Bioactivity of Leaf Extract *Hyptis suaveolens* (Bush tea) on Larvae of *Anopheles gambiae* Collected from Keffi Area, Nasarawa State, Nigeria.

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Abstract

Malaria vector control continues to be a major challenge in -Nigeria. *Hyptis suaveolens* (Bush tea) is one of the traditionally used mosquitos repellent. The effect of methanolic leaf extract of *Hyptis suaveolens*on larvae of *Anopheles gambiae* sampled from Keffi, was conducted following the world Health Organization guidelines for laboratory testing. Samples of the leaf were collected and used for test Procedures. Larvae of *A. gambiae* were exposed to 50, 100, 150,200, 250 and 0.00(control) mg/ml concentrations of leaf extract for 72 hours. Percentage mortality was calculated by using Abott's formular and lethal concentration (LC_{50}) was determined by a log dosage probit mortality. The result revealed highest and lowest mortality rate of 25.00 ± 0.00 and 1.66± 0.00 for larvae respectively. There were significant differences at ($P<0.05$) on the mortality rate on the larvae exposed to different concentrations of the plant extracts. The 72 hours LC_{50} value leaf extracts on larvae was 52.00mg/ml. The result of this study indicated that *Hyptis suaveolens* possess larvicidal repellant properties and it should be encouraged to be used in communities at zero expense.

Keywords: *Hyptis suaveolens, Anopheles gambiae,* methanol Extract, mortality.

Introduction

Mosquitoes are vectors for many diseases such as dengue fever, yellow fever, malaria, filariasis, Japanese encephalitis and other fevers. There are many types of mosquitoes living in the tropical and sub-tropical regions of the world - *Anopheles, Aedes* and *Culex. Anopheles stephensis* is one of the most common mosquitoes in Nigeria and the primary vector of malaria (Donald, 2004). The control of mosquitoes is however becoming increasingly difficult because the effectiveness of vector control has declined due to development of resistance by vectors against the synthetic organic insecticides and environmental hazards as a result of persistent use (Dolianitis and Sinclair, 2002). In the absence of prophylactic vaccine, DDT and other insecticides for effective control of mosquitoes, wide variety of plant species from various ecosystems that have a range of acute and chronic toxic effects against mosquitoes were used locally to control mosquitoes in communities (Shaalam *et. al*., 2005). Currently more than 2000 plants species have been identified as having insecticidal, repellent properties and about 344 plant products are known to possess anti-mosquito characteristics (Adda *et. al*., 2011). *Hyptis suaveolens* is one among such plant species used locally in rural areas as repellent for biological control of mosquitoes (Hemen *et. al.*, 2013). Considering the health challenges that the poor and less privileged people, mostly in tropical Africa and Nigeria, are facing, in particular, malaria, the control of this mosquito-borne disease is however becoming increasingly difficult. (Dolianitis and Sinclair, 2002). The ethanoic extract of *Hyptis suaveolens* was examined for its toxicity effect on the larvae of the yellow fever mosquito *Aedes aegypti* (Bhagwat and Umathe, 2003). Extracts of various parts of *Hyptis suaveolens* have been obtained with solvents like petroleum, chloroform, methanol, ethanol, n-hexane, and water using soxhlet extraction, cold maceration, and steam distillation methods (Edeoga *et. al.*, 2005).Consequently, interest in plant-based products has been revived because of the development of resistance, cross-resistance, possible toxicity hazards associated with synthetic insecticides and their rising cost. The phytochemical compounds obtained from the huge diversity of plants species from the tropical forests are important sources of safe and biodegradable chemicals, which can be screened for larvicidal activities (Kavendan *et. al*., 2014). A large number of plant extracts have been reported to have excellent larvicidal activities, such plants as *Azadirachta indica*, *Cymbopogan sp*, *Eucalyptus sp*, *Maculata citrodon* (Shalaam *et al*, 2005). *Draceana aborea* and *Vitex doniana* (Nnamani *et. al*., 2007). The aim of this study was to evaluate the effect of leaf extract of *Hyptis suaveolens* (Bush tea) on larvae of *Anopheles gambiae*as a means of mosquito control, determining the effect of leaf extract of *Hyptis suaveolens* against *Anopheles* mosquito larvae and examine phytochemical components of the leaf extract.

Materials and Methods

Study Area

The study was conducted in Keffi Area Nasarawa State Nigeria. Keffi Local Government Area is located in the basement complex of northern Nigeria between longitude 7^0.49'03" and latitude $8^0$46;$8^0$53 5", it stands at an elevation of 400 meters above sea level. It was created in 1976 and is undergoing rapid population growth and infrastructural development due to its proximity to the Federal Capital of Nigeria for it shares border with the centre of unity Keffi has a population density of 450-500 persons from (2006 census), making it one of the most densely populated local governments of Nasarawa state (Zurbrugg 2009).

Collection and Identification of *Hyptis suaveolens*

Hyptis suaveolens leaves were collected in the month of July, 2018 around Plant Science and Biotechnology garden in the Faculty of Natural and Applied Sciences, Nasarawa State University Keffi, Nigeria. The identification of

the plant was confirmed by Mr. Lateef Akeem at Herbarium Department of Medicinal Plant Research and Traditional Medicine, NIPRD Abuja. The voucher number NIPRD/H/6967 was deposited to the sample.

Preparation and Extraction of Plant Material

The Fresh leaves were carefully washed and raised with distilled water, they were allowed to dry under shade for a period of five days. The dried leaves of *Hyptis suveolens* were grounded in to powder using wooden pestle and mortar until the powder passed through a 0.4mmmesh sieve. The powder was stored in opaque containers inside a refrigerator until needed.

400g of dried powdered leaves of *Hyptis suaveolens* was weighed and macerated in 100% methanol and allowed to stand for 24hr in other to make sure all active ingredient from the plant get extracted. The solution was filtered using Muslin cloth and vacuum filtration. It was observed twelve hourly for period of 48 hours (two days) at Chemistry Laboratory of National Institute for Pharmaceutical Research and Development NIPRD Abuja. The filtrate was concentrated using the rotary evaporation. The sticky extract was finally poured in an evaporating dish and dried on the water bath for final drying to solid. The solid extract was weighed and collected in a sample bottle to be used for study.

Mosquito Larval collection and rearing

Larval stages of *Anopheles* mosquitoes were collected from different locations in Keffi Area. The collection was done during the month of September to October at each location, *Anopheles* larvae were collected from various breeding sites including ground pools, waste tin, puddles and majority from the shoulder of the bridge (WHO 2006). Mosquito larvae were scooped using a scoop net and later emptied in to transparent plastic bowl. Prior to introduction of larvae, the bowl were scrutinized for presence of unwanted organisms or predators; which were excluded. The mosquito larvae collected were returned in well labeled plastic bottles to the insectary laboratory at Nasarawa State University Keffi. The larvae were transferred to the containers with fresh water in order for larvae to acclimatize. Susceptibility test were done to some larvae following WHO standard operation procedure (Harbach 2012).

Identification of Larvae and Adults Mosquitoes

Third and fourth instar *Anopheles gambae* larvae were identified from the Insectry laboratory zoology Department Nasarawa State University Keffi, properly identified and separated accordingly, by using the standard taxomonic Keys (Tyagi *et. al.*, 2012).

Larvicidal Test

Bioassay for the larvicidal test was carried out following the WHO (2013) standard procedures for laboratory testing of mosquito larvicides. The methanol extracts of *Hyptis suaveolens* were evaluated using different concentrations of 50mg/ml, 100ml/mg, 150ml/mg, 200ml/mg and 250ml/mg. distilled water (100ml) to which 1ml acetone was added and used as controls(negative control) while another container with only distilled water were used as (positive control) with three replicates from each – were used. Thereafter, twenty five larvae of *Anopheles* mosquitoes were introduced to each container. Larval mortality was counted at 24, 48 and 72 hours after treatment. Larvae were considered either alive if they were clearly moving normally, or dead when there is no movement and no response (WHO 2013).

The interpretation of the mortality rate of larvae of *Anopheles* mosquito based on (WHO 2013). Susceptibility test is:

Mortality rate between 98-100% within the diagnostic time.

Mortality rate between 80 -97% suggest possible resistance.

Mortality rate < 80% indicates resistance.

The percentage mortality was calculated by employing the formula:

$$\%\ Mortality = \frac{Number\ of\ dead\ lavae\ x\ 100}{Number\ of\ lavae\ introduced}$$

Statistical Analysis

Abott's formal was applied for mortality correction whenever required. The percentage mortality were calculated and mean ± standard deviation was subjected to the analysis of variance (ANOVA) SPSS 22.0. Version. Lethal concentrations were considered 50% (LC50) mortality of mosquito larvae. The P-values < 0.05 were considered statistically significant.

Results

Table 1: Phytochemical Constituents of Leaves of *Hyptis suaveolens* (Bush tea)

Phytochemical Constituent	Status
Alkaloids	+++
Glycocides	+++
Terpenoids	++
Phenol	++
Tannin	++
Saponin	++
Flavonoids	++
Steroid/Triterpenoids	+

Key:
+++ High concentration
++ Moderate concentration
+ Low concentration

Phytochemical screening of methanol extract of dried leaves of *Hyptis suaveolens* is as presented in table 1. The analysis revealed that alkaloids and glycosides were observed to be the highest recorded (+++) whileterpenoids, phenol, tannin, saponin, flavonoids has moderate concentrations (++) and least was steroids/triterpenoids (+).

Table 2: Average cumulative percentage mortality of *Hyptis suaveolens* extract on larvae of *Anopheles gambiae*

Conc. (mg/ml)	Log Conc.	Average No. of Larvae	Percentage mortality perhours 24hrs	48hrs	72hrs	Abbot's value (72 hrs)	Probit Values
Control	0.000	25	0.00	0.00	1.32	0.00	0.0000
50	1.698	25	17.32	37.32	56.00	54.20	51.1055
100	2.000	25	34.64	49.32	66.64	66.70	5.4316
150	2.176	25	49.32	68.00	78.64	79.20	5.8134
200	2.301	25	66.64	78.64	91.70	91.70	6.3852
250	2.397	25	73.32	98.64	100.00	100.00	8.7190

Table: 2 Shows cumulative percentage mortality larvae of *Anophles gambiae* exposed for 72hrs to various concentrations of leaf methanolic extract. The *Hyptis suaveolns* achieved lowest and highest mortality rate (5.1055%) and (8.719%), at 50mg/ml and 250mg/ml concentrations respectively. However there was a significant difference among the five concentrations of leaf extract on mosquitoes larvae mortality, F (df between 4, df within 10) = 147.25, (P < 0.05) more so, post hoc testing revealed significance difference between each concentration (dosage) of *Hyptis suaveolens* methanolic extract with 50mg/ml, (M = 14.00, SD = 1.00) having fewer larval mortality after 72 hours, then 100mg/ml (M = 22.67, SD = 0.577), 150mg/ml (M = 19.67 SD = 0.577), 200mg/ml (M = 22.67, SD = 0.577) and 250mg/ml (M =

25.00, SD = 0.00) in an increasing order respectively. These findings indicated that, there was more larval mortality in 250mg/ml followed to 200mg/ml and down to the least concentration (50mg/ml) of *Hyptis suaveolens* methanolic extract respectively.

Table 3: LC_{50} of Methanolic Extract of *Hyptissuaveolens*

Time of Exposure (Hours)	LC_{50} (mg/ml)
24	160.00
48	113.00
72	52.00

Table 3: Shows lethal concentration (LC_{50}) of the extract that kills 50% of the *Anophles gambiae* larvae at different hourly. The LC_{50}value of 24hrs, 48hrs and 72hrs with 160.00mg/ml, 113.00mg/ml and 52.00mg/ml respectively. However, the lower the LC_{50} value the more effective the larvicidal activity of the extract.

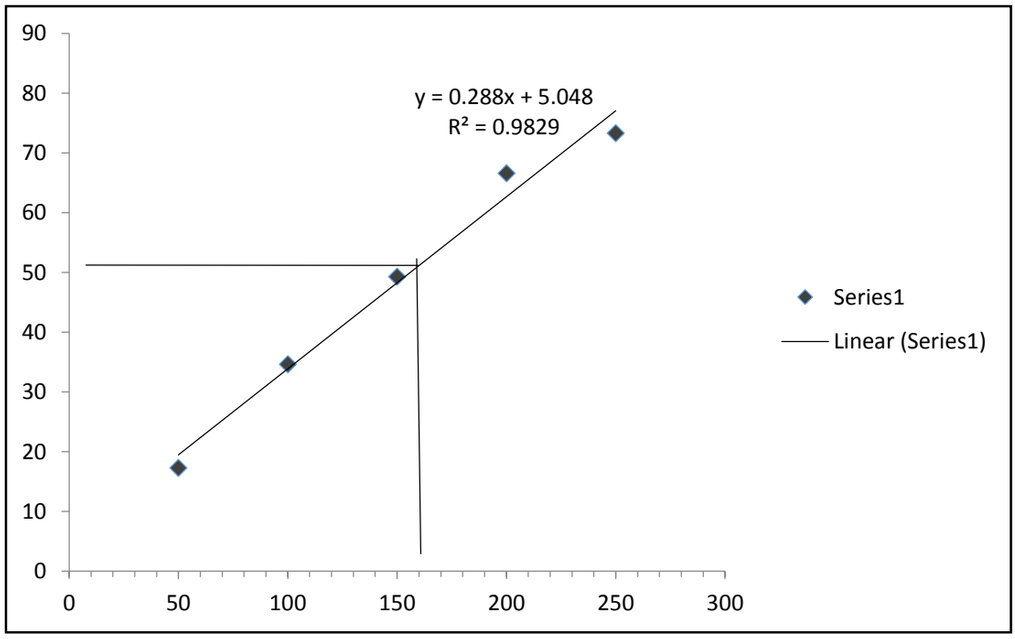

Fig. 1: Lethal concentrations of leaf extract of *Hyptis suaveolens* (Bush tea) on larvae of *Anopheles gambiae* at 24 hours

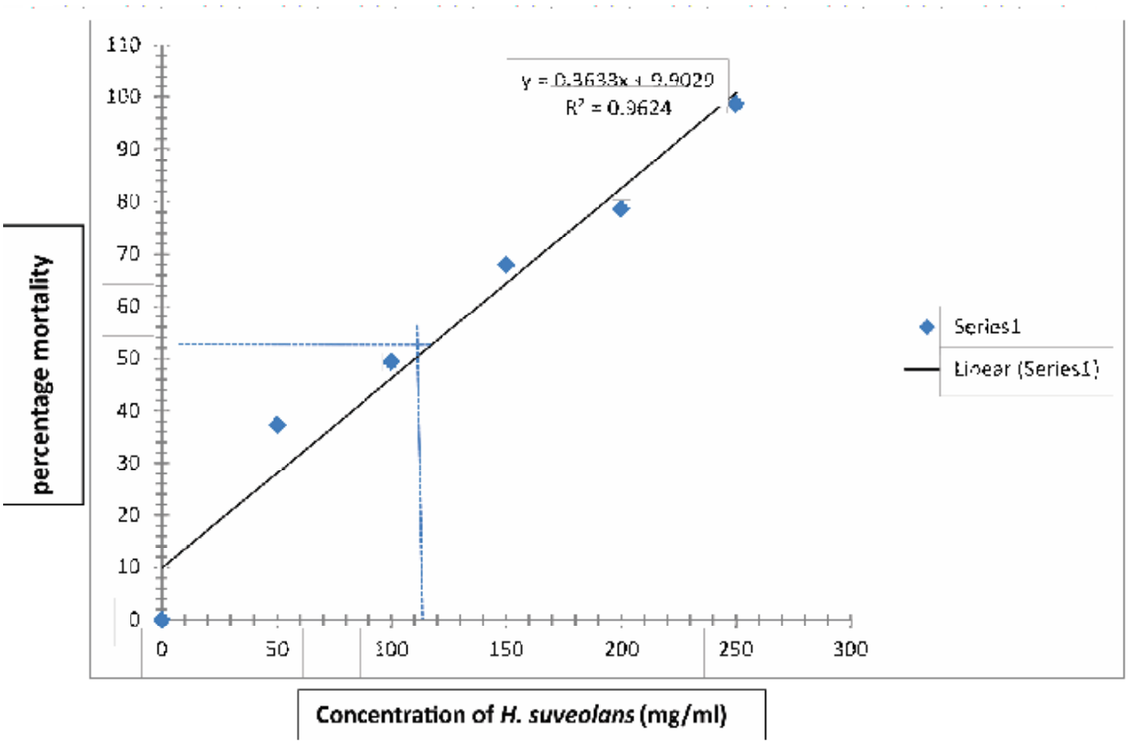

Fig 2: Lethal concentrations of leaf extract of *Hyptis suaveolens* (Bush tea) on larvae of *Anopheles gambiae* at 48 hours **Concentration of *H. suveolans* (mg/ml)**

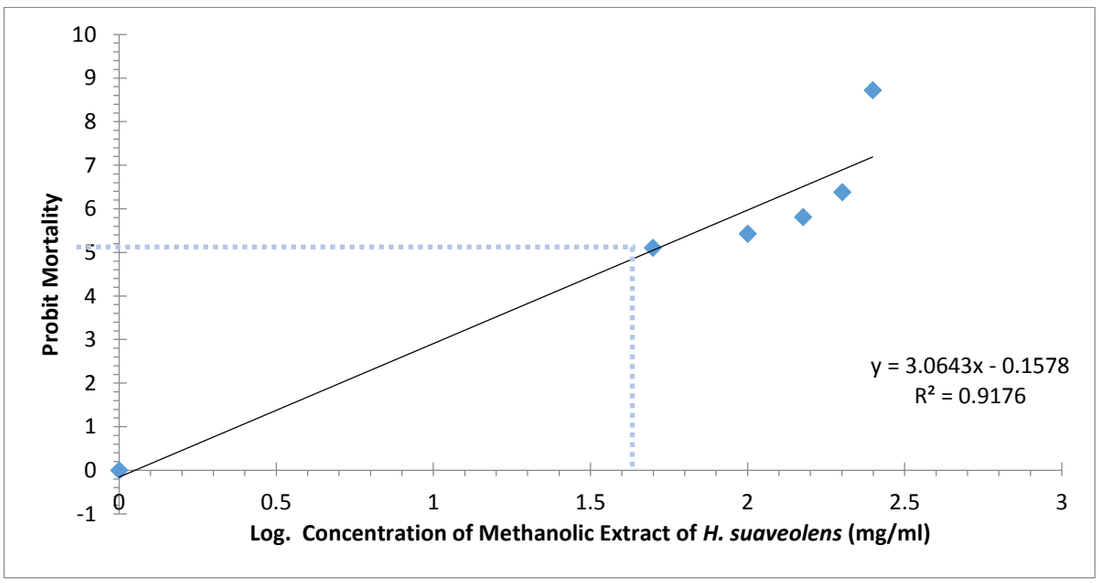

Fig. 3: Lethal concentrations of leaf extract of *Hyptis suaveolens* (Bush tea) on Larvae of *Anopheles gambiae* at 72 hours.

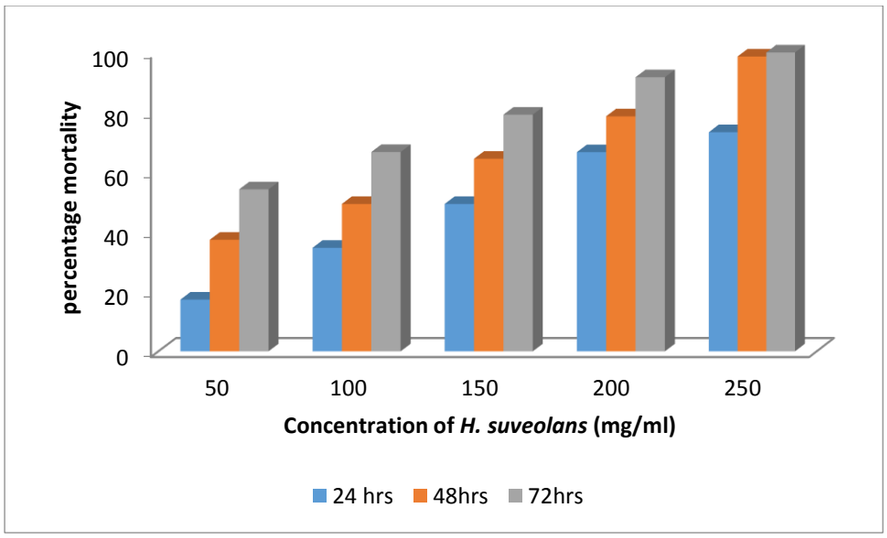

Fig. 4: Percentage mortality of A*nopheles gambiae* larvae treated with *Hyptis suaveolens* extract after 24, 48 and 72 hours.

Discussion

The presence of some bio-active components such as alkaloids, tannin, flavonoids, saponin, steroids/triterpenoids, glycosides, terpenoids andphenol in *Hyptis suaveolens* while the absence of saponin and Anthraquinones in leaf extracts is consistent with other researches such as study on the larvicidal activity of the methanolic leaf and stem/bark of *Jatropha ypticurcas, Citrus grindis and Tinospora rumphi* against the dengue vector, *Aedes aegypti* mosquito. Gutierre *et al.*,(2014) reported that the leaf and bark methanolic extrat of *Jatrapha curcas* contains alkaloids, flavonoids and steroids while the leaf and bark/stem methanolic extracts of *Citrus grindis* and *Tinospora rumphii* are rich in alkaloids saponins, tannin flavonoids and steroid. These compounds are known to possess insecticidal and larvicidal abilities of insects and other animals (Okigbo 2010).

The leaf extract of *Hyptis suaveolens* administered at different concentrations (50, 100, 150, 200 and 250 mm/ml) and time (24, 48 and 72 hours) revealed that they have good degree of toxicity with LC50 values 87.00, 100.00 and 135.00mg/ml on *Anopheles* mosquito larvae. This finding is differs with the works of other researchers such as Kochela *et al* (2010).Who reported that methanol leaf extracts of *Ficus bengalensis* has LC50 of 60.44, 76.41 and 89.55ppm on second, third and fourth insta larvae after exposure for 24 hrs. The results are comparable to earlier result of Yohanna *et al.* (2012) using *Artemisia annua* extract against *culex* mosquito larvae El-boki, (2003) using the neem *Azadiracha indica* extract against pipiens larvae andAbdullahi *et al.* (2011) using aqueos extract of leaf of *Strigaher menthila* and *Mitracarpusscaber* against *Culex quinquefasciatus* larvae. There was striking difference in the larvicidal effect of plant extract on the *Anopheles* mosquito larvae. *Hyptis suaveolens* extract at 24, 48 and 72hr had mortality up to 73.32%, 98.64% and 100.00% of *Anopheles* mosquito larvae The high mortality reported in this study is in agreement with Cruz-esrada *et. al.* (2015) who reported that the ethanolic extracts of *Acalypha, Gaimeni, Annona, Squamosa, Carlowrightia, Myrintha, Petrivena alliance* and *Trichillia arborea* (collected from different localities of the Yucatan peninsula, Mexico) at concentration of 10mg/ml cause high mortality (95 to 100%) on *B. tabali* eggs. Similarly, mortality caused by aqueous extract of this plants ranged from 98 to 100% at concentration of 3% no significant differences on mortality within the same type of extract and the chemical insecticide inaidachoprid were observed.

The overall mortality of *An. arabiensis* in diethytoluamide sprayed huts (82%) was

significantly higher than lambdacyhalothrin (76% p= 0.043) and not statistically different to pirimiphos methyl (86%, p = 0.204). mortality rates of *Cx. quinquefasciatus* in all sprayed huts were much lower than those recorded for, *An. arabiensis*. However, mortality rates associated with all sprayed huts were significantly greater than the control Kitau *et al.* (2014).

In a study carried out on sixteen aromatic plants extracts from three species belonging to the asteraceae family: (*Mantisalladuriali*, *Rhaponticumacuule* and *scorzoneraindulata*) obtained by using organic solvents of increasing polarity, they were tested for insect growth inhibition contact toxicity and antifeedant activity against larvae of confused flour beetle *Triboliumconfusumduval* (coleopteran tanebirionidae). For all extracts mortality was higher at larval stages 83%and 77% respectively by using petroleum ethal and methanol extracts of *R. communis*, suggest that *R communis* may be used in grain storage against insect pests.

However, the result of this study is at variance with Jbilou (2006) who recorded slightly low mortality (58%) of *Tribolum castermum* caused by the 10 days after treatment. At the same time mortality rates from the extracts of *Aristolechiabaefica, Jugaiva and Raphanusraphanistrum* (100mg/ml) each reached 34, 31 and 26% respectively.

Yohanna*et.al.* (2012) reported that oil from *Lantancamara* leaves produced repellence action against the bees, *Apismellifera*, house fly and tabanu species. The mosquitocidal activity of *Lantana camara* has been administered on the larval forms in laboratory. Studies by (Anyanwu, 1997) showed that this plant produced 90-100% mortality of larvae of *Aedes aegypti* and *Cx. Quiqui fasciatus*. The results of lantana leaves extract also proved that they have larvicidal properties against *Anopheles gambiae*.

This finding agree with Nath *et al.* (2006) who reported that leaf extract of *lantana camara* showed larvicidal activity against *Cx. Quiquesciatus* and *Aedes allbopicus*. Another study by (Jbilou 2006) showed the effect of the root barks of *lantana viburnoids* species against late 3rd or 4th instar larvae of *Tribolium castemum*. They reported that extracts could serve as a source of larvicidal for managing various mosquito habitats in the field. Similarly, the presence of Alkaloids, steroids, glycosides, terpenoids, phenols, saponin, flavonoids and tannin in *Hyptis suaveolens* may serve as an indicator for the plants mosquito larvicidal properties.

In conclusion, the results of this work have shown that leaf of *Hyptis suaveolens* extract have larvicdal activity against *Anopheles* mosquito, which support the ethno botanical use of larvicides. The extract can also be used for control programmes in various situations. They offer safer alternative to synthetic chemicals and can easily be obtained by individuals and communities at zero expense.

Screening of locally available medicinal plants for mosquito control would generate local employment reduced dependence on expensive and imported products and stimulate local effects to enhance the public health system.

Recommendations

Considering the insecticidal properties of *Hyptis suaveolens* leaf, it is recommended that

i. The oil can be used in subsequent work to make mosquito repellent popularly known as mosquito coil.
ii. Further studies could be done by extracting the components of the oil and test each one of themto know the particular compounds that are responsible for mosquitocidal, larvicidal and repellency properties. This information could help in determining the possibility of using the oil to make insecticides or use it as a component of fumigant to eliminate all unwanted target insects.

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References

Abdullahi, K. Abubakar, M.G., Umar, R A. Gwarzo, M.S., Mohammad, M. and Ibrahim, H.M. (2011). The Larvicidal efficacy of aqueous extracts of *Striga herrmonthica* (delile) *Benth* and *Matra carpusscaber* (Zucc) on *Culex quinquefasciatus* Larvae. *Nigeria Journal of parasitology*, 35(1): 105-10

Adda, C., Atachi, P.K and Tom O.M (2011). Potential use of the bush mint, *Hyptis suaveolens* for the control of infestation by pink stalk borer, sesamiacalanistis, maize in southern Benin,.*West Africa Journal insect science*. 11.33.

Anyanwu, G.I. and Uloko, J.I. (1997) Evaluation of insecticidal effects of *Lantana camara* (Verbanaceae) on mosquito adults and Larvae. *West African Journal of Pharmacol Drug Resource*, 13(2): 23-26.

Bhagwat D.P and Umathe, S.N (2003) Theimmunomodulatory activity of *Hyptis Suaveolens* (L.) Poit, Family –Lamiaceae *Indian Journal of Phamatology*. 35:128-36.

Cruz-Estrada A., Gamboa. Angulo M., Rocio Borges-Argez R. and Ruiz-Sanchez, E (2015). Insecticidal effects of plant extracts on immature-Wintefly *Bemisiatabaci* Genn. (Hemiptera: Aleyroideae). http://www.ejbiotechnology.infodoi: 10.225.5/Voll 6-issue 1-full text – 6. Accessed 15/12/2015 at 11:40am.

Dolianitis, C. and Sinclair, R. (2002) Optimal treatment of head lice: is a no-nit policy justified; *Clinical Dermatology*, 20: 94-96

Donald, J. (2004). Mosquitoes in Your Life**.** Associate Research Professor in Entomology [On-line].Availabl: http://www.rci.rutgers.edu/~insects/moslife.htm.

Edeoga, H.O., Okwu, D.E and Mbaebie B.O (2005), Phytochemical constituents of some Nigerian medicinal plants. *African journal of Biotechnology* 4(7): 685-688

El-Boki, M.M (2003). Latent toxicity of *Azadrrachtin* treatment on *culex pipiens* (Diptera: culicidae): *journal for eqcyptician Academic society for environmental development*3. 63-74.

Gutierrez, P.M., Antepuesto, A.N., Eugenio, B.A.L and Santos, M.F.L. (2014). Larvicidal activity of selected plant extracts against the dengue vector *Aedes aegypti* mosquito. *International Research Journal of Biological sciences*, 3(4): 23.

Hemen, T.J., Johnson, T.J. Ujah, O. F., and Udenze, E.C. (2013) Ethnobotanical effect of *Hyptis suaveolens* Plant on Mosquito Species Population in Guinea Savanna, Nigeria. *Pharma Science Monitor* 4(4); 249-254

Harbach RE (2012), *Culexpipiens:* Species *versus* species complex-taxanomic history and perspective mosquito control association 28:10-23.

Jbilou, R., Ennabili A. and Sayah, F. (2006).insecticidal activity of four medicinal plant extracts against *Tribolium castaneum* (Herbs) (*Coleoptera: Tenebrionidae*). *African Journal of Biotechnology* 2006, 5(10): 936-940.

Kavendan, K. Mahesh P., K., Subramaniam J, Murugan K and Williams J.S. (2014) larvicidal activity of indegenious plant extracts on the rural malarial vector *Anopheles culicifaciesgiles, Journal of Entomological and Acarological Research* volume 47, 1747

Kitau J., Oxborought, R. Matowo, J. Mosha, F., Magesa, S.M. and Row Land, M (2004) indoor residual Spraying with microencapsulated DEET repellent (N, N-diethyl-m toluamide) for control of *Anopheles arabiensis* and *Culex quiquefasciatus*. Parasites and Vectors 2014, http//:www.parasite and

vectors.com/content/7/1/446.Accessed 02/10/2014 at 1:23pm.

Koche, D., Shirsat, R., Imran, S. and Bhadange, D.G. (2010).Phytochemical screening of eight traditionally used ethnomedicinal plants from Akola district (MS) India. *International Journal of Pharma. and Bio Sciences*, 1(4): 253-256.

Natch, D.R., Bhuyan, M; and Goswami S. Botanical's (2006), a mosquito larvicides, *Defence Science Journal*, 56:507-511.

Nnamani, C.V., Oselebe, H.O and Ogbonna, A.N. (2007).Effects of leaf extracts of Draceana aborae L. and Vitex doniana Sweet on the larvae of Anopheles mosquito. *Animal Resaerch International*, 5(2): 184-186.

Okigbo R.N, Okeke J.J, Madu N,C. (2010) Larvicide effects of *Azardirachta Indica, Ocimum gratis simum* and *Hyptis suaveolens* against mosquito larvae. *Journal of Agricultural Technology*, 6 (4): 703-719.

Shaalam, E., Canyon, D. V., Younes, M., Abdel-wahab, M. and Mansour, A. (2005).A review of botanical phytochemicals with mosquitocidal potential. *Environment and International Health*, 15: 1149 – 1166

Tyagi, B.K., Munirathinam, A, Krisnamorthy, R. and Venkatesh, A. (2012). A field based handbook on identification keys to mosquitoes of public health importance in India (RME Madurai).

WHO, (2006): Indoor Residual Spraying: Use of Indoor Residual Spraying for Scaling Up Global Malaria Control and Elimination. WHO Position Statement (PDF) (Report). World Health Organization. 2006.

WHO (2013) Susceptibility Test Manual Instructions for Determining the susceptibility or resistance of mosquito Larvae to insecticides. WHO/VBC/75.583.

Yohanna J., Mafuyai H.B and Akinyombo, A.O. (2012).The larvicidal effects of leaf extract of *Artemisia annua* (Sweat wood-worm) on *Culex* mosquito larvae.

Zurbrugg C. (2009) Solid Waste Management in Developing and Countries. (www.sanicon.net) Accessed – on 18th July 2016.