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Activity guided fractionation of *Nicotiana tabacum* extract that is lethal on *Anopheles gambiae* larvae (Diptera: Culicidae) in Nigeria

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Abstract
Extracts of *Zingiber officinale*, *Erythrophleum africanum*, *Calotropis procera*, *Nicotiana tabacum* and *Datura metel* were assayed for lethal activity against 4th instar larvae of *Anopheles gambiae*. Activity guided fractionation of the extracts with organic solvents was employed. Each fraction obtained was formulated into 4.0mg/mL concentration and tested against 25 larvae over 12 to 72 hrs. Phytochemical screening, column and thin-layer chromatography as well as GC/MS of the extract were used for searching active ingredients. Data was analyzed through Analysis of Variance and differences between means were separated with DNMRT. Aqueous extract of *N. tabacum* exhibited highest lethal activity (100% mortality) of the larvae. Ten column fractions were determined for the extract, with Meth III fraction causing 80% mortality. GC/MS revealed that, Bicyclo (2, 2, 1) heptane 3-methylene-2, 2,-dimethyl-5-o1 acetate (62.40%) was the major compound. The compound could be responsible for lethal activity, thus, may be useful candidate for further investigation.

Keywords: *An. gambiae*, larvae, lethal activity, *N. tabacum*, phytochemicals, plant extracts

1. Introduction

Any mosquito that bites or annoys people can be considered a health problem, particularly for individuals who are allergic to mosquito bite or suffer from entomophobia. Mosquito-transmitted diseases, such as malaria, filariasis, dengue fever, chikungunya, zika, and yellow fever, pose a significant public health concern, as a relatively large proportion of the human population is exposed to these infectious diseases, especially in tropical areas [1]. Prevalence of these diseases is in more than 100 countries across the world, infecting over 700,000,000 people every year globally [2]. There are more than 3500 mosquito species in the world grouped into 41 genera and 135 subgenera [3, 4]. Of these numbers, around 73 species occur in Nigeria [5, 6] with more prominent ones belonging to genus *Aedes* (40 common species), *Anopheles* (one or more species found in each state) and *Culex* (29 species with 12 common ones) [7]. The vectors of malaria in Nigeria are *Anopheles culicifacies*, *An. stephensi*, *An. fluviatilis*, *An. minimus*, *An. gyrus* and *An. gambiae* [8]. These species are responsible for transmission in specific ecotypes with *Anopheles culicifacies* accounting for 60-70% of malaria transmission in rural and peri-urban areas [9]. But *Anopheles gambiae* commonly referred to as the African malaria mosquito, is the most common vector of human malaria in the Afro tropical Region [10].

Control efforts can be justified when a mosquito poses a nuisance or is an economic or health-related pest or vector. Most of the widely used vector interruption methods are synthetic insecticide-based. Many of them target the adult mosquitoes as adulticides such as DDT, BHC, pyrethrins, pyrethroids, chlorpyrifos, malathion, carbaryl, sumitrin etc. while others are used as larvicides against the larvae, like methoprene (IGR), Altosid, Abate, BVA oils, agnigue etc. A major challenge facing malaria control is the high spate of insecticide resistance in mosquito populations [11] with reported cases in Africa and Nigeria in particular [12]. Other problems associated with injudicious use of synthetic insecticides include non-biodegradability, harmful effects on natural enemies, and environmental pollution [13]. However, resistance to pesticides was not serious before the early 1940s when raw plant materials were the principal agents of pest control [14]. Research focus is now on new bioactive phytochemicals from the plant kingdom and an effort to determine their structure and commercial production. These phytochemicals make up to 1 per cent of world's pesticide market [15]. The current study analyzed *N tabacum* extract with highest lethal activity against *An. gambiae* mosquito through activity guided fractionation.

2. Materials and Methods

2.1 Mosquito larvae treated

Breeding sites such as gutters and man-made ditches of less than 2 meters deep were searched for the collection of mosquito larvae. Collection of the larvae was made using plastic dipper (300 mL, handle length: 11cm) by simple scooping. From the composite samples of larvae collected, species of *Anopheles gambiae* were sorted and identified in the laboratory using the keys [16] and pictorial guide [17].

2.2 Rearing of the larvae

The Larvae were fed with brewer's yeast as per WHO (2006) protocol in plastic trays inside wooden cum wire gauze cage (50 x 30 x 30cm).The cage had internal demarcation of mesh netting (3cm^2 per hole), served as pigeon box. Adults that emerged out were fed on 10% sucrose solution but females blood-fed in addition with restrained three – weeks old chick (shaved from the back and underside). In this procedure, multiple generations of the mosquito were maintained and used as larval stock for the study.

2.3 Plant materials and extraction method
Samples from five plant species were purchased within Sokoto main market (13° 00'N, 5° 14'E, alt 274m) and identified by a taxonomist at Usmanu Danfodiyo University, Sokoto Nigeria. Rhizome of *Zingiber officinale* (L.), leaves of *Erythrophleum africanum (*Welw.), *Calotropis procera* (Ait.) and *Nicotiana tabacum* (L.) while seed samples of *Datura metel* (L.) were used in this study. All the samples were air –dried under shade for 3 days. Using pestle and mortar, the rhizome and leaves were pulverized into fine powder and sieved with 1mm mesh. Coats covering seeds of *D. metel* were decorted by exerting pressure on the seeds then winnowed to obtain the seed kernels [18]. The seed kernel was pulverized into paste using an electric blender and was emulsified with 0.009mL of polysorbate (Tween-80®) [19]. Aqueous extracts of the plant materials were prepared from which 20% was used for each extract to run preliminary larvicidal test against the larvae, while remaining extracts were dried. The extract found with highest larvicidal activity was subjected to fractionation with three organic solvents in the increasing order of polarity [20]. In this case, 300 grams of each dried extract was reconstituted in 500mL distilled water and equal volume of hexane was added in a separation funnel. This was mixed vigorously for about 40 minutes and the hexane fraction was carefully separated into a beaker. Into the aqueous extract, equal volume of chloroform was added to obtain its fraction and then methanol following same procedure. All the fractions were separately evaporated to dryness on a Buchi Rotavapor (Labortechnik, Switzerland), and 4 grams was measured from each and made up to 1 L with distilled water to form 4000mg/L (4mg/mL) as stock solution. The fractions were independently tested for larvicidal activity and one with the highest was used to run column and thin-layer chromatography.

2.4 Phytochemical analysis
Phytochemical screening of the aqueous extracts was carried out using standard procedures to search for bioactive compounds as described [21].

2.5 Column chromatography of the active extract
Twenty five (25) grams of the active extract was dissolved in minimum amount of the solvent used to pack the column and added to the top of the column. The solvent was drawn from the bottom of the column until the level was just above column level. Fresh solvent was carefully added and the stopcock was open while the solvent continuously flowed through the column. A standard volume of 50mL was collected for each eluted fraction. Those fractions obtained were separately monitored through thin-layer chromatography (TLC) and determined those with similar R_f values.

2.6 TLC of column fractions
Commercially prepared silica gel plates (20x20cm) were used in this aspect. A line of adsorbent (solvent) down to the glass plate was drawn across the plate with a sharp pencil, to maintain the distance of the start line from the solvent surface and positions of initial sports constant. Ethyl acetate: methanol: water (77:13:10 v: v: v), was used as solvent system and a sample of 10µl was applied in single bands (0.5mm long and 2-3mm wide) on the surface of the plate with a suitable capillary tube. The edge of the chromatographic plate with the test samples was immersed in TLC tank, saturated with solvent vapor to attain solvent front endpoint. The plate was subsequently transferred into iodine tank for color development of the spots, then removed, inspected immediately in day light while the spots encircled with pencil. R_f values of the different fractions were determined, those with similar values combined together and solvent evaporated.

2.7 Bioassay
For each dried column fraction, a stock solution of 4000mg/L was prepared and one hundred milliliter (4mg/mL) was transferred into a beaker, also containing larval food (as above). Then, 25 fourth instar larvae was obtained from the larval stock and released in the beaker containing the extract. A similar beaker containing the same number of larvae, food and 100mL distilled water was used as control. The larvae were then maintained at 27 ± 2^0C and 75 ± 5 % RH [22]. Mean death and mortality of the larvae was monitored at intervals of 12, 24, 48 and 72 hours post-treatment. This was by counting and recording the number of dead larvae while all the treatments were replicated three times.

2.8 GC/MS analysis
Gas chromatography and mass spectrometry analysis of the most active column fraction was carried out for constituents' quantification. This was determined by GC-MS Shimadzu Model QP-2010 (Japan) under the following conditions: DB-Polyethylene glycol coated fused silica capillary column (30m length x 0.25mm ID x 0.25µm film thickness): Helium was used as the carrier gas at a flow rate of 1.6 mL/minute, at a column pressure of 100.2 Kpa. 250 ^{0}C injector temperature; 240 ^{0}C interface temperature, 200 ^{0}C ion source temperature, column temperature programmed at 60 ^{0}C with 10 ^{0}C/ minute rise to 230 ^{0}C. For GC/MS detection, ionization energy of 70ev was used. One gram (1g) of sample was taken and made up to 10mL with methanol from which 1µL was injected (split mode) in the column. Components of the sample were identified based on NIST Library.

2.9 Data analysis
Data obtained from the dose – response assays were subjected to Analysis of Variance (ANOVA) procedure using SAS v.9.1 software and significant differences in the treatment means were separated with Duncan's New Multiple Range Test (DNMRT) at 5% level. Mortality of the larvae was expressed in percent.

2.10 Ethical approval
This work has been approved by the Research and Ethics Committee (UREC), Usmanu Danfodiyo University Sokoto, Nigeria with reference No. UDUS/UREC/2019/016.

3. Results
3.1 Preliminary determination for lethal activity
Preliminary observations on the lethal activity of 20% aqueous extract of the selected plants on *An. gambiae* larvae are presented in Table 1. The results showed that, 21.67±2.3 death of larvae was recorded within 12 hrs exposure to *N. tabacum* extract. Within the same period, it was only *D.*

metel extract among others that caused larval death to a mean of 9.00±3.0. This value was significantly lower ($p < 0.05$) than of *N. tabacum*. The remaining extracts had caused similar but non-significant ($p > 0.05$) lethal effect on the larvae 12 hrs post-administration of the extracts. Death of *An. gambiae* larvae increased simultaneously with increased exposure time from 48 to 72 hrs but *N. tabacum* extract caused complete larval death (100% mortality) within 48 hrs. Although lethal effect caused by *N. tabacum* was significantly higher, that of *D. metel* was also significant compared to the extracts of *Z. officinale*, *E. africanum* and *C. procera* most of which did not differ significantly ($p > 0.05$) from control observation. Since activity guided bioassay was employed in this study, the most active extract was further tested.

Table 1: Lethal activity of 20% crude plant extracts against 4th instar of *An. gambiae* larvae

Plants extracts	**Parts used**	**Mean of dead larvae ± SD Period of exposure (hrs.)**			
		12	**24**	**48**	**72**
Z. officinale	Rhizome	1.00±1.0^{ab}	1.33±1.5^{ab}	2.67±1.2^{ag}	3.67±0.6^{g}
E. africanum	Leaves	1.00±1.0^{ab}	1.33±1.2.^{ab}	2.33±0.6^{bg}	2.67±0.6^{ag}
C. procera	Laves	1.00±1.0^{ab}	1.33±0.5^{ab}	2.00±1.0^{abg}	2.33±0.5^{abg}
N. tabacum	Leaves	21.67±2.3^{c}	24.67±0.6^{e}	25.00±0.0^{e}	25.00±0.0^{e}
D. metel	Seeds	9.00±3.0^{d}	12.67±1.2^{f}	13.67±1.6^{f}	19.67±1.5^{h}
Control	Untreated	0.33±0.6^{b}	0.33±0.6^{b}	1.33±0.6^{ab}	1.33±0.6^{ab}

Means followed by the same letter(s) within all columns are not significantly different ($p > 0.05$) with DNMR Test.

3.2 Qualitative presence of phytochemicals

Bioactive compounds present in the aqueous extracts resulting from phyto-chemical screening, is shown in Table 2. There was high presence of flavonoids and tannins in *E. africanum*, moderate to trace amount in *C. procera* but saponins and alkaloids were detected in all the extracts. The only extract found with high presence of cardiac glycosides was *E. africanum* whereas *N. tabacum* and *D. metel* had moderate amount. High amount of saponins glycosides was found in *N. tabacum* while moderate amount was present in *E. africanum.* Volatile oils were also detected in all the extracts except in *C. Procera*, while glycosides and steroids were detected in varied amounts among the extracts.

Table 2: Qualitative phytochemical contents of the plants aqueous extracts

Phytochemical contents	**Plant extracts**				
	Z. officinale	***E. africanum***	***C. procera***	***N. tabacum***	***D. metel***
Flavonoids	+	+++	++	ND	+
Tannins	ND	+++	+	ND	ND
Saponins	+	+	++	+++	+++
Alkaloids	+++	+++	+	+++	+++
Cardiac Glycosides	+	+++	+	++	++
Saponin Glycosides	ND	++	ND	+++	ND
Volatile oils	++	+	ND	+	+++
Glycosides	+	+++	+	++	++
Steroids	++	++	++	+++	+++
Anthraquinones	ND	ND	ND	ND	ND

Key: ND = Not Detected, + = present in trace amount, ++ = moderately present, +++= highly present

3.3 Lethal activity of the extract fractions

Among the organic solvent extracts of *N. tabacum,* it was the methanol extract that caused the highest lethal activity against the test larvae. The extract was therefore subjected to column and thin-layer separation, yielding ten fractions each was tested against the larvae and the results shown in Figs. 1 and 2.

All the fractions caused very low lethal effect except methanol fraction III (Meth III) observed for larval mean death ranging from 6.33±1.5 to 20.00±2.6 (80% mortality) after 72hrs of larval exposure. However, fraction II (Meth II) was the next in lethal activity against the larvae observed for 9.33±0.6 mean death of larvae (37.3% mortality) after 72hrs. Mortality of the larvae caused by exposure to fractions Meth VI and IX was similar to that observed for control as shown in Fig.2.

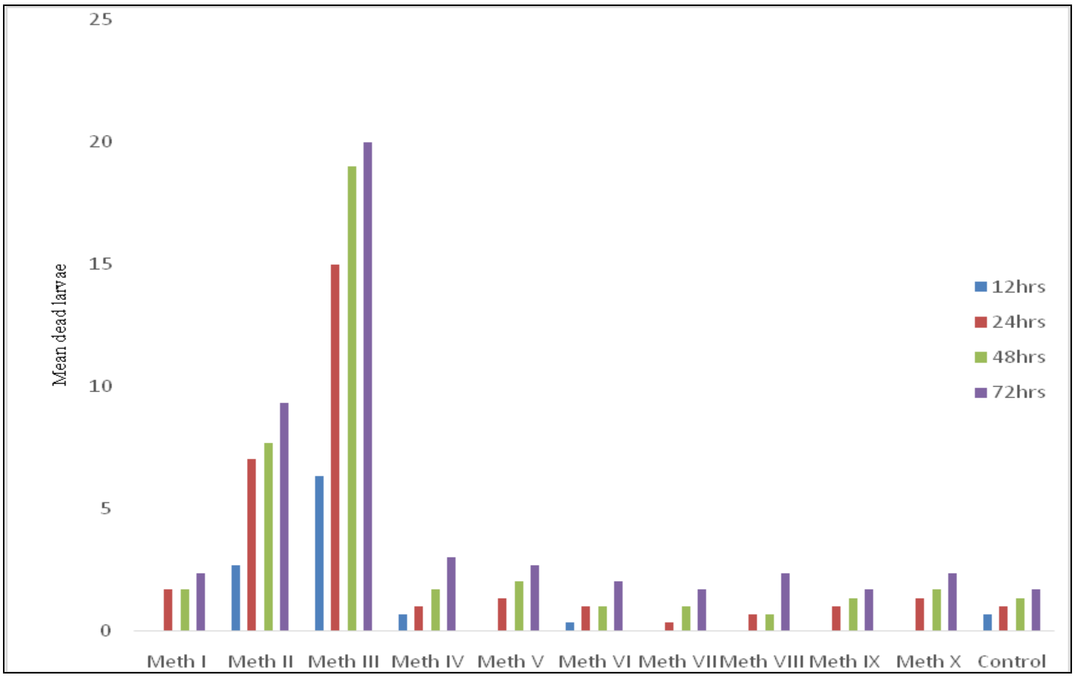

Fig 1: Lethal activity of *N. tabacum* column fractions against *An. gambiae* larvae over 12-72 hrs.

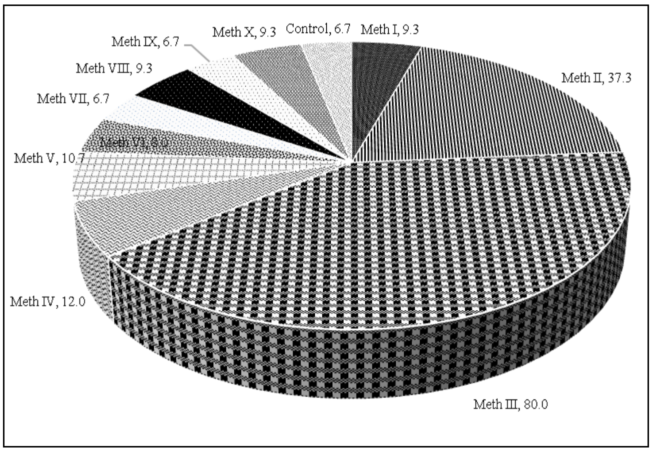

Fig 2: Percent mortality of *An. gambiae* larvae exposed to *N. tabacum* extract fractions

3.4 Result of GC/MS

The GC/MS analyses leading to the identification of compounds in Meth III fraction of *N. tabacum* extract is shown in Table 3. It was found to contain thirteen (13) compounds, consisted mainly of alicyclic alkane; Bicyclo {2, 2, 1} heptane-3-methylene-2, 2-dimethyl-5-o1 acetate (62.40%) and fatty acids (18.39%). Among the fatty acids, Octadecanoic acid (8.27%) was the major component. The fraction was also characterized by the presence of sesquiterpenes (7.50%), with Cis-z-alpha-Bisabolene epoxide in appreciable quantity (4.35%). Moreover, the sterol; gamma-Sitosterol (5.53%) and substituted aromatic (4.44%) with Butlated Hydroxytoluene (3.87%) as the major component, were also appreciable in amount. The remaining compounds were in the range of 0.4-0.6% in quantity. Retention indices, molecular weight and chemical formulae for the identified compounds are also shown.

Table 3: Composition of *N. tabacum* column fraction (Meth III) analyzed by direct GC/MS

Peaks	Compounds	Retention time	Area (%)	Molecular weight	Chemical formula
1	Butylated Hydroxytoluene	23.631	3.87	220	$C_{15}H_{24}O$
2	Diethyl Phthalate	24.429	0.57	222	$C_{12}H_{14}O_4$
3	n- Hexadecanoic acid	26.353	0.37	256	$C_{16}H_{32}O_2$
4	n-Hexadecanoic acid	27.902	5.95	256	$C_{16}H_{32}O_2$
5	Hexadecanoic acid, ethyl ester	28.129	0.62	284	$C_{18}H_{36}O_2$
6	9-Octadecenoic acid, (E)	29.027	3.80	282	$C_{18}H_{34}O_2$
7	Octadecanoic acid	29.219	8.27	284	$C_{18}H_{36}O_2$
8	gamma-Sitosterol	30.022	5.53	414	$C_{29}H_{50}O$
9	9-Octadecenoic acid (Z)-, 2,3,- dihydroxypropyl ester	30.691	0.62	356	$C_{21}H_{40}O_4$
10	Cis-9-Hexadecenal	30.950	0.49	238	$C_{16}H_{30}O$
11	Cis –Z-alpha-Bisabolene epoxide	31.363	4.35	220	$C_{15}H_{24}O$
12	Bicyclo (2,2,1) heptane -3-methylene-2,2-dimethyl-5-ol acetate	33.174	62.40	194	$C_{12}H_{18}O_2$
13	trans-Z-alpha-Bisabolene epoxide	34.748	3.15	220	$C_{15}H_{24}O$

Meth III- methanol fraction three

4. Discussion

Among the aqueous extracts of plants tested in this study for lethal activity against the 4th instar of *An. gambiae* larvae, it was the *N. tabacum* that exhibited highly significant activity. The extracts of *D. metel, Z. officinale, E. africanum* and *C. procera* were moderate to low in lethal activity against the larvae. Lethality was however, seemed to be enhanced by period of larval exposure to the extracts from 12-72 hrs. These observations conform to those reported by several authors who searched for the efficacy of crude plants extracts in the control of larval mosquitoes and other vector insects. Hot water leaf extract of *N. tabacum* at 1000ppm caused 100% mortality on the larvae of *Culex quinquefasciatus* within 12 hours of exposure [23]. The aqueous extract of *D. metel* seeds at 5ppm resulted in more than 50% mortality of the same species of larvae after 24 hours exposure [24]. Cent percent mortalities were reported [25] on *Cx. pipiens* larvae when exposed to 2% concentration of kernel bark and root extracts of *Balanites aegyptiaca* within three days. Application of 0.3% of *Solanum nigrum* crude leaf extract resulted in 100% larval mortality in *An. culicifacies*, *Cx. quinquefasciatus* and *Aedes aegypti* after 24 hours of larval exposure [26]. Mosquitoes are not the only animals affected by plant extracts, some workers found that, undiluted aqueous extract and 50% diluted aqueous extract of *N. tabacum* had immediate effect on mortality of the engorged adult *Rhipicephalus haemaphysoloids* and its fecundity production of female [27]. Conversely, another observation showed that, the seed extract of *D. alba,* a generic partner of *D. metel* was more effective than leaf extract of *N. tabacum* for piscicidal activity on trash fishes [28]. But an insecticide does not have to cause high mortality on target organisms in order to be acceptable.

Screening of the extracts shows the presence of alkaloids, tannins, saponins, flavoniods, glycosides and steroids. These compounds could be responsible or likely reasons for lethal activity. However, the reason for variations in activity of the extracts against the larvae was not certain. Higher lethal activity of *N. tabacum* may implicate the phytochemical compounds in the extract as more toxic than of the remaining extracts. Both *N. tabacum* and *D. metel* belong to the same plant family known as Solanaceae, consisting of many familiar members with promising lethal activity on mosquito. The two plants were also ranked in the first category of plants with high degree of toxicity by the US Army division of Entomology. Phytochemicals act as general toxicants against adults and larval mosquitoes; others interfere with growth and development or produce olfactory stimuli acting as repellent or attractant [29]. More specific studies demonstrated that the chemicals interfere with the proper functioning of mitochondria, specifically at the proton transferring sites [30]. They were also found to affect the midgut epithelium, gastric caeca and malpighian tubules in mosquito larvae [31]

Comparing lethal activity of the ten fractions of *N. tabacum* extract acquired through column chromatography, revealed that, fraction three (Meth III) was most active against the exposed larvae. A study has found that, only one fraction was active among five fractions of *Agave sisalana* against the larvae of *Cx. quinquefasciatus* [32]. Similarly, one fraction *of Melia volkensi* fruit kernel extract had growth inhibition activity at low concentration, whereas two other fractions had acute toxic effects on mosquito larvae [33]. Out of the four fractions of methanol extract of neem at 1mg/mL, only one fraction caused significant larvicidal activity within 24 hours against 4th instar larvae of *Cx. quinquefasciatus* [34]. Variability in lethal activity between the fractions of *N. tabacum* against the larvae, may advocate differences in their chemical composition or in the concentration of active compounds.

The GC/MS chemical profiling of *N. tabacum* Meth III fraction, indicated thirteen compounds rationed in the following chemical groups; alicyclic alkane, fatty acids, sesquiterpens, sterol and substituted aromatics. On account of compounds concentration, bicyclo (2, 2, 1) heptanes-3-methylene-2,2-dimethyl-5-o1 acetate (62.40%) in the first category, could be responsible for lethal activity. Earlier authors had attributed larvicidal activity on compounds based on concentration in active plants extracts. These include, 5-E-Ocimenone from the floral extracts of *Tagates minuta* [35], methyl 4-hydroxyl-3-(3-methyl 2 butenyl) benzoate in the *Piper* species [36], diterpenoid furans 6 alpha-hydroxyvouacapan-7 beta from seeds extract of *Pterodon spolygalaflorus* [37] and 2,3-dihydro-3,5-dihydroxy-6methyl-4 H—Pyran-4-one from *Lantana camara* leaf extract [21]. Similar compounds identified in Meth III fraction of *N. tabacum;* sesquiterpens and sterol, were implicated for larvicidal, acaricidal and herbicidal activity in some plant extracts [38]. This could suggest that multiple compounds may be at interplay for impacting lethal activity against *An. gambiae* larvae. There was the possibility of some compounds in the fraction to have acted as natural synergist for enhancing lethal activity. Synergism might inhibit the ability of mosquito larvae to employ detoxifying enzymes against natural chemicals. Natural synergism also discourages the development of resistance in the vectors [39].

Economic importance of tobacco as narcotic probably limits its usage in the area of pest control. Tobacco is grown in over 125 countries, on over 4 million hectares of land, a third of which is in China a lone [40]. Leaves production was nearly 7 million metric tons in the year 2000 [41] with more than 6000 transnational companies involved in the production and marketing for consumption [42]. Global tobacco crop is worth approximately US $ 20 billion, a small fraction of the total amount generated from the sale of manufactured tobacco products. The figure of 1-2 billion smokers worldwide is predicted to rise to 1.9 billion, consuming more than 9 trillion cigarettes by year 2025 [42]. In many countries, workers spend a significant portion of their household income on tobacco: an estimated 10% in Egypt and 11% in China [41]. A pack of international brand of tobacco will buy 1.5kg of cucumber in Georgia, a dozen eggs in Panama, four pairs of cotton socks in China, a dozen coconuts in Papua New Guinea, 1 kg of fish in France, Ghana or Moldova and 6 kg of rice in Bangladesh [43]. But, tobacco kills more than AIDS, legal drugs, illegal drugs, road accidents, murder and suicide combined [44].

Lethal activity of *N. tabacum* extracts and fraction observed in this study, may support the claim that many members of the Solanaceae plant family to which the plant belongs, is poisonous to insects. The increase in mosquito vectors and incidence of mosquito-borne diseases is rising due to climate change and water contamination [45]. The solution to the problem of mosquito-borne diseases lies ultimately with effective anti-mosquito measures be course mosquitoes are regarded as the weak link in the chain of disease transmission. Such measures include elimination of breeding sites, killing of mosquito larvae, destruction of adult mosquitoes and prevention of man-vector contact. In this effort, chemical method is particularly effective in well-defined and limited breeding sites. Many synthetic chemicals used in the control of mosquitoes are reported to cause ecological imbalance, manifested by pollution of the environment which may lead to depletion in ozone layer that shields earth from ultraviolet rays of the sun. Bio-pesticides of plant origins are considered safer in usage, without phytotoxic properties and leave no scum in the environment. Thus, herbal sources could give a lead for discovering new insecticides. Therefore, re-emphasized the need to explore the possibility of using herbal based larvicides as supplementary or complimentary measures for mosquito control.

5. Conclusion

Among the extracts tested in this study for lethal activity against *An. gambiae* larvae, it was those of *N. tabacum* that were most active. Lethal activity was observed to be enhanced by exposure of larvae to the extracts from 12-.72 hrs period. Phytochemical compounds like alkaloids and saponins determined in the extracts tested were reported in previous works for having mosquito larvicidal activity. The compound, Bicyclo (2,2,1) heptanes- 3 methylene-2,2 –dimethyl-5-o1 acetate in active Meth III fraction of *N. tabacum* had the highest percentage, thus, may be useful candidate for further investigation. Meth III fraction of *N. tabacum* could be used to control *An. gambiae* larvae, offering an alternative to rather expensive and environmentally hazardous synthetic insecticides.

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