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The field Strain of *Anopheles gambiae s.s.* Giles Larvae Resistance Ratio (RR) is Twice That of Susceptible (Laboratory Reared) Strain when Tested with Plant Crude Flower and Leaf Extracts

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Abstract: ***The long term use of many insecticides is constantly threatened by the ability of insects to evolve resistance mechanisms that render the channels ineffective. Such resistance poses a serious threat to insect pest control worldwide. Resistance may result from either an increase in the ability of the insect to detoxify the insecticide or by changes in the target protein with which the insecticide interacts and in which case metabolic or target-site resistance will arise. In resistance, those insecticides involved act on the voltage-gated sodium channel proteins found in insect nerve cell membranes. The correct functioning of these channels is essential for normal transmission of nerve impulses and this process is disrupted by binding of the insecticides, leading to paralysis and eventual death. Some insect pest populations have evolved modifications of the sodium channel protein which prevent the binding of the insecticide and result in the insect developing resistance. In this study Anopheles gambiae 3rd instar larvae both susceptible and field strains were tested against the plant extract from C. cinerariifolium (Cc), E. camaldulensis (Ec) and N. tabaccum (Nt) in the concentrations of 50, 100, 150, 200 and 300 ppm, on exposure for 24 hr to observe the out-coming resistance ratio (RR). The results indicated that all the laboratory susceptible strain showed complete larval mortality (100%) when subjected to test concentrations. The larvae showed no resistance and the RR ranged from 0.885 lowest (Cc ethanol January 2010) to 1.250 highest (Cc aqueous April 2010). Considering the field strain RR varied from 1.003 lowest (Cc ethanol January 2010) to 4.989 highest (Nt aqueous in March 2010). A majority of the field strain was susceptible to low concentrations of the extracts and hence obsessed with high RR notably N. tabaccum aqueous extract (1.981), Ec aqueous extract (1.823) and Nt hexane extract (1.561). A weak resistance was portrayed by Cc ethanol extract (RR 1.003) , Ec hexane (RR 1.005), Cc ethyl acetate (RR 1.066), Nt DCM (RR1.039) , and Nt methanol extract (RR 1.081). The crude leaf extracts which exhibited resistance close to RR1 but ,however, counted as no resistance were: Cc DCM (RR 0.996), Ec methanol (RR 0.998), Ec DCM (RR0.993) and Nt ethanol leaf extract (RR 0.999). It was observed that RR increased as the rains increased from the months of March to June 2010.***

Keywords: Resistance ratio, susceptable strain, temephos, plant extracts, *C. cinerariifolium, E. camaldulensis, N. tabaccum,* 3rd instar larvae, rain season, concentrations

1. Introduction

1.1 Insecticide resistance

Insecticide resistance defined as a genetic selection to insecticides that allows some individual insects the ability to survive previously lethal doses of insecticides. Overtime, this results in the survival of increasing numbers of resistant individual mosquitoes within the population and may impair the effectiveness of insecticide applications causing operational control failures. Resistance generally occurs in areas where insecticide exposure is frequent and / or mosquitoes are exposed at increasing doses (long-term use of the same types of insecticides). The level and mechanism(s) of resistance can be focal and often depend on many biological and operational factors, such as the flight range of the species and the frequency of applications. It should not be assumed that one location where resistant mosquitoes are found is representative of the larger region. Mosquito populations susceptibility to a particular insecticide if resistance is routinely monitored for and changes in mosquito control activities are made in a timely manner. The rate at which a population recovers is dependent on which genes are producing the resistance and their frequency in the population (Owusu *et al.* 2017); Brogdon and Chan (2014). One major method to manage insect resistance to insecticides is the consideration in the use of plant flower leaf, back or root extracts. Of the three flower and leaf extracts under study which have proven outstanding in mosquito control is *Cc.* Nicotine is cited to having high protection in mosquito control (Hunt *et al*, 2011) and effective against two insects, aphid (Toxoptera aurantii) and Khapra, (Trogoderma granarium); (Okia *et al.* 2018).

It has been reported that 447 insect species became resistant in 1986 to most groups of insecticide (organochlorine, organophosphate), carbonate, synthetic pyrethroid, fumigant), including *Bacillus thuringiensis* (Sumamrote et al. 2017); and WHO, (2013) states that a total of 68 countries have reported resistance to at least one class of insecticide, with 57 of those countries reporting resistance to two or more classes. Widespread resistance to pyrethroids has been reported for malaria vectors from numerous countries in sub-Saharan Africa as well as central and south South-east Asia (Soni *et al*. 2018). Dano *et al*.(2014) recorded that *C. Pavonana* of Lembang (Cibogo and Cikidang) had an overwhelming Resistance Ratio (RR) of 6.81 and 7.88 which exceeded 4 times.

Margaret *et al*., (2014), reported tool development for resistance management in view of *Culex quinquefasciatus* Say. In this context the Cyt1A protein of *Bacillus thuringiensis* subsp. *Israelensis* (BTI), De Barjac was evaluated for its ability to suppress resistance to *B. Sphaericus* in a highly resistant population of *Culex*

quanquefasciatus Say. A combination of *B. Sphaericus* 2362 in 10:1 ratio with a strain of BTI that only produced Cyt 1A reduced resistance by > 30,000 – fold. Resistance was suppressed completely when *B. Sphaericus* was combined with purified Cyt1A crystals in a 10:1 ratio. Synergism was observed between the Cyt1A toxin and *B. sphaericus* against the resistant mosquito population and accounted for the marked reduction in resistance. However, no synergism was observed between the toxins against a non-resistant mosquito population. These results indicated that Cyt1A could be useful for managing resistant *B. sphaericus* 2362 in *Culex* populations, and also provide additional evidence that Cyt1A may synergize toxicity by enhancing the binding to and insertion of toxins into the mosquito microvillar membrane.

The lack of resistance to BTI apparently is caused by its complex mosquitocidal proteins, which are synthesized during sporulation and assembled into separate inclusions enveloped together to form a spherical parasporal body. Four major proteins have been identified in this parasporal body: CytIA(27KDa), Cry4A(134kDa), Cry4B(128kDa), and Cry11A(66kDa). Studies have shown that the broad activity spectrum and synergistic interactions between Cyt1A and the Cry proteins, and among the Cry proteins (Wirth *et al.* 2015). Of greater relevance to the management of B. sphaericus resistance are more recent studies in which it has been shown that Cyt1A delays the development of BTI resistance in *Cx. quinquefasciatus* (Wirth *et al.* 2015), and it can suppress resistance levels hundred fold to Cry4 and Cry11A when combined with these endotoxins (Wirth *et al*. 2015).

World Health Organization confirms that insect resistance is now widespread in many malaria vectors throughout the world and is of particular concern in African vectors especially *An. funestus* (WHO. 2018). Since 2010, resistance to at least one class of insecticides has been reported in at least one malaria vector species in 60 of the 96 malaria endemic countries that conducted monitoring; also 49 countries reported resistance to at least two classes of insecticides. Resistance to all four available classes of insecticide has been reported. Resistance to pyrethroids was most commonly reported, with three quarters of countries that monitored this class in 2014 reporting resistance (Mnzava *et al.* 2015).

1.2 Insecticide Resistance mechanism

The mechanisms responsible for the now widespread frequency of resistance have also been identified. These tend to be of two main types: those mediated by changes at the target site of the insecticide [e.g knock down resistance (kdr) mutation] and those caused by increases in the rate of insecticide metabolism. However, it is likely that other, as yet unknown, resistance mechanisms are contributing to the strong resistance phenotypes seen in some populations.

Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal. The detoxification prevents the insecticide from reaching the intended site of action within the mosquito. In the case of malaria vectors, three enzyme systems are believed to be important metabolizers of insecticides: esterases, monooxygenases and glutathione S. transferases. Target site RR occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation. When this happens, the insecticide can no longer bind to the intended target site of the receptor; thus, the insect is either unaffected or is less affected by the insecticide.

In the case of DDT and the pyrethroids the mutation occurs in the sodium channel receptor, conferring what is described as "knockdown resistance"(mediated by the kdr genes). In the case of organophosphates and the carbamates, the mutation occurs in the protein acetycholinesterase (a neurotransmitter), conferring what is usually referred to as Ace-1 resistance. The gene for resistance to dieldrin (rdl) occurs in the gamma aminobutyric acid receptor and has been shown to also confer resistance to fipronil (WHO 2018).

1.3 Documented cases of resistance to pyrethrins

There are no cases of insect resistance in regards to natural pyrethrins quoted in literature. However, a number of known cases of *Anopheles* mosquitoes and other mosquito species resistance to pyrethroids are more documented as opposed to pyrethrins in many countries which include the following: urban Benin (Gnanguenon *et al.* 2015); southern Benin (Yadouleton *et al.* (2010) ; Wanjala and Kweka (2018) west Kenya i.e. Gembe east and west, Mbita and four main western islands in the Suba county, Nyanza Province (Kawada (2017; 2018); cities of Douala and Yaouunde, Cameroon (Nwane *et al*. 2013; Nkondjio *et al* (2017); Nigeria (Ol'e Sangpa *et al.* (2017); north-western Tanzania (Matowo *et al.* 2015); Ghana (Essandoh *et al*. 2013); south-west Ghana (Kudom *et al*. 2012; Awuah *et al.* (2016); Tanzania, lower Moshi, northern Tanzania (Mahande *et al*. (2012); east of Tanzania (Nkya *et al*. 2014); eastern Uganda (Ondeto *et al*. (2017) ; South Africa (Djauaka *et al*. (2016); Hargreaves *et al.* (2000); Burkina Faso (Diabate *et al.* (2014); Equatorial Guinea (Salgueiro *et al.* 2013) ; Angola (Wondji *et al.* (1212); Gabon (Diegbe *et al.* (2017); Irving and Wondji (2017) ; Ethiopia (Fettene *et al.* (2013 and Tesfahuneygn and Gegreegziabher 2018); Cote d`voire (Fodjo *et al*. (2018) and Congo Brazaville (Koekemoer *et al*. 2011). Mosquito resistance to *Ec* and *Nt* extracts are not documented.

2. Methods and materials

These experiments were performed between January and July 2010 at Moi University, Eldoret, Kenya.

In January and February 2010 field larvae were collected from the wetlands (swamps) since the two months were dry months. In March, April, May and June (wet months) 2010, larvae were collected from drainage ditches, hoof prints, swamps, open containers (plastics and metallic), farow ditches, and used tyres. These were months experiencing plenty of rainfall. Since WHO standard dipper was not available, *A. gambiae s.s* **Giles** larvae were collected using an improvised dipper as used by Emedi *et al.* (2012). A laddle was made from an empty 350ml sized water bottle by

longitudinally cutting an opening to make an oval hole half was long from the bottom. Larvae were collected randomly so that the 3rd instar could be separated from the rest in the laboratory. Also there was fear as water is disturbed mosquitoes tend to submerge to the bottom of pools. Larvae were collected, placed in bowled-flat based cylinder, its mouth covered with stockenett and as many as possible larvae were collected. The larvae were dipped into 300 ml bottles containing fresh water from the habitat of the larvae and transported them to the insectary for bioassays.

In the laboratory, the field generation larvae were identified and separated using taxonomic keys of Gillies and Coetzee (1987). For this identification a x50 or x100 magnifying glass was used in the observation of the prominent keys i.e dypeals, saddle hair, thorax, abdomen, mesople and mesopleural hair.

The field generation larval were fed on green algae collected from their habitat because this was one of the common foods for the larvae and an abrupt change of food could affect the larvae. The larvae were maintained in similar rearing conditions as the laboratory species (28+20C, 75+5% Relative Humidity and 12:12h (Light: Dark periods). In the absence of a humidifier humidity was maintained by use of wet towels spread over rearing cages but leaving one side of the cage uncovered with the towels (Imam *et al.* 2014 and Baughman *et al.* (2017).

Tests for RR were carried out following WHO (2013) procedures.

Overall resistance was determined as follows: LC_{50} of field strain divide by LC_{50} of laboratory strain then categorizing RR into 3 levels: Slightly resistant ($1 < RR \leq 5$); moderately resistant ($5 < RR \leq 10$); (RR>10) (Rodnguez *et al.* 2007). This was interpreted that values of RR greater than 1 was an indication of resistance and values less than or to 1 were considered susceptible. The results were also counterchecked using WHO (2013) method of determining RR at the prescribed diagnostic dose and diagnostic time and this was: Susceptible = 100 – 98% mortality; Possibly resistant = 98 – 90% mortality and Confirmed resistant = < 90% = (hence, more testing required). Acute oral toxicity was analyzed through Probit-log to obtain percent mortality.

3. Results

The results of the RR of the susceptible and field strain of the larvae is shown in Tables 1 and

Table 1.: Resistance ratio (RR) of the field strain larvae in the months of January – June 2010.
Table 1 shows RRs for the field strain of the larvae. Larvae succumbed to very high concentrations of the extracts in all months and resulted to RR varying from 1.003 (*Cc* ethanol) in the month of January 2010 to 4.989 (*Nt* aqueous) in the month of *May 2010*. The highest RRs were recorded in the months of March, April, May and June 2010 probably when the larvae were exposed to temephos swept into the larvae habitats from agricultural lands by runoff.

	Jan	Feb	Mar	Apr	May	June
C.cinerariifolium						
Ethanol	1.003	1.015	1.160	1.175	1.180	1.195
Methanol	1.018	1.045	1.145	1.185	1.194	1.198
DCM	1.092	1.084	1.144	1.165	1.172	1.175
Hexane	1.050	1.115	1.310	1.340	1.290	1.298
Ethyl acetate	1.140	1.175	1.195	1.290	1.296	1.255
Aqueous	1.155	1.185	1.418	1.435	1.385	1.295
E. camaldulensis						
Ethanol	1.080	1.145	1.278	1.165	3.200	3.155
Methanol	1.090	1.165	1.280	1.530	2.240	2.130
DCM	1.060	1.085	1.165	1.190	3.240	2.240
Hexane	1.085	1.080	1.288	1.462	4.122	1.320
Ethyl acetate	1.069	1.162	1.380	1.395	2.285	2.265
Aqueous	1.108	1.110	1.318	1.395	3.226	2.460
N. tabaccum						
Ethanol	1.094	1.078	1.800	1.920	2.720	2.660
Methanol	1.096	1.140	1.298	2.295	2.345	3.136
DCM	1.055	1.075	1.295	2.220	2.270	2.255
Hexane	1.085	1.354	2.390	2.415	2.432	2.425
Ethyl acetate	1.075	1.085	1.216	2.240	2.292	2.265
Aqueous	1.130	1.225	4.989	5.560	4.915	4.975

Table 2: Resistance ratio (RR) of the laboratory reared larval in the months of January to June 2010.
Resistance ratio of the susceptible larvae varied from 0.140 (*Ec* hexane) in February 2010 to 1.98 (*Ec* aqueous) in May 2010. All the concentrations attained 100% larval mortality.

	Jan	Feb	Mar	Apr	May	June
C. cinerariifolium						
Ethanol	0.885	0.888	0.896	0.890	0.945	0.942
Methanol	0.987	0.992	0.988	0.998	0.920	0.999
DCM	0.855	0.850	0.880	0.960	0.972	0.985
Hexane	1.144	1.070	1.043	1.046	1.020	1.049
Ethyl acetate	0.966	0.968	0.978	0.980	0.988	0.985
Aqueous	1.153	1.160	1.160	1.250	1.146	1.145
E. camaldulensis						
Ethanol	0.990	0.989	0.995	0.999	1.125	1.135
Methanol	0.892	0.898	0.856	0.905	0.984	0.998
DCM	0.886	0.868	0.894	0.920	0.955	0.986
Hexane	1.025	0.140	1.080	1.135	1.130	1.115
Ethyl acetate	1.155	1.155	1.166	1.175	1.178	1.170
Aqueous	1.168	1.182	1.190	1.196	1.198	1.185
N. tabaccum						
Ethanol	0.955	0.960	0.982	0.992	0.998	0.900
Methanol	0.994	0.906	0.910	0.955	0.970	0.995
DCM	1.005	1.012	1.050	0.999	1.108	1.115
Hexane	1.085	1.035	1.149	1.151	1.162	1.155
Ethyl acetate	1.168	1.055	1.165	1.175	1.175	1.171
Aqueous	1.188	1.105	1.085	1.175	1.156	1.140

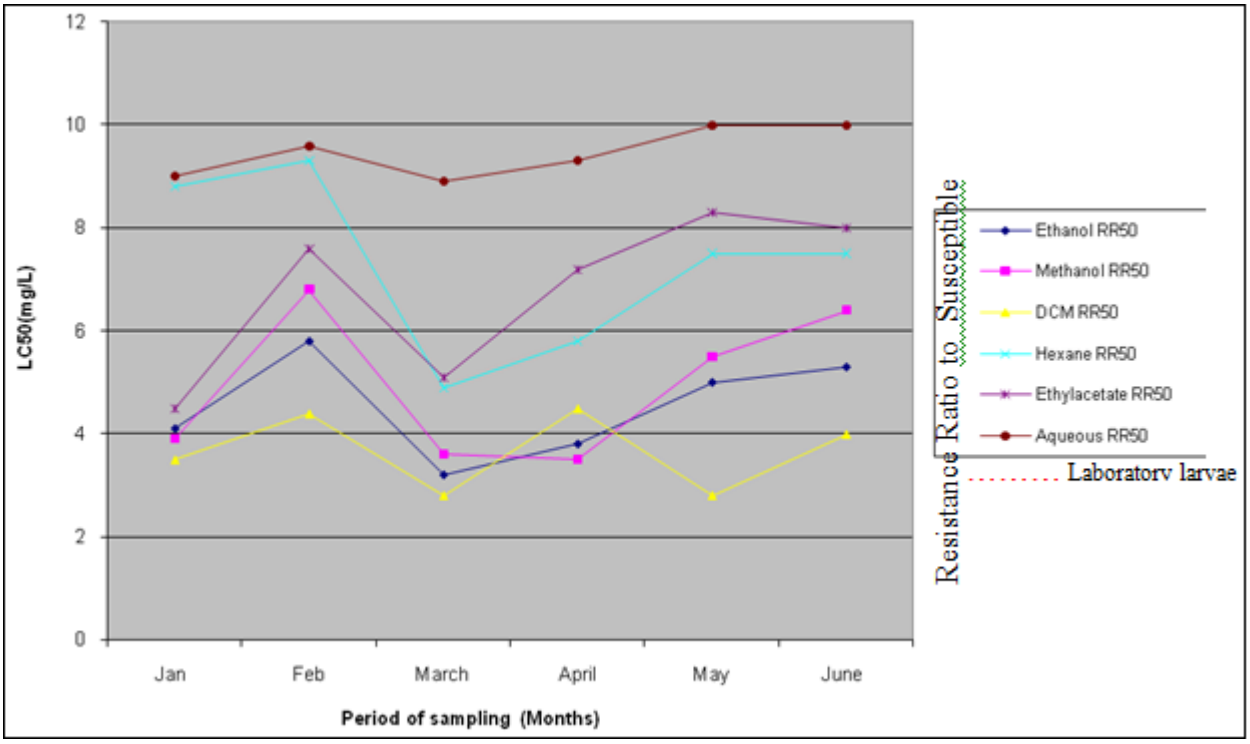

Figure 1: *C. cinerariifolium* crude leaf extracts resistance ratio – January to June 2010

In Fig.1 the tests indicated 100% larval mortality at the concentrations ranging from 0.850 fold (*Cc* DCM, January 2010) to 1.250 fold (*Cc* aqueous, April 2010). In the order of their best top 5 extracts in their larval RR lowest to highest were: *Cc* DCM (0.850 fold); February 2010); *Cc* ethanol (0.850 ppm, January 2010); *Ec* DCM (0.886 ppm, January 2010); *Ec* methanol (0.905 ppm, April 2010); and *Cc* hexane (1.020, May 2010).The individual extract mortality to these 5 were 100%, 100%, 100%, 100%, and 88% respectively.

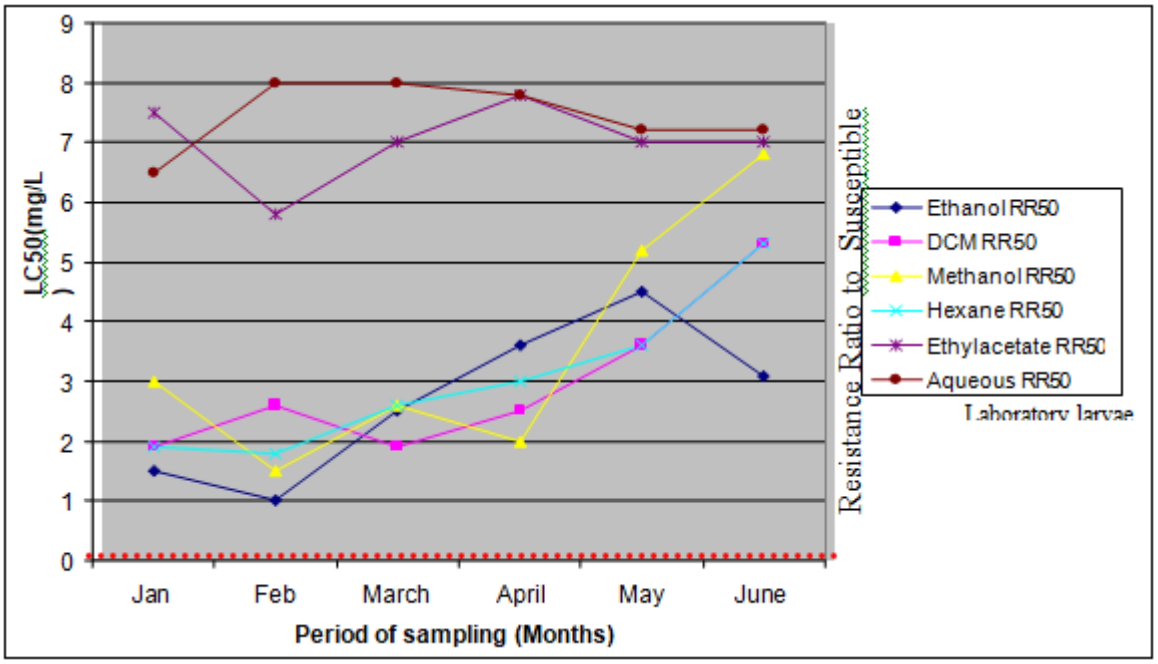

Figure 2: *E. camaldulensis* crude leaf extracts resistance ratio – January to June 2010

Fig. 2 shows varying RR of Ec extracts from 0.868 fold (*Ec* DCM, February 2010) to 1.198 fold (*Ec* aqueous, May 2010). In their best top 6 extracts lowest to highest RR folds were: Cc DCM 0.850 , Cc ethanol 0.855, *Ec* methanol 0.856, *Ec* DCM 0.868, *Ec* methanol 0.905, and *Nt* aqueous 1.085. The larvae indicated RR of a succeeding order successfully from dry to rainy months.

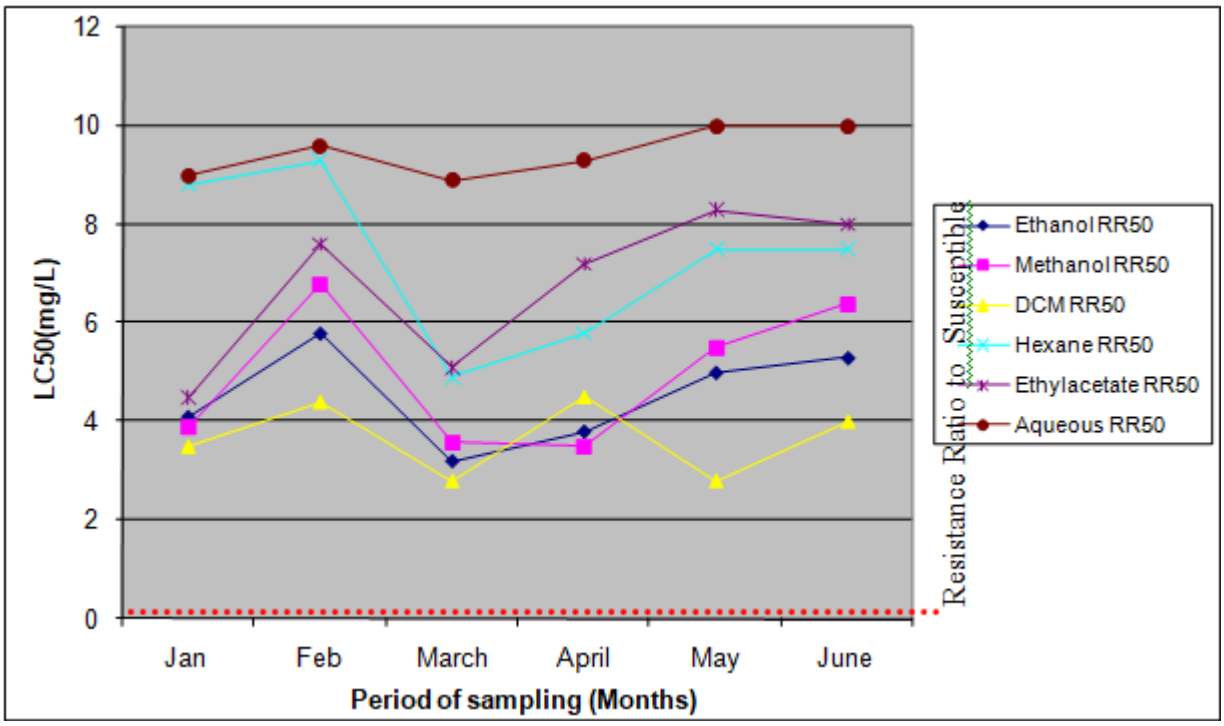

Figure 3: *N. tabaccum* crude leaf extracts resistance ratio (RR) – January to June 2010

In Figure 3, the RR ranged from 0.906 fold (Nt methanol, February 2010) to 1.175 fold *Nt* ethyl acetate (April/May) and *Nt* aqueous 1.75 fold in May 2010. The broad ranges in terms of the extracts succeeding order were: *Nt* methanol 0.906 fold, February 2010; *Nt* ethanol and *Nt* methanol both 0.955 fold in January and April 2010; *Nt* DCM 1.005 in January; *Nt* hexane 1.035 fold; *Nt* ethyl acetate 1.055 in February and *Nt* aqueous was 1.085 in March 2010.

Resistance of the extracts to the field strain larvae

Unlike the susceptible strain of larvae, the field strain larvae indicated high resistance to the extracts. The RR ranged from 1,003 fold (*Cc* ethanol, January 2010) to 5.560 fold (*Nt* aqueous, March 2010). Forty three extracts (RR ranging from 1.003 fold to 1.198 fold) exerted low RR to the larvae while 65 extracts (RR ranging from 1.225 fold (*Nt* aqueous, February) to 5.60 fold (*Nt* aqueous March 2010) yielded high RR to the larvae. A majority of extracts with low RR fall in the moths of January and February 2010. A limited number were in the month of March, probably at the beginning of the month when rains were just starting. It is possible that the high RR in the months of March to June 2010 was influenced by rains of which the runoff swept temephos used in agriculture into the larval habitats causing prior larvae exposure thereby increasing the resistance of the larvae to the extracts.

4. Discussion

Both strains were subjected and exposed to the diagnostic dose of the crude flower and leaf extracts of the three plants for 24 hr. The results of the two larvae were then compared WHO (2013) (WHO, 2005) and as adapted by Rocha *et al*. (2015), Grisales *et al*. (2013), Mulyatno *et al*, (2012) and Johan and Shahid (2012).

The results indicated that all the laboratory susceptible strain showed complete larval mortality (100%) when subjected to test concentrations. The larvae showed no resistance and the RR ranged from 0.885 lowest (*Cc* ethanol January 2010) to 1.198 highest (*Ec* aqueous May, 2010).

However, the field strain larvae showed a significant increase in resistance towards LC_{50} values particularly in the months of March, April, May and June 2010. This was possibly because of heavy rains in these months which contributed to temephos addition into larval habitats by runoff and becoming magnified in the mosquito larvae or their parents thus elevating larvae resistance to extracts. While in the dry seasons (January and February) when the agriculturally used chemicals were low in waters and this decreased larvae resistance to the extracts.

There was no marked difference in the resistance pattern to those resistance producing extracts in LC_{50} values (susceptible and field strains) where the two strains only produced resistance at a lower rate.

The results indicate a rather strong resistance to *Nt* aqueous (1.981), *Ec* aqueous (1.823) and *Nt* hexane extract (1.561). A weak resistance was portrayed by *Cc* ethanol flower extract (1.003) , *Ec* hexane (1.005), *Cc* ethyl acetate (1.066), *Nt* DCM (1.039) , and *Nt* methanol leaf extract (1.081). The crude leaf extracts which exhibited no resistance to the laboratory reared larvae strain and which susceptibility was high were *Cc* DCM (0.996), *Ec* methanol (0.998), *Ec* DCM (0.993) and *Nt* ethanol leaf extract (0.999).

Importantly, results indicated presence of cross-resistance among the field strain in 24 hours post-recovery period. Probably this was due to the selection of a certain insecticide of one or more genes which would generally extend to other compounds that share either a metabolic pathway or a target

site (Subbiah *et al.* (2009; Wirth *et al*., (2000). More so one obvious reason for this is that different groups of genes can be selected with one insecticide (Hitayati *et al*. 2011; Karunamoorthi and Sabesani, 2012). The field strain on the other hand has a high probability of previous exposure to temephos and may therefore be expected to exhibit higher tolerance for temephos. Variation in the resistance was seen to occur in crude leaf extracts administered and may be this could be contributed by heterozygous genes in the population which caused quick dilution of resistant genotypes resulting in the decline of resistance level Low *et al.*; (2013); (Selvi *et al.*, 2010). Among other common factors enhancing resistance are impacts from pyrethroids used in household insecticides, fogging for mosquito control and agricultural practices (Kudom *et al* 2011). On the researcher's personal survey of the site of mosquito collection it was observed that other on-site factors could also contribute to this resistance such as smokers and tobacco leaf brokers and *eucalyptus* timber users (carpentry workshops) of which waste products is saw dust which pollutes wetlands through runoff. Both these factors could emit nicotine and *eucalyptus* oil respectively to pollute the malara vector habitats and developing resistance to malaria vector mosquitoes

Indeed, some previous works demonstrate that oils containing mainly oxygenated compounds have a higher persistence and lose their activity more slowly than those with a high content of hydrogenated compounds (Rathore and Nollet, (2012); IS Global Barcelona Institute of Global Health (2017); Liao *et al.* (2017). Apparently, due to very short persistence time demonstrated by the oils of the three plants investigated in this study, may have a high content of hydrogenated compounds as opposed to oxygenated compounds.

Detection of resistance of biopesticides in malaria vectors will help public health personnel to formulate appropriate steps to counter reductions in effectiveness of control effort that may accompany with the emerging problems of insecticide resistance. Further, more cross-resistance or resistance as a result of agricultural uses of insecticides may evolve and adversely impact the options to switch an alternative method or insecticides for disease control and hence focus to plant extracts and other biological control agents.

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