabat *et al.*, 2015).

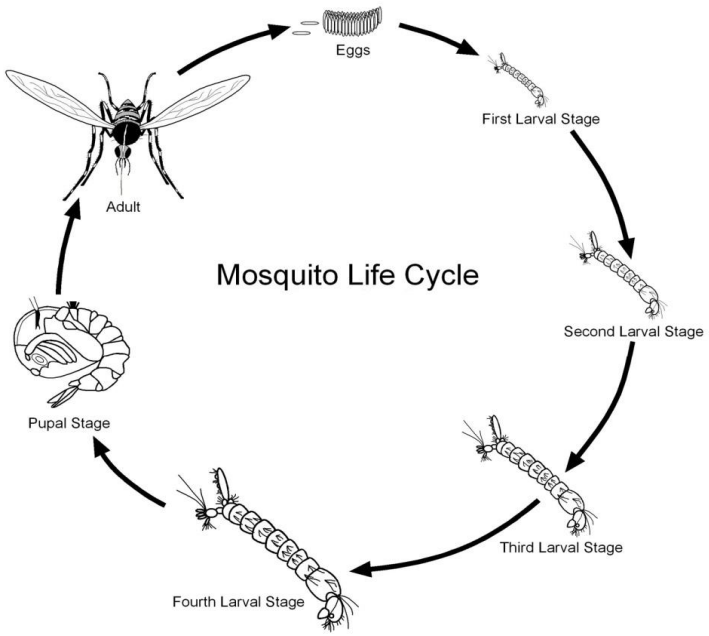


Figure 2.3: Mosquito lifecycle

(Adapted from http://www.nsmad.com/about-mosquitoes/mosquito-biology/).

Mosquitoes develop through two distinct biphasic ecologies, aquatic stages represented by egg, four larval phases and pupal stages while terrestrial ecology dominated by adult stage.

2.7 Developmental physiology of mosquito larvae

Insect development is very complex. The insect growth, development, metamorphosis and reproduction are under genetic, hormone and metabolic control systems (Flatt *et al.*, 2005; Terashima *et al.*, 2005; Badisco *et al.*, 2013). The developmental timing and transition from larval stages to pupal eclosion is under stringent regulatory checkpoints (Rewitz *et al.*, 2013). Primarily, the two developmental neurohormones, 20-hydroxyecdysone (20E) and sesquiterpenoid juvenile hormone (JH), are tightly linked to metabolic control systems for successful insect development cycle (Flatt *et al.*, 2005). These neurohormones are biosynthesized and secreted into the hemolymph to control a cascade of physiological signals that mediate molting (Takaki &

Sakurai, 2003). In insects, growth to a target size following accumulation of adequate nourishment in the fat body induces pulses of ecdysteroid hormone (ecdysone) triggering metamorphosis (Mirth & Riddiford, 2007). This actually determines the resultant adult size. Environmental sensory signaling networks especially IIS and target of rapamycin (TOR) pathway play a key role in insect development (Xu *et al*., 2012; Hatem *et al.*, 2015). They regulate developmental timing by regulating the biosynthesis of developmental hormones upon sensing ideal conditions else induce diapause (Mirth & Shingleton, 2012). Various studies have argued that the neuroendocrine system mounts complex insect size-related and nutritional signals in conjunction to environmental cues, to timely coordinate ecdysone production and secretion (Edgar, 2006; Shingleton, 2011; Koyama *et al*., 2013). Shiao *et al* (2008) argued that the juvenile hormone connects larval nutrition with TOR in mosquitoes. Thus, insulin-like growth factor family regulates growth rates and period by impinging on the endocrine system that coordinates the onset of insect metamorphosis (Colombani *et al*., 2005; Edgar, 2006; Mirth & Riddiford, 2007). Warren *et al* (2006) reported that during the end of third instar stage, three low-level pulses accompanied by a high level peak of ecdysone mediate some physiological changes that are required for transformation of a feeding larva into a pupa. A series of reactions involving Rieske oxygenase and enzymes encoded by Halloween genes including cytochrome P450s induce ecdysone production in the prothoracic gland (Ono *et al.*, 2006; Niwa *et al.*, 2010). It has been documented that, the cytochrome P_{450} enzymes which are up-regulated for xenobiotic metabolic activities synergize the activity of neurohormones for proper growth and development (Warren *et al.*, 2006; Rewitz *et al.*, 2006; Iga & Kataoka, 2012). They also contribute to the adaptation of plant allelochemicals and insecticide resistance (Scott, 1999). The low titers of ecdysteroid during pupal stage inactivate *Corpora allata* thereby initiating pupal metamorphosis into adult mosquito (Takaki and Sakurai, 2003).

2.8 Insect stress responses, survival and life history trade-offs

Insects face the challenge of balancing between physiology and adaptation to heterogeneous environments (López-maury *et al*., 2008). In the event of striving for survival, insects remodel their physiology by allocating resources to sustain vital processes and shut down other metabolic processes (Zera & Harshman, 2001; Ricklefs & Wikelski, 2002). Adaptation has biological costs paid later in life. Constraints in competing for limited resource allocation force insects to forgo some traits in favour for others; survival versus fitness.

Neuropeptide hormones in conjunction with antioxidant systems interplay to alleviate stress in insects (Felton & Summers, 1995; Perić-Mataruga *et al*., 2006). Juvenile Hormone (JH) regulates many insect processes at physiological conditions in addition to regulation of metamorphosis, molting and reproduction (Noriega, 2014). In disturbed homeostasis induced by various exogenous stressors including toxic insecticides, JH plays a role in modulating various metabolic processes aimed at adaptation (Noriega, 2014). Additionally, adipokinetic hormones (AKHs) have been reported to be the main anti-stress hormones that prevent synthetic processes but rather enhance mobilization of energy stores through regulation of uncoupling proteins (UCPs) for stress alleviation (Figure 2.4) (Kodrík *et al*., 2015). The expression levels of AKH in the hemolymph following stress induction has been reported to initiate a cascade of reactions *via* cyclic AMP (cAMP) and phosphokinase C (Bednářová *et al*., 2013) upon binding to G-protein coupled receptors situated on the membranes of target cells (Staubli *et al.*, 2002; Caers *et al.*, 2012). Another study by Bednářová *et al*., (2015) reported that the employment of Forkhead box class O transcription factor (FoxO) by AKH exert protection effect against oxidative stress to the organism. FoxO's role in controlling transcriptional changes in response to stress is centrally due to its cytosolic localization and subsequent translocation into the nucleus thus acting as a transcriptional factor (Bednářová *et al*., 2015). This may induce diapause, slowed metabolism and expression of defense proteins such as heat shock proteins, cytochrome P_{450} monooxygenases and glutathione related proteins (Hahn & Denlinger, 2011; King & MacRae, 2015).

Cellular adaptation to environmental stress relies on a wide range of regulatory mechanisms that are tightly controlled at several levels, including transcription (Savolainen *et al.*, 2013). Adaptive responses constitute changes in gene expression whereby the stressors interact with nuclear receptors (Bednářová *et al.*, 2013). Though, adaptation to novel ecological conditions may confer some degree of advantage, fitness costs paid by the vector actually affect disease transmission dynamics (Mireji *et al.*, 2010). These include reduced egg viability, larval and pupal survivorship, adult emergence, fecundity and net reproductive rates (Mireji *et al.*, 2010).

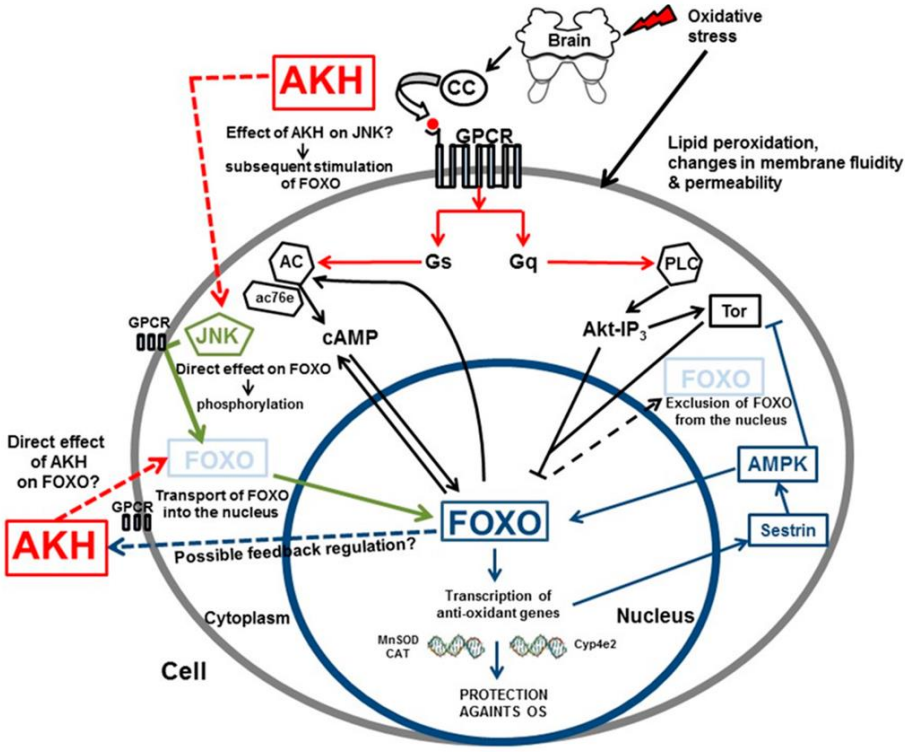


Figure 2.4: Insect hormonal regulation of stress.

Environmental stress is perceived by sensory systems and signals sent to insect brain for decoding. Binding of stressors to G-protein coupled receptors initiate a cascade of reactions involving Forkhead transcription factor (FOXO) recruited by AKH to mediate stress responses. Adapted from Kodrík *et al.*, (2015)

2.9 Analysis of gene expression by *q*PCR

The recent advances in molecular biology and "omics" technologies have made it possible to study gene expression variation in organisms exposed to challenging environments (Orlando *et al.*, 1998). In this study, *q*PCR was deployed to analyze differentially expressed genes in *An. gambiae s.s* larvae exposed to sublethal doses of *C. sinensis* extract which are associated with lifecycle responses (i.e. survival and development). *q*PCR has been quite instrumental in scientific studies due to its robustness and accuracy of predicting abundance of mRNA (Bustin, 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental insects

Mosquitoes used in this study were obtained from *icipe*'s Arthropod Rearing and Quarantine Unit (ARQU). This Kisumu mosquito strain originated from *icipe's* Thomas Odhiambo Campus (Mbita), in Suba District near Lake Victoria and has been established in the insectary since 2003. To simulate natural conditions, the larvae were reared under laboratory conditions of water temperature (28 ± 2˚C), relative humidity of 55-60% and 12:12 h (light: dark) photoperiod. The larvae were reared in large plastic pans (37 × 31 × 6 cm) L × W × H containing distilled water at densities of 200-300 per 3 L and fed on artificial diet Tetramin® fish food (Tetra GmbH, Melle, Germany). The rearing water was replaced with fresh water and diet after every two days. Pupae were held in plastic cups and transferred into standard 30 × 30 × 30 cm rearing cages. Adults were provided with 10% sucrose solution contained in a glass tube (2 × 8 cm) connected to a paper tube as a wick. Female mosquitoes were blood-fed on restrained Swiss albino mice about 4-5 days post-emergence and provided with oviposition plastic containers (11.5 cm in diameter and ~ 6.2 cm in depth, lined interiorly with a piece of filter paper as oviposition site) for egg collection 2–3 days after blood meal. The eggs were hatched under insectarium conditions ready for colony cycle maintenance.

3.2 Collection of plant material and extraction

Fresh immature shoot leaves of tea (clone TRFK 6/8) were bought from Limuru Archdiocesan Farm, Limuru, Kenya (longitude: 01˚07'10S, latitude: 36˚39'37E, elevation: 2,225 m above sea level) in February, 2016. TRFK 6/8 is endowed with properties such as; high black tea quality (fast fermentability and high levels of polyphenols), low yielding and susceptible to water stress with low levels of caffeine (1.7%). The field study did not involve endangered or protected plant species thus no

specific regulatory permission was required during sampling. However, permission to use the plant for this study was granted by Limuru Archdiocesan farmers. The clone under study was authenticated by Mr. Peter Kihara. The tea leaves were first sundried for 1 h then shade-dried at 25 ± 2˚C to a constant weight with intermittent turnings to promote aeration process. The dried tea leaves were ground into powder using an electric grinder (Model 5657; Retsch GmbH, Haan, Germany). The pulverized leaf powder (500 g) was soaked in 2 L of methanol: water (9:1 *v/v*) for 72 h with daily agitation to extract all the nonpolar and polar bioactive agents. The extract was filtered through Whatman® No.1 filter paper and the excess solvent removed using rotor evaporator (Laborota 4000 efficient, Heidolph instruments GmbH & Co. KG, Germany). The residual extract was lyophilized to dryness in a freeze-dryer (Labconco stoppering tray dryer, Labconco Corporation, USA) programmed to an average temperature of 13˚C, vacuum pressure of 998×10^{-3} millibars and collector at -40˚C. The resultant dry extract was stored at -20˚C ready for bioassays.

3.3 Effects of tea leaf extract on the survival and development of *An. gambiae* s.s larvae

To evaluate the effects of the tea extract on survival and development of *An. gambiae* s.s larvae, the extract was reconstituted in absolute ethanol (Fisher Scientific, Loughborough, UK) to required concentrations. The assay was conducted following WHO (1996) and WHO (2005) guidelines for testing mosquito larvicides. The extract (250 mg, 125 mg, 50 mg, 25 mg and 12.5 mg) was separately re-suspended in 1 ml ethanol (Fischer Scientific, Loughborough, UK) and each solution diluted with 499 ml distilled water to make 500 ppm, 250 ppm, 100 ppm, 50 ppm, and 25 ppm. These solutions were dispensed into five beakers each containing 100 ml of test solution. Batches of twenty (20) late early third/early fourth instar larvae (L3/L4) (Kisumu strain) were put into each beaker. For negative experimental control, 1 ml of ethanol (Fisher Scientific, Loughborough, UK) was diluted in 499 ml and dispensed into 5 beakers each containing 20 larvae. The assays were conducted at water temperature 28 ± 2˚C, relative

humidity of 55-60% and photoperiod of 12L:12D. The mortality rates were recorded every 24 h for 72 h. Images of morphological defects induced by the extracts were captured with light microscopy (Leica Corporation, Heerbrugg, Switzerland).

3.4 Bioassay-guided fractionation of tea extract and larvicidal assays

The extract was subjected to gradient-column elution for semi-purification. Silica-packed column (40 × 330 mm) (PYREX®, Corning Inc., USA) was conditioned with n-hexane (Sigma Aldrich, USA) for 3 h. The extract (35 g) was loaded onto the packed silica (Kiesegel 60 M (0.04-0.63 mm), Macherey-Nagel GmbH & Co. KG, Düren, Germany) then eluted with gradient of n-hexane and ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) and finally with methanol. Fractions were chromatographed on thin layer chromatography (TLC) silica plates (ALUGRAM® Xtra SIL G/UV_{254} (0.2 mm), Macherey-Nagel GmbH & Co. KG, Düren, Germany) developed with *n*-hexane and ethyl acetate (1:2 *v/v*) as mobile phase. The plates were air-dried, sprayed with 30% sulfuric acid and baked in oven for detection under UV lamp ($\lambda_{254–365}$ nm). Fractions with similar retention factor (R_f) values were pooled together, rotor evaporated, reconstituted to various doses in absolute ethanol and screened for activity against mosquito larvae.

3.5 LC/ESI-Qtof/MS analysis

About 1.5 mg of the methanolic fraction was re-dissolved in 1 ml of LC-MS grade CHROMASOLV methanol (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 14,000 rpm for 5 min, after which 0.2 µl of the supernatant was automatically injected into LC/ESI-Qtof/MS. The chromatographic separation was achieved on a Waters ACQUITY UPLC (ultra-performance liquid chromatography) I-class system (Waters Corporation, Milford, MA, USA) fitted with a 2.1 mm × 50 mm, 1.7 µm particle size Waters ACQUITY UPLC BEH C18 column (Waters Corporation, Dublin, Ireland) heated to 40˚C and autosampler tray cooled to 5˚C. Mobile phases of water (A) and acetonitrile (B) each containing 0.01% formic acid were employed. The following

gradient was used: 0–5 min, 10% B; 5–7 min, 10–60% B; 7-10 min, 60–80% B; 10–15 min, 80% B; 15-18 min, 100% B; 18–20 min, 100% B; 20–21.5 min 100-10% B; 21.5–25 min 10% B. The flow rate was held constant at 0.4 ml min^{-1}. The UPLC system was interfaced with electrospray ionization (ESI) to a Waters Xevo QToF-MS operated in full scan MS^E in positive mode. Data were acquired in resolution mode over the *m/z* range 100–1200 with a scan time of 1 sec using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature 100˚C and desolvation temperature of 350˚C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25–45 eV was applied in the T-wave collision cell using ultrahigh purity argon (≥ 99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; $[M+H]^+$ = 556.2766) was sampled at 10 sec intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50–1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 2-propanol/water (*v/v*). MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using mono-isotopic masses with a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms was set as follows: carbon ≤ 100; hydrogen ≤ 100; oxygen ≤ 50; nitrogen ≤ 6; sulfur ≤ 6 (Jared *et al.*, 2015). The LC/ESI-Qtof/MS data acquisition and analysis were based on the following parameters: mass accuracy (ppm) = 1 000 000 × (calculated mass-accurate mass) ÷ calculated mass; fit conf % is the confidence with which the measured mass (accurate mass) matches the theoretical isotope models of the elemental composition in the list; elemental composition is a suggested formula for the specified mass. This reflects a summation of the quantities of elements, isotopes or superatoms that can compose the measured data, calculated using the following atomic masses of the most abundant isotope of the elements: C=12.0000000, H=1.0078250, N=14.0030740, O=15.9949146, S=31.9720718. The empirical formulae generated were used to tentatively predict

chemical entities based on the online databases (Chemspider, Metlin (Smith, 2005) and published literature (Tavares *et al*., 2012; Lambert *et al.*, 2015).

To confirm the presence of proanthocyanidins, vanillin-HCl assay was performed as described in Butler *et al*., (1982). A small amount 0.5% vanilin was solubilized in 2 ml methanol. The mixture was added to 4% HCl and heated in water bath set at 20˚C for 30 min. About 1.5 mg of the test sample was added into the screening solution, incubated for 30 min at room temperature and the color change noted.

3.6 Primer design

Genes selected for the study included *AgamJHAMT*, *AgamILP1*, *AgamCYP6M2* and *Hsp70* based on the induced phenotypic traits following larval exposure to bioactive fraction of tea extract and their relevance in choice is explained here-in. *AgamJHAMT* encode for a vital enzyme that catalyzes the rate limiting step in JH biosynthesis and thus a key regulator of metamorphosis in insects (Shinoda & Itoyama, 2003). *ILP1* is a member of the regulatory signal transduction peptides that perceives sensory signals from the environment and enroutes them for decoding into behavior responses. During juvenile stages, insulin-like signaling nexus nutritional-dependent body size to neuroendocrine cells for regulating metamorphosis (Flatt *et al*., 2005). Until the insect attains the adequate size, insulin-like peptides trigger the secretion of insect developmental hormones from their respective glands (Hatem *et al*., 2015). Xenobiotic exposure to insects induces expression of cytochrome p450 genes and heat shock proteins for cell protection against severity effects (David *et al*., 2006). *CYP6M2* is among the enzymes involved in insecticide detoxification. In this study, relative expression analysis of the selected genes was used to demonstrate the genetic changes of mosquito larvae in response to tea extract exposure. Based on the induced mosquito phenotypes, primers targeting these probable responsive genes were designed *in silico* using Primer3 (version 0.4.0) for α-tubulin, insulin-like peptide gene 1 (*AgamILP1*), heat shock protein 70kDa (*Hsp70*), *CYP6M2* and JH acid methyltransferase (*AgamJHAMT*)

sequences downloaded from NCBI (www.ncbi.nlm.nih.gov/) (Table 3.1). Gene-specific primers had annealing temperatures ranging from 54.3˚C–62.5˚C and the sizes of amplification products ranged between 146-245 bp.

Table 3.1: Primers for *q*PCR studies

Gene	Accession number		Primer sequences (5'-3')	Product size (bp)
α-tubulin (Internal reference)	**AGAP010971**	Fwd Rev	CAATGAGGCGATCTACGACA TACGGCACCAGATTGGTCT	171
Insulin-like peptide gene 1	**AGAP010605**	Fwd Rev	GCTTCTGCTCGTTCTGCTCT ACTTTTTGCGGAATCCATTG	152
Heat shock protein 70kDa (*Hsp70*)	**AGAP004581**	Fwd Rev	ACGCCAACGGTATTCTGAAC ACAGTACGCCTCGAGCTGAT	197
CYP6M2	**AGAP008212**	Fwd Rev	AGGTGAGGAGAGTCGACGAA ATGACACAAACCGACAAGG	235
JH acid methyltransferase	**AGAP005256**	Fwd Rev	GAAGGGCTGGACAATTTGAA TTCTCTTCGCTGCCAGTTTT	146

Fwd – Forward primer, Rev- Reverse primer

3.7 Total RNA extraction

Mosquito larvae were anaesthetized by chilling them on ice for 20 min followed by two washes in phosphate buffered saline (PBS, pH 8.0). RNA extraction materials (microfuge tubes, pipette tips and pestles) were treated with 0.1% diethyl pyrocarbonate (DEPC) for 1 h and autoclaved to destroy residual DEPC. Total RNA was extracted from pools of five replicates (n = 5) of untreated and treated larvae samples after 72 h post exposure using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Briefly, the larvae homogenate was re-suspended in 550 ml of cold phosphate buffer saline (PBS, pH 7.4) and centrifuged at 300 × g for 5 min at 4˚C. The supernatant (250 µl) was transferred into a clean Eppendorf tube containing 750 µl TRIzol reagent (Invitrogen, Carlsbad, CA) and incubated at room temperature for 10 min to dissociate nucleoprotein complexes.

For phase separation, 200 µl of molecular biology grade chloroform was added to the supernatant and vortexed vigorously for 5 sec. The samples were incubated at room temperature for 10 min and centrifuged at 12000 × g for 15 min at 4˚C. The clear upper aqueous solution containing the RNA was carefully transferred into RNase free 1.5 ml Eppendorf tube. To precipitate the RNA, 500 µl of molecular biology grade absolute isopropanol was added to the samples and incubated for 1 h at -20˚C. The samples were centrifuged at 12000 ×g for 20 min at 4˚C. A white pellet formed at the bottom of the tube was air-dried after removal of supernatant. The RNA pellet was washed twice with 1 ml of ice cold 70% ethanol by centrifugation at 7500 × g for 10 min at 4˚C. Ethanol was then discarded and the remaining traces of ethanol were removed by pipetting. The RNA pellet was air-dried for 10 min on a clean bench. Finally, RNA pellet was dissolved in 30 µl of RNase free water and stored at -80˚C until required.

The extracted RNA was quantified using Nanodrop reader (Thermo scientific, UV-VIS spectrophotometer, California, USA). Blanking was done with 1 µl nuclease free water prior loading 1 µl of RNA sample. Data was exported onto Microsoft Excel spreadsheet for further analysis.

Integrity of isolated RNA was studied on a 1.2% non-denaturing agarose gel stained with 0.03 mg/µl ethidium bromide. Five microliters of RNA sample were mixed with 2 µl of RNA loading dye and loaded into the wells of agarose gel alongside a 1kb RNA ladder (Thermo Scientific, USA). The gel was run for 1 h at 80 V and gel results visualized under UV transilluminator.

3.8 cDNA synthesis

First strand cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription kit as per manufacturer's instructions (Applied Biosystems, USA). Briefly, the DNase treatment reaction consisted of 1 ng RNA template, 1× Reaction Buffer with $MgCl_2$, 6 µl of RNase-free DNase I, and topped up with nuclease free water to make a total volume of 60 µl. The reactions were incubated at 37˚C for 30 min, followed by

addition of 6 µl 50 mM EDTA and incubation for a further 10 min at 65˚C. Subsequently, cDNA synthesis was conducted by addition of 1× reaction buffer, 0.5 µl RNase inhibitor, 1× 100 mM dNTPs mix, 1× RT random primers, 1 ng RNA template and 0.5 µl MultiScribe® MuLV reverse transcriptase in a total reaction volume of 10 µl, under cycling conditions:- 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. The cDNA generated was stored at -20˚C ready for RT-*q*PCR analysis.

3.9 Gene expression analysis

Expression profiles of *CYP6M2* (AGAP008212), *Hsp70* (AGAP004581), *Insulin-like peptide gene 1* (AGAP010605) and *JH acid methyl transferase* (AGAP005256) in *C. sinensis*-treated larvae and their respective controls were separately assessed with Fast SYBR® green detection dye using Stratagene Mx3005P real time *q*PCR system (Agilent technologies, USA). Amplification of the aforementioned genes was performed using GenScript designed oligonucleotide primers in Table 3.1. Quantification of cDNA samples (3 µg) was performed in triplicates with 6.25 µl of SYBR® green master mix (Thermo scientific, USA) in presence of 0.5 picomoles of respective primers and α-tubulin set as internal reference gene (Table 1). The cycling conditions were programmed as: - initial step of 95˚C (10 min), 40 cycles of 95˚C (15 sec), 59˚C for 30 sec, 72˚C for 30 sec followed by 1 cycle of 95˚C (30 sec), 55˚C (1 min), and 95˚C (30 sec) for all sample genes. The gene expression levels of the samples were determined using $2^{-\Delta\Delta Ct}$ method following normalization with internal control.

3.10 Statistical analysis

The experimental data were entered in Microsoft Excel 2010 and exported into R software version 3.2.3 (R Team, 2014) for analyses. Corrected mortality rates are expressed as % mean ± S.D for each dose tested. The test concentrations were $\log_{10}$-transformed to reduce variations prior to fitting a dose-response model for estimating lethal dose concentrations. Non-linear regression using *glm* function in R with *probit* link and quassi binomial distribution error was used to estimate the lethal concentrations

of crude extract and its active fraction. The LC_{50} of both the crude extract and its active fraction were estimated from the *glm* output using the *dose.p* function in MASS Package in R. Data from RT-*q*PCR were normalized by α-tubulin gene (internal reference) and the expression fold changes obtained by $2^{-\Delta\Delta CT}$(Livak & Schmittgen, 2001). ΔC_T was defined as the value of subtracting the C_T value of endogenous control from the C_T value of the target messenger RNA (mRNA). The significance difference between treatment means was determined using analysis of variance (ANOVA) while that of gene expression levels determined by Student's t-test with values of $P \leq 0.05$ considered significant. Graphs were designed using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, California, USA).

CHAPTER FOUR

RESULTS

4.1 Phytochemical analysis

Bioassay-guided fractionation of *C. sinensis* extract yielded 11 fractions. Upon screening for activity, only the polar methanolic fraction 11 showed the maximum larval mortality equivalent to that attained by crude extract at 500 ppm (Appendix 2). Analysis of this fraction using LC/ESI-Qtof/MS predicted abundance of condensed tannins (oligomerics) with *m/z* $[M/H]^+$ 593.2830 (15.2641%) and retention time (R_t) of 16.15 min (Table 4.1 & Figure 4.1). Other prominent mass spectrum peaks that denote bioactive compounds were *m/z* 195.0919 ($C_8H_{10}N_4O_2$) (**2**), 303.0516 ($C_{15}H_{10}O_7$) (**3**), 287.0566 ($C_{15}H_{10}O_6$) (**4**), 903.2551 ($C_{42}H_{46}O_{22}$) (**5**) and 887.2620 ($C_{42}H_{46}O_{21}$) (**6**). A database search tentatively identified the compounds represented by peak 2 (Rt 3.06 min) as Caffeine, peak **3** (Rt 4.42 min) Quercetin, peak **4** (Rt 5.10 min) Kaempferol, peak **5** (Rt 8.56 min) Kaempferol 3-[2"-(6"-coumaroylgluocysyl)-rhamnoside]-7-glucoside and peak **6** (Rt 8.56 min) Kaempferol 3-rhamnosyl-(1-3)(4"-p-coumarylrhamnosyl)(1-6)-glucoside (Table 4.1 and Figure 4.1). A red color change obtained from vanillin-HCl assay confirmed the presence of proanthocyanidins (Appendix 3).

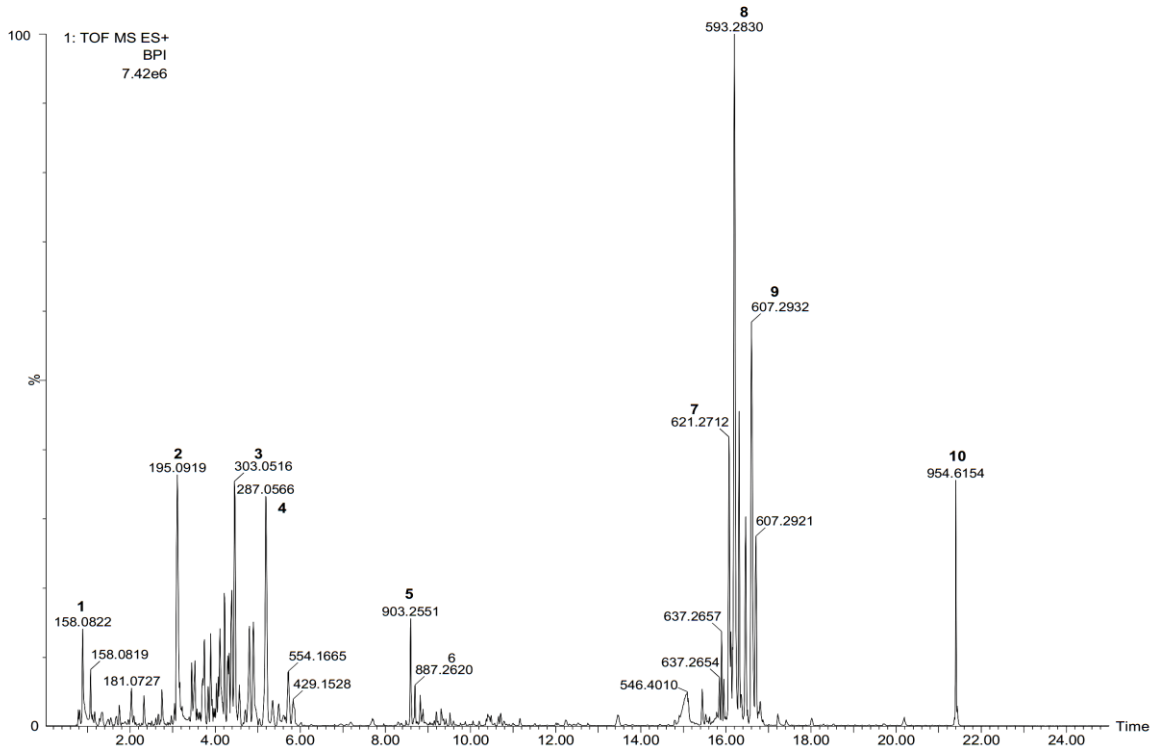


Figure 4.1: LC/ESI-Qtof/MS chromatographic analysis of methanolic fraction of *Camellia sinensis* leaf extract.

Six major bioactive compounds were detected as follows:- **2**-Caffeine, **3**-Quercetin, **4**-Kaempferol, **5**- Kaempferol 3-[2"-(6"-coumaroylgluocysyl)-rhamnoside 7-glucoside, **6**- Kaempferol 3-rhamnosyl-(1-3)(4"-p-coumarylrhamnosyl)(1-6)-glucoside, **8**-Proanthocyanidin. Proanthocyanidin *m/z* 593.2830 was the abundant compound (15.2641%).

Table 4.1: Mass spectrometry data and identification results of the major constituents of *Camellia sinensis* bioactive fraction by LC/ESI-Qtof/MS.

Peak No.	R_t (min)	*m/z* $[M+H]^+$	Peak area (%)	Chemical formula	Tentative identification
1	0.84	158.0822	2.1269	-[a]	Unknown[b]
2	3.06	195.0919	7.0454	$C_8H_{10}N_4O_2$	Caffeine
3	4.42	303.0516	4.9190	$C_{15}H_{10}O_7$	Quercetin
4	5.10	287.0566	6.0235	$C_{15}H_{10}O_6$	Kaempferol
5	8.52	903.2551	1.4543	$C_{42}H_{46}O_{22}$	Kaempferol 3-[2"-(6"-coumaroylgluocysyl)-rhamnoside 7-glucoside
6	8.56	887.2620	0.6415	$C_{42}H_{46}O_{21}$	Kaempferol 3-rhamnosyl-(1-3)(4"-p-coumarylrhamnosyl)(1-6)-glucoside
7	16.00	621.2712	6.3769	-[a]	Unknown[b]
8	16.15	593.2830	15.2641	$C_{31}H_{28}O_{12}$	Proanthocyanidin
9	16.52	607.2932	9.4702	$C_{36}H_{38}N_4O_5$	Phenyl peptide
10	21.44	954.6154	2.3624	-[a]	Unknown[b]

[a]- represents missing chemical formula for the compounds 1, 7 and 10
[b]-"Unknown" under tentative identification column means that compound was unidentifiable from searched databases and published literature.

4.2 Effect of tea extract and its active fraction on survival and development of *An. gambiae* s.s larvae

An. gambiae s.s larvae were exposed to increasing doses of *C. sinensis* extract and effects of the extract on larval survival and development evaluated. The experimental findings showed that the extract evoked toxicity to the developing larvae in a dose-dependent relationship (Table 4.2; Figure 4.2). At 250 and 500 ppm, the extract evoked > 90% mortality within the first 24 h post exposure. Median lethal dose (LC_{50}) of the crude extract at 24 h was 117.15 ppm (95% confidence interval 112.86-127.04) and reduced sharply to 87.11 ppm at 48 h and 72 h post exposure. Moderate mortality was observed in larvae exposed to 100 ppm. A 100% larval survival was observed in control and exposure at 50 ppm and 25 ppm. However, these sublethal doses induced developmental period extension and growth disruption effects (Figure 4.3). Additionally, mosquito larvae exposed to sublethal doses exhibited delayed molting, delayed pupation

by 7-10 days and delayed toxicity-induced killing. Similar observations were observed on exposure to proanthocyanidins-rich bioactive fraction. Larval mortality equivalent to 500 ppm of crude extract, which was 100%, was exerted at 25 ppm within 24 h post exposure to bioactive fraction terminating mosquito development prematurely (Table 4.2). Effective dose ranged between 3.60-5.52 ppm (95% CI 0.29-9.65). Sublethal doses (1, 2.5 & 5 ppm) protracted larval development inducing growth disruption effects similar to those evidenced at 25–100 ppm of crude extract (Figure 4.3). ANOVA test showed significant difference between the treatment means (ANOVA, $P < 0.001$).

Table 4.2: Acute toxicity of crude tea (*C. sinensis*) extract and active fraction on exposure to L3/L4 instars of *An. gambiae* s.s for 24 h, 48 h and 72 h post-exposure.

	Larval mortality rates (% Mean ± S.D)*						Lethal Concentrations (ppm)	
Time interval	**Concentrations**						**LC_{50}**	**95% CI**
Crude extract								
	500 ppm	**250 ppm**	**100 ppm**	**50 ppm**	**25 ppm**	**Control**		
24 h	100±0.00	91±9.62	39± 6.52	0±0.00	0±0.00	0±0.00	117.15	112.86-127.04
48 h	100±0.00	98±2.24	62±10.37	0±0.00	0±0.00	0±0.00	87.11	82.57-112.82
72 h	100±0.00	100±0.00	84±11.94	0±0.00	0±0.00	0±0.00	87.11	82.57-112.82
Active fraction								
	25 ppm	**10 ppm**	**5 ppm**	**2.5 ppm**	**1 ppm**	**Control**		
24 h	100±0.00	69±17.10	42± 8.37	25±14.58	0±0.00	0±0.00	5.52	2.68-9.65
48 h	100±0.00	78±2.24	56±12.94	32±14.40	0±0.00	0±0.00	4.45	1.55-8.71
72 h	100±0.00	88±9.08	70±11.18	38±14.40	0±0.00	0±0.00	3.60	0.29-8.71

LC- lethal concentration, S.D – Standard Deviation, CI- Confidence interval, LC_{50} – lethal concentration that killed 50% of test mosquito larvae population, * - Mean values are significantly different $p \leq 0.05$

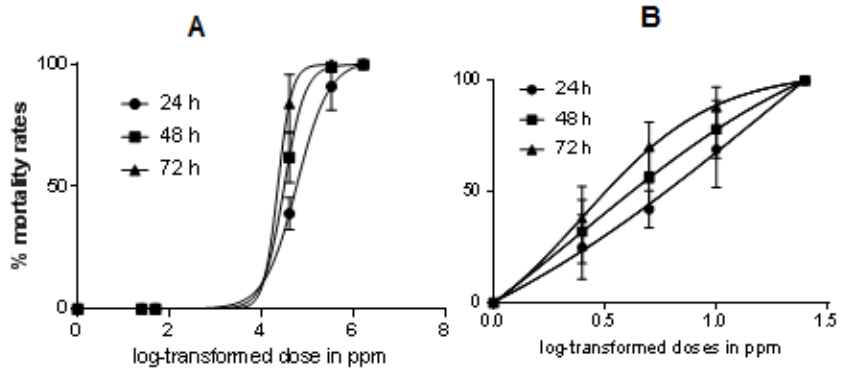


Figure 4.2: Dose response curves for crude tea extract and bioactive fraction at 24 h, 48 h and 72 h post exposure relative to test control.

Doses of the extract are log-transformed. **A:** Dose response fitted model of *An. gambiae* s.s larvae to crude extract of *C. sinensis* at different exposure time periods (24 h, 48 h and 72 h). **B**: Dose response fitted model of *An. gambiae* s.s larvae exposed to bioactive fraction at different time periods (24 h, 48 h and 72 h). Each point on the curve represents percentage mean (± S.D) larval mortality of five replicates for a particular dose.

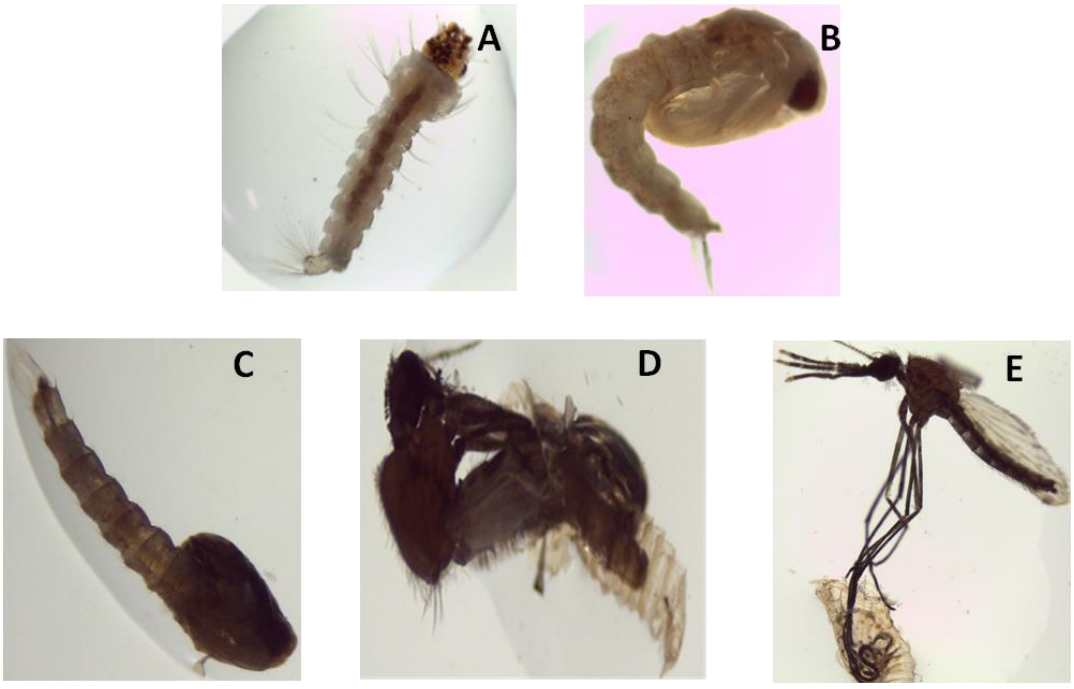


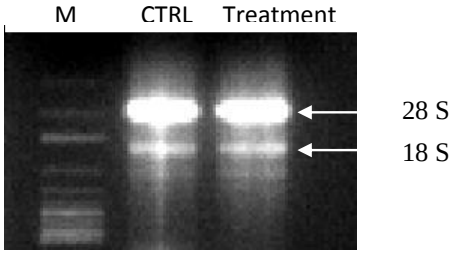
Figure 4.3: Growth disruption effects of *C. sinensis* on *An. gambiae* s.s larvae.

A: Normal fourth instar larvae, **B:** Normal larval-pupal intermediate**, C:** Abnormal larval-pupal intermediate, **D:** Arrested emergence, **E:** Failed emergence and adult legs stuck in pupal caste during eclosion (light microscopy magnification 25×). The extract induced growth reducing effects in *An. gambiae* s.s mosquito larvae.

4.3 Effects of tea extract on *An. gambiae* s.s larval gene expression

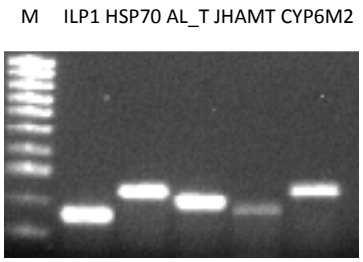
Exposure to sublethal doses of *C. sinensis* bioactive fraction differentially regulated larval genes associated with various physiological processes relative to test controls (Table 4.3 & Figure 4.4). Generally, nearly all tested genes were considerably suppressed compared to controls (Table 4.4 & Figure 4.4). The fraction significantly repressed expression levels of *AgamILP1* (AGAP010605) (t = -22.626, $P < 0.001$), *AgamJHAMT* (AGAP005256) (t = -110.21, $P < 0.001$) and *CYP6M2* (AGAP008212) (t = -179.12, $P < 0.001$) with up-regulation of *HSP70* (AGAP004581) (t = -80.007, $P < 0.001$; Table 4.3). *CYP6M2* was the most downregulated gene (Δ fold 621.6678) followed by *AgamJHAMT* (Δ fold 265.0278) and slightly reduced *AgamILP1* (Δ fold 9.7136). Transcripts encoding 70 kDa heat shock protein were up-regulated by magnitude of 159.7863 fold. *q*PCR amplification profile of the tested larval genes is presented in Appendix 5. Furthermore, agarose gel photos showing successful total RNA isolation and gene-specific primer optimization are presented in Figure 4.4.

A



1.5% non-denaturing gel showing 28S and 18S bands of RNA samples

B



1.5% Agarose gel showing various gene amplicons during primer optimization

Figure 4.4: 1.5% agarose gels of larval RNA samples and primer optimization. A: M: Molecular marker; CTRL: Control

Table 4.3: Relative mean (± S.D) expression levels of various genes in *An. gambiae* s.s larvae in relation to α-tubulin (endogenous reference gene)

Sample/Gene	*AgamILP1*	*AgamJHAMT*	*HSP70*	*CYP6M2*
Control	3.03 ± 0.066	0.76 ± 0.07	5.13 ± 0.152	-2.15 ± 0.07
Treatment	6.30 ± 0.241*	8.81 ± 0.107*	-2.19 ± 0.045*	7.13 ± 0.122*

***ILP1*-Insulin like peptide gene 1, *HSP70*- Heat shock protein 70 kDa, *CYP6M2*- Cytochrome p450 family 6 subclass M2, *JHAMT*- Juvenile hormone acid methyltransferase, S.D- Standard deviation, *-Mean expression values are statistically significant different relative to controls (Student t-test, $P < 0.001$)**

Table 4.4: Normalized expression levels of various genes in *An. gambiae* s.s larvae exposed to *C. sinensis* extract relative to test controls

Gene ID	$^{\Delta}C_{T\ (control)}$	$^{\Delta}C_{T\ (treatment)}$	$^{\Delta\Delta}C_{T}$	Fold change ($2^{-\Delta\Delta}C_{T}$)
AgamILP1	3.03	6.31	3.27	-9.6465
AgamJHAMT	0.76	8.81	8.05	-265.0278
Hsp70	5.13	-2.19	-7.32	159.7863
CYP6M2	-2.15	7.13	9.28	-621.6678

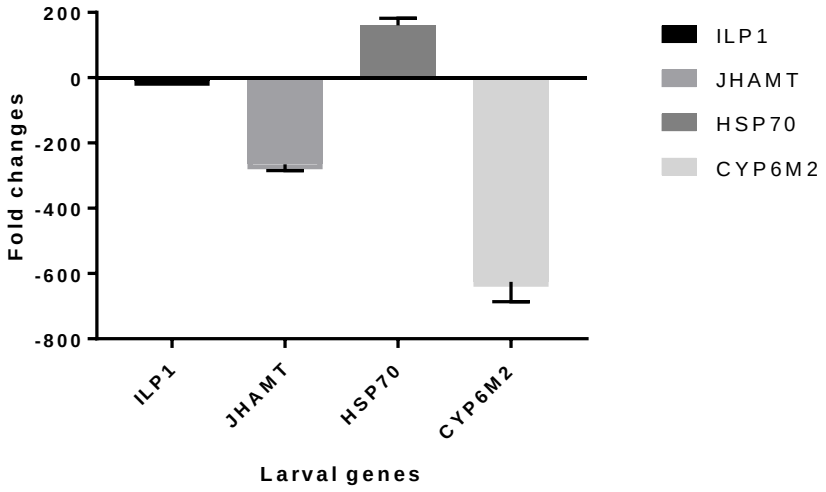


Figure 4.5: Effect of tea proanthocyanidin-rich fraction on larval gene expression.

Treated *An. gambiae* s.s larvae exhibited significant downregulation of genes involved in signal transduction (*ILP1*), JH biosynthesis (*JHAMT*) and xenobiotic metabolism (*CYP6M2*) relative to test controls (Student t-test, $p < 0.05$). *Hsp70* was observed to be significantly upregulated in treated larvae in comparison to test control group (t-test, $p = 0.00045$).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

With an aim to evaluate whether tea (*C. sinensis*) could be a potential source of mosquito control agents, this study investigated its efficacy against the aquatic immature stages of *An. gambiae* s.s. This is the first study reporting the bio-efficacy of tea leaf extract against malaria vectors. Strikingly, tea extract significantly reduced larval survival by evoking > 90% larval mortality at 250 ppm and 500 ppm within 24 h post-exposure and induced growth disruption effects at sublethal doses. The appreciable toxicity of crude extract suggested presence of active phytochemicals which necessitated further fractionation to isolate the bioactive principles. Fractionation of the tea extract led to a polar portion with six major known bioactive compounds tentatively identified as; proanthocyanidins (condensed tannins), caffeine, quercetin, kaempferol and its *o*-glycosides.

Previous studies reported bioactivity of tea against *D. melanogaster*, *Aedes aegypti*, and *Ae. albopictus* (Ranaweera, 1996; Zabar *et al*., 2013; Lopez *et al.*, 2015). Consistent with these findings, tea phytochemicals demonstrated bio-efficacy against mosquito larvae. Contrary to our dosage, high doses of the tea extracts (10 mg/ml-75 mg/ml translating to 10,000–75,000 ppm) were used to achieve equal larvicidal potency. This could be attributed to difference in susceptibility of test insects, variation of geographical location of the test plant, extraction method and difference in abundance of bioactive principles. The aforementioned studies attributed toxicity effects to (-)-epigallocatechin-3-gallate (EGCG), the abundant polyphenolic constituents of *C. sinensis*. Surprisingly, no traces of catechins were detected within the bioactive fraction tested in this study possibly due to oxidative fermentation reactions. Proanthocyanidins were the most abundant compounds detected and strongly associated with the observed effects. However, proanthocyanidins (condensed tannins) are polyphenolic products of

catechins epimerization playing defensive roles against fungal and herbivory attacks in tea (Dixon *et al.,* 2005). Previous reports confirmed that condensed tannins (abundant bioactive compounds) evoke toxicity to insect herbivores eliciting defensive response (Barbehenn & Peter Constabel, 2011). Tannins are bitter polyphenols with mouth puckering effect associated with feeding deterrence. Additionally, they have been reported to be prone to oxidation at high pH of insect guts resulting in production of semi-quinones radicals and highly reactive oxygen species, evoking toxicity (Barbehenn & Peter Constabel, 2011). It was observed in this study that exposure of mosquito larvae to tea extract deterred feeding evidenced by presence of feed remnants at the bottom of test beakers, and killed the larvae terminating their development immaturely. On the other hand, tannins are good antioxidants and mediate life extension properties (Amarowicz, 2007). In this regard, sublethal exposure of mosquito larvae resulted in protracted developmental period. The results of this study show similarity to those obtained by Molan *et al.*, (2003) and (Molan & Faraj, 2010) who studied the effects of condensed tannins on nematodes. The other bioactive compounds could have contributed towards exerting synergistic or racemic mixture effects to proanthocyanidins. Caffeine has been reported to interfere with mosquito larval development (Laranja *et al*., 2003). The hydroxylflavones, quercetin and kaempferol, induce cell cycle arrest by inhibiting CDC25A tyrosine phosphate at G2/M phase and/or inducing apoptosis (Aligiannis *et al*., 2001). In nature, polyphenolic compounds form part of defense against fungal attacks and insect herbivory in plants (War *et al*., 2012). They provoke feeding deterrence with intense disorganization of midgut epithelia cells upon ingestion, which concomitantly reduces insect survival and development (Procópio *et al*., 2015). The fact that tea polyphenolic compounds exert anti-carcinogenic effect by inducing cell cycle arrest, apoptosis and growth inhibition could also be implicated in this study (Gupta *et al*., 2000; Khan *et al*., 2006). The post-embryonic stages of insects comprise of cell proliferation and DNA replication events preceding growth and morphogenetic organization (Lee & Orr-Weaver, 2003). As proanthocyanidins are pro-oxidants and pro-apoptotic molecules that astringently precipitate cellular proteins

(Frazier *et al*., 2010), they could presumably halt these events in developing insects resulting to death. Also, the compounds bind to nucleic acids, increasing topoisomerase II DNA cleavage activity, inducing DNA breaks and reduced cell survival (Kuzuhara *et al*., 2006; Timmel *et al*., 2013). Taken together, these mechanisms could be attributable to the impaired larval development and toxicity.

Interestingly, proanthocyanidins-rich fraction at sublethal dose of 5 ppm induced effects similar to those exerted by insect growth regulators (IGRs) (Staal, 1975; Dhadialla *et al.*, 1998; Tunaz & Uygun, 2004). Treatment with proanthocyanidins-rich fraction resulted in protracted larval phase developmental period by 7-10 days, delayed molting, and pupal eclosion and in addition, this treatment arrested adult emergence. These findings correlate with those obtained by Lopez *et al*., (2015) that tea polyphenolic constituents significantly delayed *D. melanogaster* larval development and maturation. The identified bioactive compounds lack structural similarity to insect development hormones, ecdysteroid 20-hydroxyecdysone (20-E) and sesquiterpenoid juvenile hormone (JH). However, the presence of flavonoid-like polyphenols in larval breeding water could have modulated signaling networks thus interfering with larval development (Mitchell *et al.*, 1993; Cameron *et al.*, 2008). Of importance is the insulin/insulin-like pathway, a regulatory signaling pathway that coordinates insect growth and metamorphosis by regulating biosynthesis of development hormones (Riehle & Brown, 1999; Edgar, 2006; Pérez-Hedo *et al*., 2013). Growth and development transitions in immature insects are orchestrated by morphological and ultra-structural changes regulated by coordinated actions of JH, ecdysone and eclosion hormones (Rewitz *et al.*, 2013). Insulin/insulin-like signaling interplay between the developmental events to ensure static allometry in holometabolous insects (Shingleton *et al.*, 2007; Shingleton *et al.*, 2008; Mirth & Shingleton, 2012; Nijhout *et al.*, 2014). Hence, any exogenous agent that interferes with either the signaling networks or homeostasis of the insect developmental hormones result in abnormal growth and development as observed in Figure 4.3. Similar to other plant-based polyphenols studied, implication of

insulin/insulin-like pathway modulation has been associated with life extension in developing *D. melanogaster* larvae (Schriner *et al*., 2014).

In many organisms, cellular toxicity and adaptive responses are directly linked across transcriptome, proteome and phenotype (Diz & Rolán-Alvarez, 2012; Pillai *et al.*, 2014). To survive in heterogeneous environments, organisms inductively remodel their genome to accommodate the stressor effects (Causton *et al.*, 2001; de Nadal *et al.*, 2011). This is characterized by tuning gene expression levels (López-maury *et al.*, 2008). Likewise, insects show great phenotypic plasticity in accommodating environmental stress. Gene expression profile revealed that the extract significantly modulated nearly all the studied larval genes, providing clue on the probable molecular targets of the extract. Genes associated with JH biosynthesis (*AgamJHAMT*), signal transduction (*AgamILP1*) and xenobiotic detoxification (*CYP6M2*) appeared repressed relative to test controls with concomitant up-regulation of *Hsp70*. Downregulation of *AgamILP1* correlated to repressed expression of *AgamJHAMT* (Sheng *et al.*, 2011). This is true because the two genes are co-expressed in the same gland-*corpora allata* of insects- to regulate various physiological functions *viz* metamorphosis (Jindra *et al*., 2013), stress regulation (Rauschenbach *et al*., 2014), mating (Kuo *et al*., 2012), oocyte maturation (Gulia-Nuss *et al*., 2011), vitellogenesis (Badisco *et al.*, 2013; Hansen *et al.*, 2014) ageing and metabolism (Enell *et al.*, 2010). Deletion of insulin-like peptides has been reported to cause growth defects and retardation in *D. melanogaster* (Rulifson *et al.*, 2002; Zhang *et al.*, 2009). In this regard, repressive effect exerted on insulin signal transduction pathways negatively affects JH-dependent downstream processes (Flatt, 2005; Tu *et al.*, 2005). More importantly, insulin sensory systems gauge insect larval nutrient-dependent body size and environmental quality to activate ecdysone and JH producing glands hence influencing developmental timing (Edgar, 2006; Koyama *et al.*, 2013; Hatem *et al*., 2015). Inputs from these signals may induce or delay production of developmental hormones depending on the suitability of the prevailing conditions (Tennessen & Thummel, 2011; Rewitz *et al.*, 2013). Findings from this study indicate that direct effects of proanthocyanidins-rich fraction on larval insulin cascade could have

restricted dietary feeding limiting development progression a similar phenomenon reported on *D. melanogaster* (Schriner *et al*., 2013) with simultaneous repression of JH biosynthesis from *Corpora allata.*

Juvenile hormone acid O-methyltransferase (JHAMT), an enzyme that catalyzes the rate limiting step of methylation in JH biosynthesis, is the master regulator of insect metamorphosis (Shinoda & Itoyama, 2003; Defelipe *et al*., 2011). In this study, transcripts encoding JHAMT appeared significantly repressed suggesting reduced hemolymph JH titers required to regulate larval development. These findings directly correlate with the observed delayed molting, larval progression and protracted developmental period towards pupation (7-10 days) relative to test controls that quickly pupated and adults emerged. The dramatic repression of *CYP6M2* co-ordinatively with reduced expression of *AgamILP1* could have meant downstream repression of *AgamJHAMT*. This is because cytochrome p450 monooxygenases epoxidize *AgamJHAMT* substrates prior methylation step (Noriega, 2014) thus reduced expression of upstream genes required for JH biosynthesis translates to down regulation of downstream genes. Consistent with the present findings, compounds with juvenoid activity or inhibitory effect on JH biosynthesis pathway enzymes prolong larval phase development and block pupal commitment towards adult eclosion (Riddiford & Ashburner, 1991). Similarly, knockdown of *AgamJHAMT* gene has been reported to adversely affect metamorphosis suggesting that its repression negatively affects developmental physiology (Minakuchi *et al.*, 2008). Shinoda & Itoyama,(2003) reported that expression of *AgamJHAMT* rapidly rises during the initial stages of final larval instar and then declines towards pupation to allow metamorphosis. However, the findings from this study illustrate that the expression of this gene was significantly repressed at this larval instar. The diminished expression of *AgamJHAMT* translates to the observed abnormal larval-pupal intermediates and disrupted emergence induced by ecdysone following dysregulation of JH levels.

Xenobiotic exposure induces up-regulation of detoxification enzymes and heat shock proteins (David *et al.*, 2006). However, CYP6 enzyme-encoding genes (*CYP6M2*) were observed to be markedly down-regulated on larval exposure to *C. sinensis* extract. The two systems form the first line defense to counteract the pleotropic effects exerted by stressors (King & MacRae, 2015). The polyphenolic compounds within the tea extract are strong antioxidants. However, proanthocyanidins (condensed tannins) are highly vulnerable to oxidation resulting to highly reactive free radicals that erode insect midgut epithelia to mediate defense mechanisms (Barbehenn & Peter Constabel, 2011). While *CYP6M2* genes are highly expressed to detoxify xenobiotics within the midgut and fat body of insects, the experimental exposure of mosquito larvae to proanthocyanidins-rich fraction demonstrated their dramatic down regulation by 621.6678 fold. This great fold change could have been due to overwhelmed incapacity to adequately quench these oxidants. The larvae were then exposed to significant high levels of oxidative stress as depicted by higher expression levels of *Hsp70*. However, the larvae clearly demonstrated extraordinary stress tolerance capacity. These findings agree with those reported by Lopez *et al.*, (2015). Studies have documented that disruption, RNA*i* knockdown and/or genetic ablation of *D. melanogaster* insulin/insulin-like peptide genes resulted in remarkable oxidative stress tolerance (Broughton *et al.*, 2005) which equally agree with current findings. Resistance to oxidative stress has been implicated to increased survival. Downstream of IIS is transcription factor dFOXO, a negative regulator of IIS, that controls cellular responses such as stress, apoptosis, cell cycle control, DNA damage and repair (Jünger *et al*., 2003). Blockade or repression of IIS translates to unphosphorylated state of dFOXO, which then translocate into the nucleus inducing expression of genes that enhance longevity and stress tolerance (Bednářová *et al*., 2015). Taken together, these mechanistic observations seem functionally associated with extended larval development and perturbed metamorphosis exerted by *C. sinensis* extract.

The ultimate goal of vector control is to reduce mosquito vectorial capacity to at least less than 1 malaria reproduction rate (MalERA, 2011). Larviciding complements the current vector control tools by suppressing both indoor and outdoor feeding mosquito populations (Walker & Lynch, 2007). Thus, introduction of xenobiotic compounds that disturb homeostasis of larval environment reduces vector competence and vector capacity by interfering with life history attributes. This study provides evidence showing that exposure of tea extract and its proanthocyanidins-rich fraction to mosquito larvae can contribute significantly in reduction of mosquito population and malaria transmission rates.

5.2 Conclusion

In addition to health effects of tea, the plant leaf extract has the potential of controlling the developmental stages of mosquitoes. Treatment of *An. gambiae s.s* larvae with tea extract reduced their survival and perturbed development progression at LC_{50} 117.15 ppm for crude extract and 5.52 ppm for bioactive fraction. From the results, it is evident that tea chemical constituents comprising of proanthocyanidins, quercetin, caffeine, kaempferol and kaempferol *o*-glucosides were responsible for larvae-killing toxicity, growth disruption effects and developmental period extension in addition to modulating various larval genes.

5.3 Recommendations

This study recommends that:-

(i) Tea chemical constituents could be tapped for mosquito larval control.
(ii) Different bioactive chemical constituents identified to be tested singly and/or in combinations to evaluate their contributions to overall activity against mosquito larvae.
(iii) Semi-field and field trial evaluations to be performed to ascertain the full potential of the plant extract and its bioactive constituents in controlling mosquitoes including the effects on adult life-history traits.

(iv) In this study, environmental safety of the extract especially to aquatic non-target species was not assessed hence recommended.

(v) Up-scaling of the bioactive product through synthetic chemistry to ease large scale application.

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APPENDICES

Appendix 1: Publications

Muema *et al. Parasites & Vectors* (2016) 9:512
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Parasites & Vectors

RESEARCH Open Access

Potential of *Camellia sinensis* proanthocyanidins-rich fraction for controlling malaria mosquito populations through disruption of larval development

Jackson M. Muema[1*], Joel L. Bargul[1,2], Steven G. Nyanjom[1], James M. Mutunga[3] and Sospeter N. Njeru[4]

Abstract

Background: *Anopheles arabiensis* and *A. gambiae* (*sensu stricto*) are the most prolific Afrotropical malaria vectors. Population control efforts of these two vectors have been hampered by extremely diverse larval breeding sites and widespread resistance to currently available insecticides. Control of mosquito larval stages using bioactive compounds of plant origin has the potential to suppress vector populations leading to concomitant reduction in disease transmission rates. In this study, we evaluated the efficacy of *Camellia sinensis* crude leaf extract and its fraction against the larvae of *A. arabiensis* and *A. gambiae* (*s.s.*).

Methods: Late third/early fourth instar larvae (L3/L4) of *A. arabiensis* and *A. gambiae* (*s.s.*) were exposed to increasing doses of *C. sinensis* leaf extract and its active fraction for 72 h, with mortality rates recorded every 24 h in both control and test groups. Ultra performance liquid chromatography electron spray ionization quadruple time of flight coupled with mass spectrometry (UPLC/ESI-Qtof/MS) was used to determine the main active constituents in the fraction.

Results: The major bioactive chemical constituents in the *C. sinensis* leaf extract were identified to be proanthocyanidins. The extract significantly interfered with larval survival and adult emergence in both species (ANOVA, $F_{(5,24)} = 1435.92$, $P < 0.001$). Additionally, larval exposure to crude extract at 250 ppm and 500 ppm for 24 h resulted in larval mortality rates of over 90 % in *A. gambiae* (*s.s.*) and 75 % in *A. arabiensis*. A relatively lower concentration of 100 ppm resulted in moderate mortality rates of < 50 % in both species, but induced growth disruption effects evident as abnormal larval-pupal intermediates and disrupted adult emergence. The estimated LC_{50} concentrations of the crude leaf extract against *A. arabiensis* and *A. gambiae* (*s.s.*) larvae at 24 h were 154.58 ppm (95 % CI: 152.37–158.22) and 117.15 ppm (95 % CI: 112.86–127.04), respectively. The bioactive polar fraction caused 100 % larval mortality in both vector species at 25 ppm.

Conclusions: Our findings demonstrate the potential of green tea extract and its active constituents in disrupting mosquito larval development. This could contribute to the control of mosquito populations and improved management of malaria.

Keywords: *Camellia sinensis*, Proanthocyanidins, *Anopheles gambiae* (*sensu stricto*), *Anopheles arabiensis*, Larvicidal activity, Vector control

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PLOS ONE

RESEARCH ARTICLE

Green tea proanthocyanidins cause impairment of hormone-regulated larval development and reproductive fitness via repression of juvenile hormone acid methyltransferase, insulin-like peptide and cytochrome P450 genes in *Anopheles gambiae sensu stricto*

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Appendix 2: Larvicidal screening for bioactive fraction of tea extract for 24 h

Fraction	**Control setup**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**
% larval mortality	0	0	0	0	0	0	0	1	0	0	5	100

Same dosage of 30 ppm was used for all tested fractions**. F1-9:1** *n*-hexane/ethyl acetate**; F2-4:1** *n*-hexane/ethyl acetate**, F3- 7:3** *n*-hexane/ethyl acetate**; F4-3:2** *n*-hexane/ethyl acetate**; F5 – 1:1** *n*-hexane/ethyl acetate**; F6-2:3** *n*-hexane/ethyl acetate**; F7-3:7** *n*-hexane/ethyl acetate**; F8 -1:4** *n*-hexane/ethyl acetate**; F9-1:9** *n*-hexane/ethyl acetate**; F10 – 100%** Ethyl acetate**; F11-100%** methanol

Appendix 3: Validation of proanthocyanidins using Vanilin-HCl assay

Red color indicates presence of proanthocyanidins in the bioactive fraction

Appendix 4: Quality control analysis of isolated RNA samples

Sample ID	Yield	A_{260}/A_{280}
Control sample	13433.3 ng/µl	1.9
Treated sample	11696.7 ng/µl	1.96

Appendix 5: RT-*q*PCR amplification profile of larval genes