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ORIGINAL ARTICLE

Comparative Larvicidal Properties and Detoxification Machinery of Bioactive Fractions of Leaf Extracts of *Hyptis Suaveolens* and *Chromolaena Odorata* on *Anopheles gambiae s.l* from North West Nigeria

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Abstract

Malaria is endemic in Nigeria and the impact of chemical insecticides is being undermined by widespread resistance in mosquito vectors. This calls for development of alternative bio-insecticidal approach. The aim of this work was to determine the active fractions of leaves of Hyptis. suaveolens and Chromolena. odorata with insecticidal potential on the larva of Anopheles gambiae s.l. Leaf extracts of the two plants were screen for phytochemicals and the active fractions were subjected to Gas Chromatography-Mass Spectroscopy (GC-MS) analysis. In addition, the detoxification enzymes (Glutathione S-Transferase, esterase and cytochromes P450) as well Glutathione levels on the susceptible and resistant individuals of the larvae were biochemically assayed. Larval bioassay was carried out by the standard procedure described by World Health Organization (WHO). The phytochemicals detected include flavonoids, tannins, saponins, terpenoids, alkaloids, phenols and phlobatannins. Butanol fracton of H. suaveolens displayed higher larvicidal activity (LC50 2167.92ppm) followed by the aqueous fraction (LC50 2613.01ppm). Both butanol and aqueous fractions of C. odorata also displayed larvicidal activities (LC50 3117.97ppm; LC50 3497.27ppm) respectively, but were lower than that observed in Hyptis suaveolens. Biochemical assay of the detoxification enzymes showed significance difference at $P < 0.05$ between the resistant and susceptible larvae for all enzymes. There was however no significant difference in the levels detoxification enzymes in both resistant and susceptible individuals for butanol and aqueous fractions of H. suaveolens when compared with their corresponding fractions of C. odorata at $P < 0.05$ respectively. The observations recorded in this study showed a promising larvicidal potential of these two plants which if further characterized could offer a promising novel bioinsecticide compound that could substitute the present classes of chemical insecticides used in malaria vector control.

Keywords: *Anopheles gambiae*; *Hyptis suaveolens*; *Chromolena odorata*; Bioactive fractions

Introduction

Malaria is caused by *Plasmodium* parasites that are spread through the bite of Anopheline mosquito vectors and of the five parasite species that cause malaria in humans, *Plasmodium falciparum* is the deadliest (WHO, 2016). In 2015, there were an estimated 212 million cases and 429 000 malaria deaths and nearly half of the world's population was at risk of malaria (WHO, 2016). Sub-Saharan Africa continues to carry a disproportionately high share of the global malaria burden as the region was home to 90% of malaria cases and 92% of malaria deaths with young children, pregnant women and non-immune travellers from malaria-free areas particularly vulnerable when infected (WHO, 2016). In high transmission area, children under the age of 5 are particularly vulnerable to infection, illness and death. More than two-thirds (70%) of all malaria deaths occurred in this ages (WHO, 2016).

The resistance of *An. gambiae s.l* to common classes of insecticides in northern Nigeria is well documented (Imam and Deeni, 2013; Ibrahim, *et al.,* 2014; Umar *et al.,* 2014; Alhassan *et al.,* 2015). In the southern part of Nigeria, many studies have also established the resistance of *An. gambiae s.l* to common classes of insecticides (Awolola *et al.,* 2007; Oduola *et al.,* 2012). The mechanisms responsible for insecticide resistance in insects are complex and include behavioral and/or physiological changes of mosquitoes leading to insecticide avoidance, altered penetration, sequestration, target site alteration or bio-degradation. In mosquitoes however, resistance is mainly associated with target site modification and metabolic resistance (David *et al.*, 2013). Of these, P450s are the primary enzyme family associated with resistance to most insecticides including pyrethroids, the most widely used class of insecticide for vector control as elevated levels of P450 activity are frequently observed in pyrethroid-resistant malaria vectors in Africa (Matambo *et al* 2010; Djouaka, 2008). Esterase hydrolysis of pyrethroids leading to detoxification is also believed to act as a cause of metabolic resistance in some instances (Vulule *et al*, 1999; Somwang *et al*, 2011) while Glutatione S-Transferases (GSTs) are regularly found overexpressed in pyrethroid-resistant strains (Lumjuan, 2011). GST acts by catalyzing the nucleophilic addition of reduced glutathione (GSH) to electrophilic substrates, leading to the formation of water soluble conjugates (Booth *et al*, 1961).

The control of mosquito-borne diseases is becoming increasingly difficult because the effectiveness of vector control has declined due to development of resistance by vectors against the currently used organochlorine, organophosphorus (malathion), carbamates (carboxyl) and pyrethroid insecticides (Doliantis and Scinclair, 2002). Furthermore, chemical insecticides adversely affect the environment and damage biological systems (Amer and Mehlhorn, 2006). It is well established that repeated use of synthetic chemical insecticides for mosquito control has led to interference in the natural biological control eco-systems, which might have led to resurgences in the target mosquito populations and has consequently resulted in the development of resistance (Constant *et al.,* 2012), and undesirable effects on non-target organisms (Frederick, 2014). A growing alternative to the conventional chemical control is the utilization of natural products from plants, fungi and other natural ingredients (De-Morais *et al*., 2007). In many parts of the world, plant-derived products have been used to repel or kill mosquitoes and other domestic insect pests and solvent extracts and essential oils of many plants have shown varying levels of insect-repellent properties (Omolo *et al.,* 2004). Further, unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavioural and physiological processes of insects (Anupam *et al.,* 2012).

Mosquitoes are most efficiently and economically destroyed when they are in the larval stage and are concentrated in their breeding site thus preventing the larvae from becoming adult mosquitoes (CDC, 2004). Several previous studies (Jagruti *et al.,* 2014) have reported the ovicidal larvicida and insect repellant properties of solvent-based extracts of various plants including *Chromolena odorata* on many insect species. However, few if any studies have studied the larvicidal or insecticidal potentials of *Chromolena odorata* and *Hyptis suaveolens* despite the observed cultural practice involving these two plants in many parts of Nigeria using fumes from dried leaves of these plants as mosquito repellant. The aim of this research

therefore was to determine the active fractions of leaf extracts of *Chromolaena odorata* and *Hyptis suaveolens* on the larva of *Anopheles gambiae s.l.* from Auyo Local Government Area, Jigawa State, Nigeria.

Materials and Methods

Collection of Plant Materials and Preparation of Extracts

Fresh leaves of *C. odorata* were collected in Lokoja (7.8023°N, 6.7333°E), Kogi State, between May and September 2016, while the leaves of *H. Suaveolens* were harvested from Getso town (11.8751°N, 7.9715°E, Gwarzo LGA in Kano State between June and August 2016. The plants were identified at the Herbarium of Biological Science Department of Bayero University Kano. While the accession number for *C. odorata* is BUKHAN 0137, that of *H. Suaveolens* is BUKHAN 0416. After identification both leaves were dried in a shade and ground in a mortar to form fine powder.

Four hundred grams of powdered *C. odorata* and *H. suaveolens* was extracted separately in l L of distilled water, concentrated by evaporation and freeze dried. 5g of the aqueous crude extract of each plant was dissolved in 150ml distilled water and then successively partitioned in 150ml n-hexane, chloroform, ethyl acetate and n-butanol respectively. 1g of the aqueous and n- butanol fractions were dissolved in 100ml of water and acetone respectively to make the stock solution and various concentrations of 1000, 3000, 5000, 7000, and 10000 parts per million(ppm) obtained through serial dilution were used for the larvicidal assay. The extracts were stored at 4°C.

Collection and Identification of Mosquito Larvae

Mosquito larvae of *Anopheles gambiae s.l* were collected from breeding sites in Auyo LGA (12.21°N, 9.59°E), northeast of Dutse, Jigawa State from July to October 2016. Late 3rd and early 4th instar larvae were used to screen for the larvicidal activity of the extracts. Morphological keys of Gillies and Coetze (1987) were used for morphological identification.

Phytochemical Screening

Standard qualitative methods were adopted for the phytochemical screening. Chemical tests were carried out on the aqueous extract for the qualitative determination of phytochemical constituents as described by Harborne (1973), Trease and Evans (1989), Sofowora (1993) and Ajaiyeobu (2002).

Larvicidal Bioassay

Standard protocol as described by WHO (2005) was employed for the larvicidal bioassay. Twenty (20) larvae were collected in small containers with 249ml water, to which 1.0 ml of the portioned extract was added. The control was set up with dechlorinated water. Experiments were carried out with a series of concentrations ranging between 1000ppm to 10000ppm of the extracts. The numbers of dead larvae were counted after 24 hours of exposure and the percentage mortality was reported from the average of three replicates using the formula below. LC_{50}, and LC_{90} values were also determined.

$$\%Mortality = \frac{\%\,Test\ Mortality\ -\ \%\,Control\ Mortality}{100\ -\ \%\,Control\ Mortality} \times 100 \qquad (1)$$

Biochemical Assay

Preparation of Homogenate
WHO (1998) method was used for the preparation of the homogenate. Twenty mosquito larvae were homogenised in ice-cold sodium phosphate buffer (0.1M; pH 7.2) in 1.5ml microfuge tubes with Pellet Pestle Motor. The homogenization was carried out on ice. The homogenate was centrifuged for 1 minute at 12000 rpm and the supernatants used for the assays.

GST Assay

GST was determined by the method of Habig (1974). To 200µl of 1-chloro-2, 4-dinitrobenzene (CDNB)/ glutathione working solution in a microplate was added 20µl of the homogenate in three replicates. The mixture was allowed to stand at room temperature for 20 minutes and absorbance was taken at 340nm as an end point.

Esterase Assay

The esterase was determined by the method described by Faiz (2007). Twenty microliter of the homogenate in replicates was put in separate microplate wells. 200µl of PNPA working solution was added and read at 405nm continuously for 2 minutes.

Cytochrome P450 (Monooxygenase) Assay

This was determined using the method of Brogden (1998). Twenty microliter of the homogenate was put in separate wells of a microplate containing 80 µl of 0.625M potassium phosphate buffer (pH 7.2) in replicates and to which was added 200µl of the mixture containing methanol solution of tetramethylbenzidine with 0.25M sodium acetate buffer (pH 5.0). To each replicate of the above mixture was added 3% hydrogen peroxide and the mixture allowed to stand for 2 hours at room temperature after which absorbance was read at 650nm. A standard curve of cytochrome c was used to determine a crude estimate of the amount of monooxygenase present and expressed as equivalent units of cytochrome P450.

Total Protein

This was done following the method of Bradford (1976). Ten microlitre of homogenate in replicates was put in separate wells of a microplate. 300µl of Bio Rad was added to each well. Absorbance was taken after 5minutes at room temperature. Estimation of the protein was done from the supernatant of whole body homogenate as per Lowry *et al*. (1951), method and bovine serum albumin (BSA) was used as the standard

Glutathione Assay

This was determined by the method described by Ellman, **(**1959). 20µl of NADPH Generating mix and 20µl of the in replicates were mixed in a microplate well. 160µl of the Reaction mix was added to each well and incubated at room temperature for 10 minutes to generate NADPH. 20µl of the sample solution and the standard solutions were added to different wells respectively and incubated at room temperature for 5-10 minutes then 20µl of the substrate solution was added to each mixture and further incubated at room temperature for another 5-10 minutes. Absorbance was taken at 415nm and the concentration of the GSH was determined using a computational formula.

Statistical Analysis

For the larvicidal bioassay, the percent mortalities were corrected using Abbott's formula and the average larval mortality was subjected to probit analysis to calculate LC_{50} and LC_{90}, 95% confidence limits and Chi squared test values using the SPSS software version 20. (SPSS Inc IBM Statistics, SAS Institute United States). The biochemical statistical analysis was done using GraphPad Instat3 (Version 3.05, Graphpad Inc, United States) Software. P value <0.05 was considered statistically significant.

Results

The result of phytochemical screening of the crude extracts of the leaves of *H. suaveolens and C. odorata* is presented in Table 1. As shown in the table, phenols, flavonoids, alkaloids, saponins, phlobatamins, tannins, and terpenoids are present in the extracts of both plants while cardiac glycosides and steroids are absent.

Table 1. Phytochemical contents of the aqueous leaf extract of *Chromolaena odorata* and *Hyptis Suaveolens*

	Chromolaena odorata	***Hyptis Suaveolens***
Phenols	+	+
Flavonoids	+	+
Alkaloids	+	+
Saponins	+	+
Phlobatannins	+	+
Tannins	+	+
Cardiac Glycosides	–	–
Steroids	–	–
Terpenoids	+	+

KEY: += PRESENT; - = ABSENT

Figure 4.1 shows the result of larval mortality of *An. gambiae s.l* after treatment with different concentrations (1000ppm, 3000ppm, 5000ppm, 7000ppm and 10000 ppm) of the extracts. Percentage mortality for butanol and aqueous fractions of *H. suaveolens* were 35 and 30 respectively while those of *C. odorata* were 28 and 25 respectively.

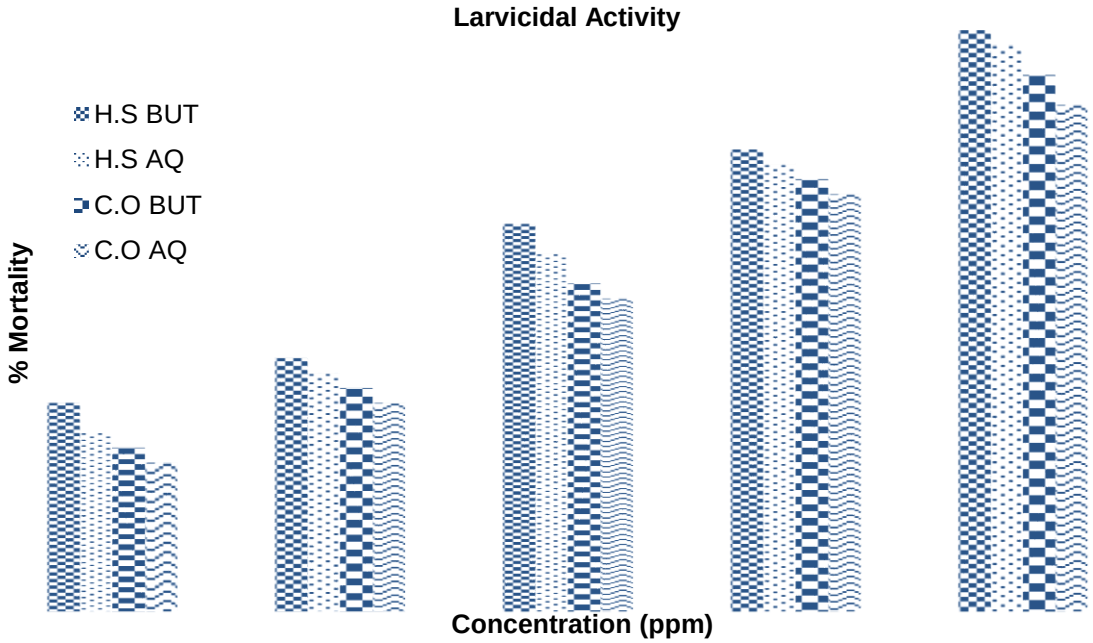

Figure 1. Larvicidal Activity of Aqueous Extracts of Leaves of *C.odorata* and *H.suaveolens* against the Larva of *An. gambiae s.l.* H.S: *Hyptis suaveolens*; CO: *Chromolaena odorata*; BUT: butanol; AQ: aqueous.

Table 2 shows the toxicity effects of aqueous and butanol fractions of the leaves of *C. odorata* and *H. suaveolens*.

Table 2. Toxicity of Aqueous and Butanol Fractions of the Aqueous Extracts of Leaves of *H. Suaveolens* and *C. Odorata* on *An. gambiae s.l* Larvae

Plant Extract	LC_{50} (ppm)	LC_{90} (ppm)	95% Confidence Interval		Slope	SD±SE	X^2	df
			LFL	UFL				
H.S. Butanol	2167.92	7307.60	1482.09	3609.53	2.045	0.489±0.09	0.438	3
H.S Aqueous	2613.01	8553.77	1695.21	4204.14	1.950	0.513±0.10	0.544	3
CO Butanol	3117.97	9283.08	1871	4988.61	1.756	0.569±0.11	0.650	3
CO Aqueous	3497.27	9707	2070.82	5797.63	1.650	0.606±0.11	0.732	3

KEY
H.S: *Hyptis suaveolens*; CO: *Chromolena odorata*; LFL: Lower fiducial limit; UFL: Upper fiducial limit; SD: Standard definition; SE: Standard Error; X^2- Chi square; df: degree of freedom.

Figures 4.2, 4.3 and 4.4 show the result of the enzyme activities for both the resistant and susceptible strains of the larvae of *An. gambiae s.l.* The result indicates that the enzyme activities for both the susceptible and resistant individuals were significantly higher at $P< 0.05$ than the control.

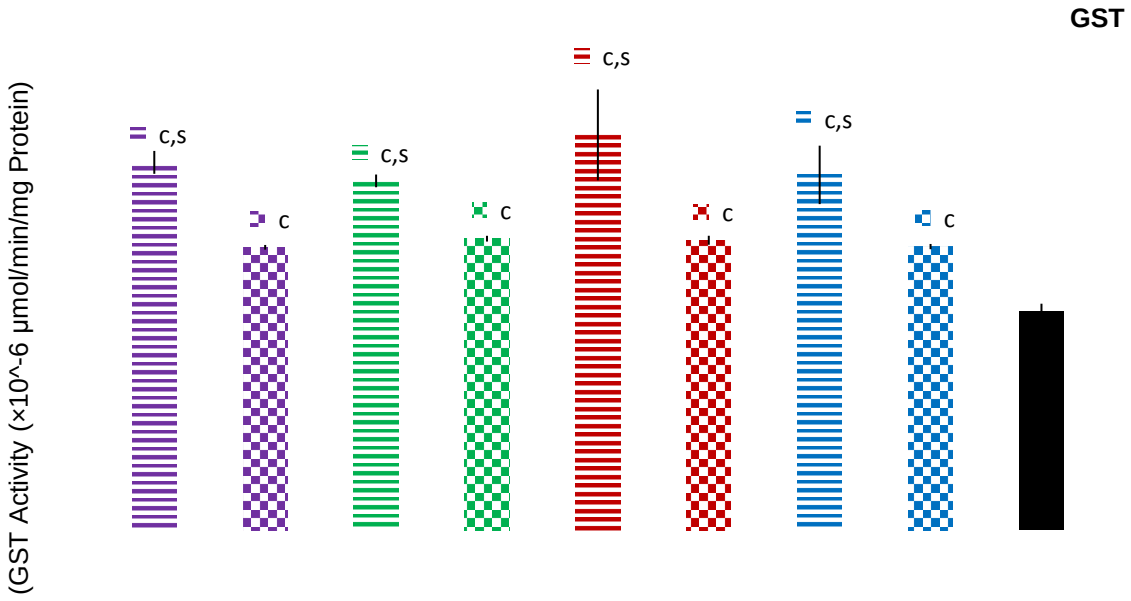

Figure 4.2. Total Glutathione-S-Transferace of the Resistant and susceptible individuals of the larvae of *An. gambiae s.l.*

Values are presented as mean ± standard deviation (n=3). Readings with letter 'c' indicates significant difference (P<0.05) when campared with control; those with's' are significantly different (P<0.05) with their susceptible counterparts under the same extract for aqueous C. odorata, butanol C. odorata and aqueous H. suaveolens,

Figure 4.2. Glutathione-S-Transferase activities of the Resistant and Susceptible strains of the larvae of *An. gambiae s.l.* after treatment with extracts of the two plants.
Values are presented as mean ± standard deviation (n=3). Readings with superscript 'c' indicates significant difference (P<0.05) when compared with control; those with 's' are significantly different (P<0.05) with their susceptible counterparts under the same extract for aqueous C. odorata, butanol C. odorata aqueous, H. suaveolens, butanol H. suaveolens respectively.

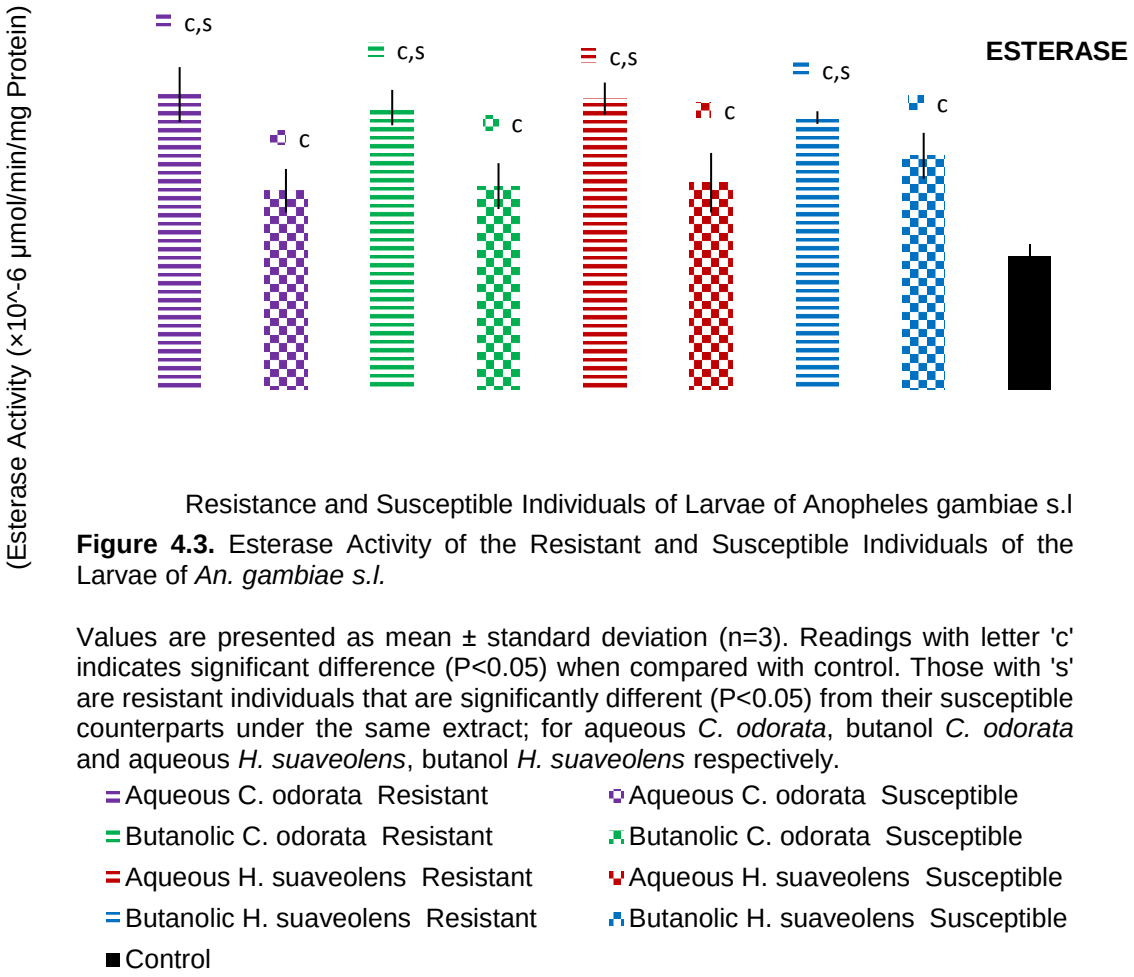

Figure 4.3. Esterase Activity of the Resistant and Susceptible Individuals of the Larvae of *An. gambiae s.l.*

Values are presented as mean ± standard deviation (n=3). Readings with letter 'c' indicates significant difference (P<0.05) when compared with control. Those with 's' are resistant individuals that are significantly different (P<0.05) from their susceptible counterparts under the same extract; for aqueous *C. odorata*, butanol *C. odorata* and aqueous *H. suaveolens*, butanol *H. suaveolens* respectively.

Figure 4.3. Esterase Activity of the Resistant and Susceptible strains of the larvae of *An. gambiae s.l.* after treatment with extracts of the two plants.

Values are presented as mean ± standard deviation (n=3). Readings with superscript 'c' indicates significant difference (P<0.05) when compared with control. Those with's' are resistant individuals that are significantly different (P<0.05) from their susceptible counterparts under the same extract; for aqueous *C. odorata*, butanol *C. odorata*, aqueous *H. suaveolens*, butanol *H. suaveolens* respectively.

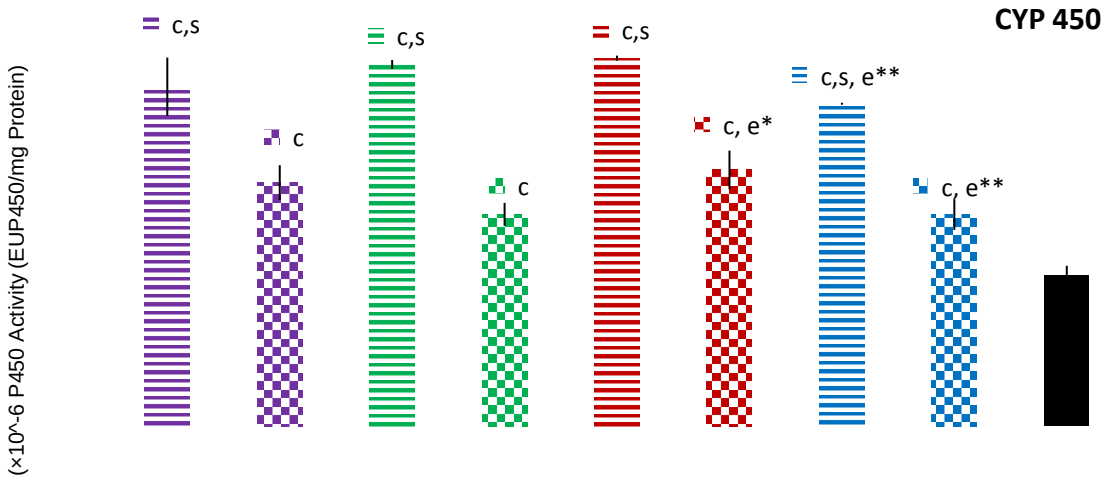

Figure 4.4. Cytochromes P450 enzyme activity of the resistant and susceptible individuals of the larvae of *An. gambiae s.l.*

Values are presented as mean ± standard deviation (n=3).Readings with letter 'c' indicates significant difference (P<0.05) when compared with control; those with 's' are significantly different (P<0.05) with their susceptible counterparts under the same extract; while those bearing 'e*' and 'e**' are resistant individuals that are significantly different (P<0.05) from their respective counterparts under the same outcome (resistant or susceptible) for aqueous *C. odorata*, butanol *C. odorata* and aqueous *H. suaveolens*,

Figure 4.4. Cytochromes P450 enzyme activity of the resistant and susceptible strains of the larvae of *An. gambiae s.l.* after treatment with extracts of the two plants.
Values are presented as mean ± standard deviation (n=3).Readings with superscript 'c' indicates significant difference (P<0.05) when compared with control; those with 's' are significantly different (P<0.05) with their susceptible counterparts under the same extract; while those bearing 'e*' and 'e**' are resistant individuals that are significantly different (P<0.05) from their respective counterparts under the same outcome (resistant or susceptible) for aqueous *C. odorata*, butanol *C. odorata* aqueous, *H. suaveolens* butanol, *H. suaveolens* aqueous respectively.

The result of glutathione level is presented in Figure 5. The result shows an increased GSH level in resistant individuals when compared with the control. The levels of GSH for *H. suaveolens* were significantly higher compared with those of the *C. odorata* for both aqueous and butanol fractions.

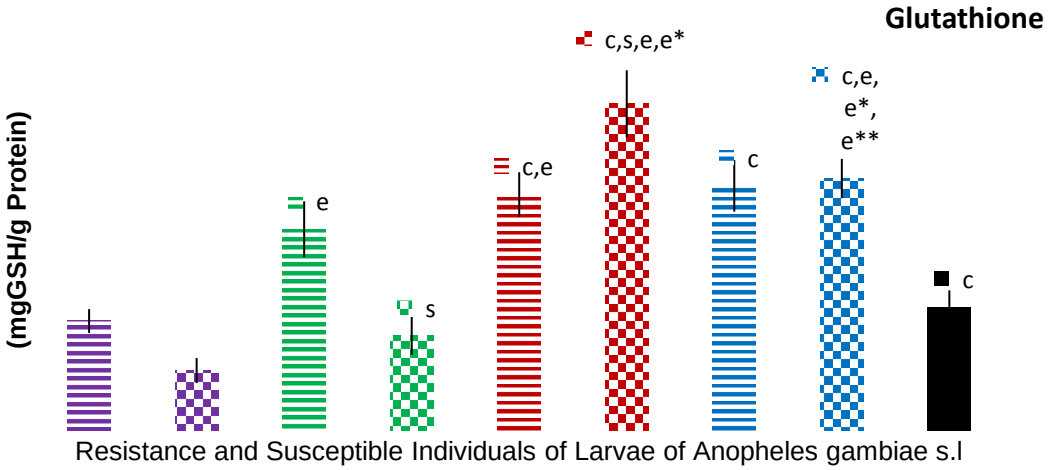

Figure 4.5. Glutathione level in the resistant and susceptible individuals of the larvae of *An. gambiae s.l.*

Values are presented as mean ± standard deviation (n=3).Readings with letter 'c' indicates significant difference (P<0.05) when compared with control; those with 's' are significantly different (P<0.05) with their susceptible counterparts under the same extract; while those bearing 'e','e*' and 'e**' are significantly different (P<0.05) with their respective counterparts under the same outcome (resistant or susceptible) for aqueous *C. odorata*, butanol *C. odorata* and aqueous *H. suaveolens*, butanol *H. suaveolens* respectively.

Figure 4.5. Glutathione level in the resistant and susceptible individuals of the larvae of *An. gambiae s.l.* after treatment with extracts of the two plants.
Values are presented as mean ± standard deviation (n=3). Data with superscript 'c' indicates significant difference (P<0.05) when compared with control; those with 's' are significantly different (P<0.05) with their susceptible counterparts under the same extract; while those bearing 'e','e*' and 'e**' are significantly different (P<0.05) with their respective counterparts under the same outcome (resistant or susceptible) for aqueous *C. odorata*, butanol *C. odorata* and aqueous *H. suaveolens*, butanol *H. suaveolens* respectively.

Table 3 provides the result of the GCMS of the aqueous and butanol fractions of *C. odorata* and *H. suaveolens*. It shows that for all the fractions, there is presence of a compound with retention time of 22.967 and mass-charge ratio of 264 which is Oleic acid (octadec-9-enoic acid). An additional compound was shown to be present in the butanol and aqueous fractions of *H. suaveolens* which had retention time of 26.764 as well as mass to charge ratio of 256. This reveals the presence of palmitic acid (hexadecanoic acid), another fatty acid.

Table 3. Compound Composition of Aqueous and Butanol Fractions of Leaves of *Chromolaena Odorata* and *Hyptis Suaveolens* by GC-MS

	Retention Time	Formula	Mass-charge (m/z) ratio
***C. odorata* Aqueous**			
Octadec-9-enoic acid	22.967	$C_{18}H_{34}O_2$	264
***C. odorata* Butanol**			
Octadec-9-enoic acid	22.967	$C_{18}H_{34}O_2$	264
***H. suaveolens* Aqueous**			
Hexadecanoic acid	26.764	$C_{16}H_{32}O_2$	256
Octadec-9-enoic acid	22.967	$C_{18}H_{34}O_2$	264
***H. suaveolens* Butanol**			
Octadec-9-enoic acid	22.967	$C_{18}H_{34}O_2$	264
Hexadecanoic acid	26.764	$C_{16}H_{32}O_2$	256

Discussion

The present study revealed that the aqueous and butanol fractions of *H. suaveolens* possess higher toxic effects on *An. gambiae s.l* than those of *C. odorata.* Phytochemical analysis revealed the presence of tannins, saponins, flavonoid, terpenoids, phenols, phlobatannins and alkaloids and the larvicidal activity of these plants could be attributed to the presence of these phytochemicals. Phytochemicals are secondary metabolites that function in plant defense system and their presence has been associated with various properties including insecticidal (Udebuani, *et al*, 2015). Also, previous studies have demonstrated toxicity of the extracts of the leaves of *C. odorata* and *H. suaveolens* against different species of mosquito larva (Appadural *et al*, 2014).

Furthermore, GC-MS characterization of the bioactive fractions revealed the presence of oleic acid, palmitic acid as the dominant fatty acid. Unsaturated fatty acids have been reported to show greater toxicity than saturated fatty acids on mosquito larva (Harada, *et al.,* 2000). Quantitative structure-activity relationship, (QSAR) of phytochemicals toward mosquito has been well documented (Wang, *et al.,* 2012). Perumalsamy *et. al* (2015) reported that QSAR of oleic acid indicates that structural characteristics, such as the degree of saturation, the side chain length and geometric isomerism play a role in the fatty acid toxicity to mosquito larva. The insecticidal potency of the essential oil of *H. suaveolens* is well documented (Owolabi *et. al.,* 2014).

The continuous and improper use of chemical insecticides has resulted in the development of insect resistance to insecticides. One of the prominent strategies deployed by insects to combat xenobiotic challenges is through metabolic detoxification. This is achieved by up-regulation of genes leading to the induction and synthesis of detoxification enzymes in response to xenobiotics. Observations in this study showed that there was significance increase, in the levels of GST, cytochromes P450 monooxygenase and esterases when compared with the control as well as between resistant and susceptible individuals. This difference could be linked to the induction of the detoxification enzymes in response to the action of phytochemicals contained in the extracts. Furthermore, increased activity of detoxifying enzymes is correlated with overexpression of associated genes in insecticide resistant populations of insects (Ranson *et al*., 2001; Wondji *et al*., 2009). Thus it can be implied that the resistant individuals survived through this mechanism.

Glutathione is important in cellular defense against toxic compounds. It is involved in phase II reaction which is essentially conjugation and redox reactions. Products of phase I reaction, after undergoing biotransformation which render them suitable for conjugation with

polar moiety are passed on to phase II thus making the entire molecule water-soluble (Timbrell, 2009). The result of glutathione levels (Figure 5) showed that the glutathione levels for *H. suaveolens* treated larvae were higher for both aqueous and butanol fractions in both the resistant and the susceptible strains when compared with the corresponding fractions of the *C. odorata*. The GSH level in all the resistant individuals was relatively high suggesting that the pathway for the detoxification was largely through the phase I reaction and possibly cytochromes P450 systems. As observed earlier, there is possible induction of cytochromes P450 particularly in the resistant individuals thereby detoxifying the toxicant. Previous investigation by Imam and Deeni (2014) showed a relationship between increased levels various environmental xenobiotics and the corresponding increase on P450 monoxygenase activity on the larvae of *An. gambiae s.l*. and opined that since detoxification systems are conserved in insects, there is a possible induction of these enzymes in response to exposure to xenobiotic products. Similar findings have been established for many other insect species (Adekunle *et al.*, 2010).

Conclusion

The findings in this work reveal that the partially purified aqueous and butanol fractions of leaf extracts of *H. suaveolens* and *C. odorata* possess larvicidal activity, with fractions of H suaveolens displaying higher activity. The activities were found to be dose dependent. Detoxification machinery (Glutathione and the detoxification enzymes) of the extracts treated larvae were found to be pronounced than the control. This indicates that these two plants contain phytochemicals that possesses potential larvicidal activities which if harnessed could play a vital role in the development of novel bioinsecticides for control and management vector borne diseases.

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