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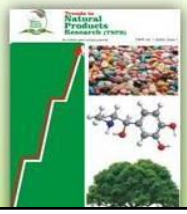

# Phytochemical and Larvicidal Evaluation of *Vernonia Glaberrima* Welw. Ex O. Hoffm. (Asteraceae) Leaves on *Aedes* Mosquito Larvae

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**Abstract**
Malaria, a parasitic infection transmitted by the anopheles mosquito, poses a significant global health threat especially in the tropical regions. Larvicidal agents can be used to kill the larva of the mosquito before they grow into adult. This study investigated the larvicidal activity of *Vernonia glaberrima* on *Aedes* mosquito larva. The leaves of *Vernonia glaberrima* were collected, macroscopically examined, dried and pulverized. Extraction of the pulverized leaf was done by cold maceration using methanol. Fractionation of the methanol extract was carried out via Column chromatography using n-hexane, ethyl acetate and aqueous methanol. Larvicidal assay was done using WHO method. Vacuum liquid chromatography was performed on aqueous methanol fractions with dichloromethane: methanol solvent system. Thin-layer chromatography was performed to pool the aqueous methanol sub-fractions to four sub-fractions. The larvicidal activity study was repeated on the aqueous methanol sub-fractions. The fractions were evaluated using GC-MS analysis to identify the constituents. The macroscopic evaluation reveals green leaves with characteristic smell, smooth texture and bitter taste. The secondary metabolites present in the leaves of *Vernonia glaberrima* were flavonoids, steroids, saponins, alkaloids, glycosides, terpenoids, phenols, hydrogen cyanide and tannins. The quantitative phytochemical screening showed relatively high concentrations of tannins (13.3 mg/g), phenol (12.7 mg /g) and flavonoids (9.9 mg/g), moderate amounts of alkaloids (6.4 mg/g), glycosides (5.1 mg/g) and terpenoids (5.6 mg/g), while saponins (0.6 mg/g), steroids (0.4 mg/g) and hydrogen cyanide (0.4 mg/g) were relatively low. All the fractions were effective against larvae of *Aedes aegypti*. Aqueous methanol fraction was the most efficacious with $LC_{50}$ of 2.807 whereas methanol fraction was the least efficacious with $LC_{50}$ of 25.876. Sub-fraction 3 of aqueous methanol fraction showed 75 % larvicidal activity. Many compounds including 2, 4-Di-tert-butylphenol, Gamma-terpinene and 14-Octadecenoic acid were identified by the GS-MC analysis. This study shows that the leaves of *Vernonia glaberrima* have potent larvicidal activity which can be harnessed for the control of malaria.

**Keywords**
*Vernonia glaberrima,* larvacidal activity, *Aedes aegypti*, Phytochemical

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## Introduction

Malaria is a parasitic infection transmitted through the bite of infected female anopheles mosquitoes that leads to acute life threatening diseases and poses a significant global health threat (Talapko *et al*., 2019). According to WHO, approximately 250 million malaria cases and 608 thousand malaria deaths were reported in 2022, 95% of these malaria-related deaths occur in Africa. The highest number of death was recorded in Nigeria, followed by Congo and Uganda and then, Mozambique. In addition, children under five years are mostly affected by the disease as about 80% of the deaths occur in children (WHO, 2023). Symptoms of malaria includes fever, body weakness, chills, headaches, abdominal pains, nausea and vomiting. The five major plasmodium species which are implicated in malaria are *P.vivax, P.falciparum, P.ovale, P.malaria, P.knowlesi* (Ashley and Phyo, 2018). In the prevention and treatment of malaria, the *Plasmodium*, the human host, the mosquito vector and the given environment are targeted (Castro, 2017). Vector control is an important strategy in the control of malaria as it is an excellent preventive measure against the infection and also reduces the disease transmission. The female anopheles' mosquito is the major vector of malaria. Transmission of malaria parasite can be controlled in different stages of the mosquito's lifecycle. Larvicidal agents can be used to kill the immature larva of the mosquito before they grow into adult mosquitoes (Choi and Wilson, 2017). Commonly used larvicidal agents are synthetic insecticides including difubenzuron and methoprene *(Milugo et al.,* 2021). However, there is an increasing development of resistance to common synthetic insecticides and this poses a big threat to malaria control programs (Mekuriaw *et al.*, 2019). Plants like *Allium sativum, Zingiber officinale and Phyllantus embilica* have been reported to have larvicidal activity against mosquito larva (Vasincu *et al.*, 2022).

*Vernonia glaberrima* is a much-branched small shrub that is usually up to 1 m tall. It is usually leafless or with new leaves just appearing at flowering time. The leaves are elliptic to lanceolate with more or less dentate-serrulate margins. The capitula are numerous in panicles or pseudumbels on short lateral branches, white, and sweetly scented. This plant is found in various types of woodland and is distributed in tropical regions of the world, especially in Africa and South America. *Vernonia glaberrima* has been reported to contain lupeol and 5-methylcoumarin-4-β-glucoside with analgesic, anti-inflammatory, antidiabetic, antiparasitic and anticancer activities (Abubakar et al., 2022; Alhassan et al., 2018; Yusuf et al., 2020). This study aimed to investigate the mosquito larvicidal activity of *Vernonia glaberrima* leaf.

## Materials and Methods

### *Plant Collection*

The leaves of *Vernonia glaberrima* were collected from Agbani, Enugu State located in the southeastern part of Nigeria in July 2023 and identified by Mr. Felix Nwafor, a taxonomist in University of Nigeria, Nsukka, Enugu State, Nigeria.

### *Macroscopic Evaluation*

The morphological characteristics of the fresh leaves of *Vernonia glaberrima* including the size, shape, type of venation, margin, base, and apex of the leaf, as well as the organoleptic characteristics, such as colour, smell, and taste of the leaf, were assessed.

### *Plant Extract Preparation*

The extraction of *V. glaberrima* leaves was carried out by cold maceration method as described by Mukherjee *et al*. (2019). Briefly, 300 g of the powdered leaves of *V. glaberrima* was immersed in 1.5 litre of methanol. The resulting mixture was filtered first using a muslin cloth. The filtrate was subsequently filtered using Whatman size 1 filter. The residue was washed with 600 ml of methanol and the marc was discarded. The filtrate was transferred into a beaker and then concentrated in a rotary evaporator and finally evaporated to dryness over a water bath to obtain the dried extract.

### *Preliminary Qualitative Phytochemical Tests*

Preliminary qualitative phytochemical analysis of the crude extract were carried using standard methods (Harbone, 1998; Evans, 2002) .

### *Quantitative Phytochemical Analysis*

Quantitative phytochemical analysis of the crude extract was carried out using standard procedures (Sofowora, 1982; Harbone, 1998; Evans, 2002).

### *Fractionation of Crude Extract*

The fractionation process was carried out using Column Chromatography Dry-Packing method (Odoh *et al.*, 2022). The dried extract was dissolved in methanol and a given quantity of silica gel was added to the extract solution and mixed thoroughly after which, the mixture was spread out on a dry surface to allow the methanol to evaporate leaving a fine smooth dried analyte-stationary phase mixture. The bottom end of a clean glass column was covered

with cotton wool and packed with the analyte-stationary phase. The glass column was then mounted on a retort stand. Cotton wool was placed on the top of the column to prevent disturbance of the stationary phase by the mobile phase. The mobile phase (n-hexane) was gently poured into the column and the elution process began. The eluents were used in the order of increasing polarity starting with n-hexane, ethyl acetate, n-butanol and aqueous methanol. The eluate of each solvent was collected, poured into beakers, labelled and allowed to evaporate in a water bath after which the dried extract was weighed.

*Mosquito Larvicidal Assays*

Standard WHO larvicidal protocol with slight modification was employed for the larvicidal assay (WHO, 2013). The larvae of aedes mosquito were bred under standard conditions at the National Arbovirus and Vectors Research Centre Enugu State. They were housed in a plastic bowl containing stagnant water covered with net, at room temperature. The larvae were acclimatized to normal laboratory conditions prior to study. Twenty (20) healthy and wriggling early fourth instars mosquito larvae were introduced into plastic bowls containing 4ml of distilled water. Stock solutions (100%, 50%, 10%) of the plant fractions were made using Tween 80 as emulsifier to facilitate the dissolving of materials in water for n-hexane and ethyl acetate fractions which were insoluble in water. Different concentrations of the fractions were introduced into the bowls containing the mosquito larva. The experiments were carried out at 25 ± 2 °C. A set of controls using water only (negative control) and permethrin (positive control) were included for comparison. 1 ml of Tween 80 in 99 ml of tap water was set up as compounders' reference for each replicate.

The number of dead and living larvae in each bowl after 24 h and 48 h of exposure was recorded. A larva was considered dead (moribund mosquito larvae) if it became immobile, did not show the characteristic diving reaction to disturbance, was in an unnatural position or was not capable of rising to the surface after probing with a syringe needle. The mortality was determined using Abbott's13 formula as follows.

$$\%\ Mortality = \frac{Number\ of\ dead\ larva}{(Number\ of\ larva\ alive) + (Number\ of\ dead\ larva)} \times 100$$

*Sub-fractionation*

The fraction which gave the best activity was further sub fractioned by gradient elution using Vacuum Liquid Chromatography in the proportion 100% methanol, 70% methanol: 30% dichloromethane, 50% methanol: 50% dichloromethane, 30% methanol: 70% dichloromethane, 100% dichloromethane. The sub fractions were dried over a water bath and tested for larvicidal activity as follows;

The study was carried using four fractions of the aqueous methanol subfractions. Tween 80 (2 ml) and 1 ml of distilled water was used to dissolve each of the n-hexane subfractions. Each fraction (0.1 g) was transferred to four different beakers and was made up to 50 ml with distilled water. The negative control was prepared by mixing 2 ml of Tween 80 and 1 ml of distilled water in a beaker and was made up to 50 ml with distilled water. For positive control, 3 ml of n-hexane solvent was poured in a beaker and was made up 50 ml with distilled water. Twenty (20) Aedes mosquito larva were inoculated in each of the six beakers. Identification of lifeless larvae was made every 30 minutes interval for 24 h.

*Gas Chromatography-Mass Spectrometry*

The plant extract (1 g) was mixed with 25 ml of ethanol in a test tube. The test tube was heated in a hotplate at 60 $^0$C for 90 min. After which the mixture was transferred to a separating funnel and washed successively with 20ml of ethanol, 10ml of cold water, 10 ml of hot water and 3ml of hexane. The extracts were mixed together and washed thrice with 10ml of 10%v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1 ml of pyridine. 200 µl was measured and added into a vial for analysis.

*Quantification by GC-MS*

The evaluation of the plant extract for bioactive compounds was performed using a BUCK M910 Gas chromatography equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). The carrier gas was Pure helium gas (99.995%) and flows at a rate of 1 mL/min. The initial temperature was set at 50 – 150 °C with increasing rate of 3 °C/min and holding time of about 10 min. The temperature was later increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in a splitless mode. Relative quantity of the chemical compounds present in the extract were expressed as percentage based on peak area produced in the chromatogram.

*Identification of chemical constituents*

Bioactive compounds present in the extract were identified based on GC retention time on HP-5MS

column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems).

*Statistical Analysis*

The percentage of mortality data was subjected to ANOVA procedure using Statistical Package for Social Sciences (SPSS 17.0). The student-Newman-Keuls (SNK) test at p = 0.05 was used for mean separation.

**Results**

*Result of the Macroscopic Evaluation*

The macroscopic evaluation reveals green leaves with characteristic smell, smooth texture and bitter taste (Table 1).

**Table 1**. Macroscopic characteristics of *Vernonia glaberrima* leaves

| Parameter | Result |
|---|---|
| Colour | Green |
| Odour | Characteristic |
| Texture | Smooth |
| Taste | Bitter |

*Phytochemical Analysis*

The secondary metabolites present in the extract were flavonoids, steroids, saponins, alkaloids, glycosides, terpenoids, phenols, hydrogen cyanide and tannins (Table 2). The quantitative phytochemical evaluation revealed relatively high concentrations of tannins (13.3 mg/g), phenol (12.7 mg /g) and flavonoids (9.9 mg/g). Moderate amount of alkaloids (6.4 mg/g), glycosides (5.1 mg/g) and terpenoids (5.6 mg/g) while saponins (0.6mg/g), steroids (0.4 mg/g) and hydrogen cyanide (0.4 mg/g) were relatively low (Table 3)

**Table 2.** Qualitative phytochemical constituents

| Constituents | Result |
|---|---|
| Saponins | + |
| Tannins | + |
| Steroids | + |
| Glycosides | + |
| Terpenoids | + |
| Alkaloids | + |
| Hydrogen cyanide | + |
| Phenols | + |
| Flavonoids | + |

**Table 3.** Quantitative phytochemical evaluation

| Constituents | Concentration (Mg/G) |
|---|---|
| Saponins | 0.597 ± 0.03 |
| Tannins | 13.327 ± 0.34 |
| Steroid | 0.423 ± 0.01 |
| Glycosides | 5.105 ± 0.17 |
| Terpenoids | 5.557 ± 0.01 |
| Alkaloids | 6.381 ± 0.15 |
| Hydrogen cyanide | 0.353 ± 0.01 |
| Phenol | 12.742 ± 0.29 |
| Flavonoid | 9.862 ± 0.03 |

*Larvicidal activity of the fractions*

All the fractions were effective against larvae of *Aedes aegypti* as presented. A probit analysis showed that aqueous methanol fraction was the most efficacious ($LC_{50}$ = 2.807; $LC_{90}$ = 20.629); followed by n-hexane ($LC_{50}$ = 4.768; $LC_{90}$ = 29.299) whereas methanol fraction was the least efficacious ($LC_{50}$ = 25.876; $LC_{90}$ = 56.922). A chi-square analysis showed that the differences between mortalities in different concentrations were not significant (P > 0.05) in all the extracts (Table 4). The larvicidal activity of the aqueous methanol sub-fractions 1,2 and 4 was 10%, 5%, and 30% respectively, while sub-fraction 3 had 75% larvicidal activity (Table 5).

**Table 4.** The larvicidal activities of the fraction

| Extract/Fractions | Conc (%) | Mortality (Mean ± SD) | $LC_{50}$ (LCL – UCL) | $LC_{90}$ (LCL – UCL) | Slope ± SE | $\chi^2$ |
|---|---|---|---|---|---|---|
| Methanol extract | 10 | 0.33 ± 0.58 | 25.876 | 56.922 | 3.743 ± 0.691 | 0.974 |
| | 50 | 14.85 ± 4.45 | (17.668 -35.362) | (41.236 – 92.969) | | |
| | 100 | 10.33 ± 9.72 | | | | |
| Aqueous Methanol | 10 | 8.67 ± 8.08 | 2.807 | 20.629 | 1.479 ± 0.690 | 0.431 |
| | 50 | 11.77 ± 10.28 | (0.000 – 8.221) | (4.899 – 313.074) | | |
| | 100 | 12.90 ± 11.19 | | | | |
| Ethyl acetate fraction | 10 | 4.90 ± 7.89 | 4.768 | 29.299 | 1.625 ± 0.606 | 0.002 |
| | 50 | 8.33 ± 9.71 | (0.088 – 10.511) | (14.800 – 52.843) | | |
| | 100 | 9.23 ± 9.91 | | | | |
| N-hexane fraction | 10 | 3.80 ± 3.85 | 11.137 | 17.859 | 6.250 ± 15.967 | 0.000 |
| | 50 | 13.35 ± 11.55 | (0.00 -0.000) | (0.00 -0.000) | | |

| | | |
|---|---|---|
| | 100 | 13.35 ±11.55 |

**Table 5.** Larvicidal activity of aqueous methanol sub-fractions

| No of dead larvae | Time (min) | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 370 | 400 | 420 | Mortality (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Subfraction 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 10 |
| | Subfraction 2 | - | - | - | - | - | - | - | - | 1 | 1 | 1 | 1 | 1 | 1 | 5 |
| | Subfraction 3 | 6 | 7 | 7 | 8 | 11 | 11 | 11 | 11 | 13 | 13 | 14 | 14 | 15 | 15 | 75 |
| | Subfraction 4 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 6 | 6 | 6 | 30 |
| | Negative Control | - | - | - | - | - | - | - | - | - | - | 1 | 1 | 1 | 1 | 5 |
| | Positive Control | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 100 |

*GS-MC Analysis of Aqueous Methanol Sub-fraction*

Gas chromatography-mass spectrometry was performed to identify compounds present in the extract which might be responsible for the larvicidal activity (Figure 1). Multiple compounds were in abundances (Table 6). 2, 4-Di-tert-butylphenol, Gamma-terpinene and 14-Octadecenoic acid had the highest peak area which shows they are mostly responsible for the activity of the plant leaves. Other compounds present in the extract were dodecane, tetracane, docosene and carene (Table 7).

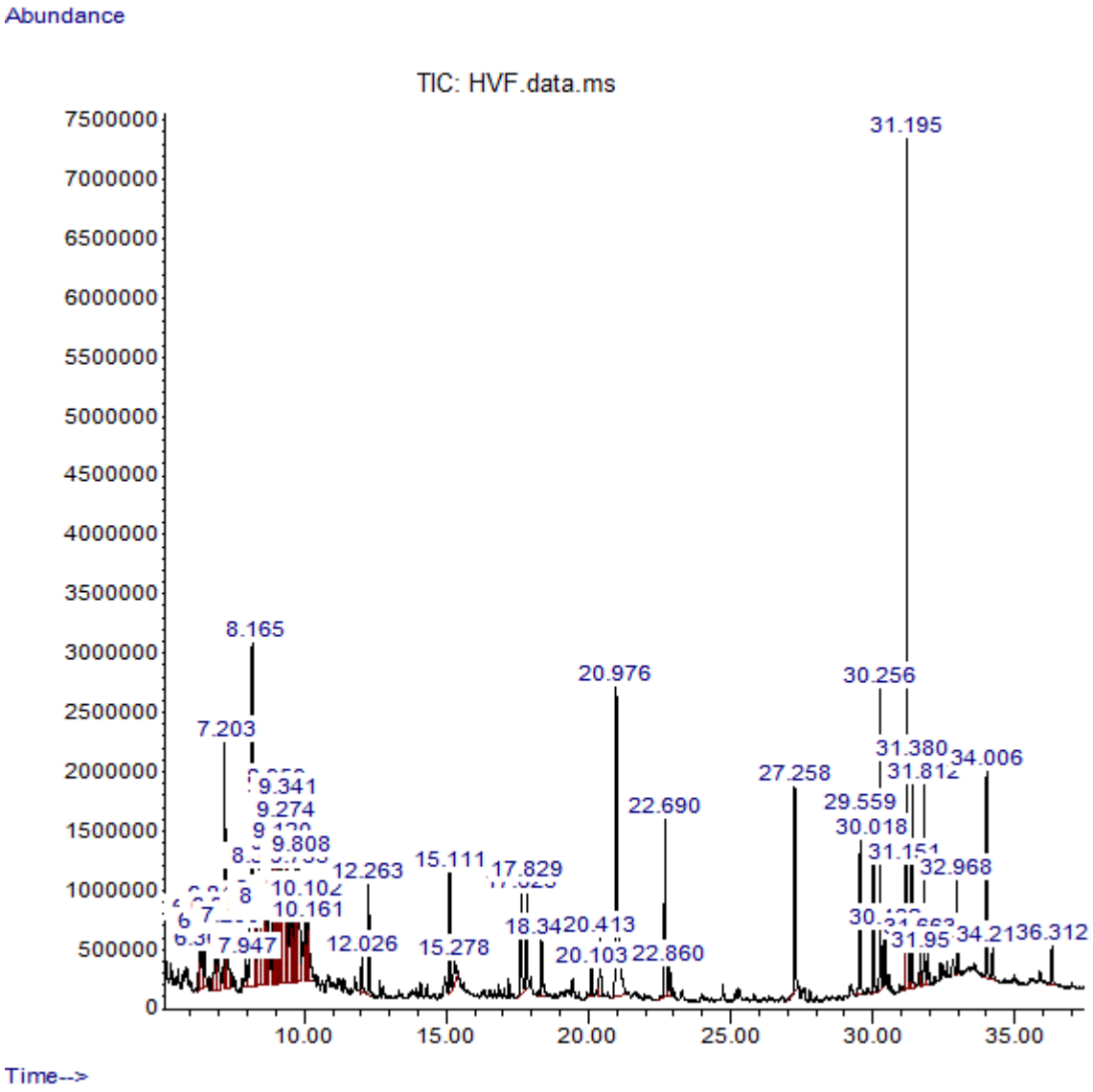


**Fig 1.** GC MS Chromatogram of *Vernonia glaberrima*

**Table 6**. GC-MS analysis of aqueous methanol sub-fraction of *V.glaberrima*

| Peak Number | Retention Time | Peak Area (%) | Compound | Quality |
|---|---|---|---|---|
| 4 | 6.846 | 1.22 | 1,2 dichlorobenzenes | 95 |
| 5 | 6.957 | 1.25 | (+)-2-Carene | 92 |
| 6 | 7.203 | 3.24 | O-cymene | 96 |
| 9 | 8.165 | 6.34 | Gamma-terpinene | 97 |
| 33 | 12.263 | 1.48 | Dodecane | 95 |
| 36 | 17.625 | 1.43 | 7-tetradecane | 90 |
| 37 | 17.829 | 1.32 | Tetradecane | 93 |
| 41 | 20.976 | 7.66 | 2,4-Di-tert-butylphenol | 96 |
| 42 | 22.690 | 2.73 | Oxalic acid | 90 |
| 44 | 27.258 | 2.78 | 1-octadecene | 94 |
| 45 | 29.559 | 1.80 | Hexadecanoic acid | 95 |
| 46 | 30.018 | 1.61 | Dibutyl phthalate | 97 |
| 47 | 30.256 | 2.67 | Cycloeicosane | 93 |
| 50 | 31.195 | 6.81 | 14-Octadecenoic acid | 98 |
| 51 | 31.380 | 1.66 | Methyl stearate | 97 |
| 53 | 31.812 | 1.65 | 1-Docosene | 98 |
| 56 | 34 | 1.88 | Bis(2-ethylhexyl) phthalate | 91 |

**Table 7.** Activities of compounds detected in aqueous methanol sub-fraction of *V.glaberrima*

| Compounds | Class | Activities |
|---|---|---|
| Carene | Monoterpene | Antimicrobial, antioxidant, semiochemical and funigent properties, larvicidal activity |
| Cymene | Monoterpene | Antimicrobial, antioxidant, anti-inflammatory, antidiabetic, analgesic, antinociceptive, vasorelaxant and neuroprotective. |
| Gamma-terpinene | Monoterpene | Antioxidant, antinociceptive, Larvicidal |
| Dodecane, tetracene, hexadecane, docosene | Alkanes | Pheromonal activity, larvicidal, antibacterial and antifungal, nematicide, antioxidant |
| 2,4-Di-tert-butylphenol | Phenols | Antibacterial, antifungal, anticancer, antioxidant, larvicidal |
| Oxalic acid | Dicarboxylic acid | Antioxidant, antibacterial |
| Hexadecanoic acid | Fatty acid | Antioxidant, hypo cholesterol, nematocidal, pesticide, anti-inflammatory |

**Discussion**

This study investigated the larvicidal activity of *Vernonia glaberrima* leaves on fourth instar A*edes aegypti* larva. The knowledge of the macroscopic characteristics of a plant can be useful during collection to authenticate the plant. The leaves of *Vernonia glaberrima* leaves were green in colour with characteristic smell and bitter taste. Plant extraction is a key step in herbal medicine research. The bioactive compounds of *Vernonia glaberrima* leaves were extracted by cold maceration technique using methanol as the solvent, whereby the pulverized leaves were immersed in the organic solvent for 72 days at room temperature with intermittent shaking (Abubakar and Hague, 2020). It is a simple and widely used extraction technique for leaves. Moreso, many researchers have reported the efficiency of this technique in extracting the active constituents of *Vernonia glaberrima* leaves (Abdullahi *et al*., 2015, Odoh *et al*., 2022). Phytochemical analysis of the plant extract revealed the presence of many secondary metabolites including flavonoids, steroids, saponins, alkaloids, glycosides, terpenoids, phenols, hydrogen cyanide and tannins. These metabolites are responsible for the pharmacological activities of the plant. The leaf extract was found to contain high amounts of phenols and flavonoids. This result is in agreement with the findings of previous studies. Lupeol, a phenolic compound extracted by chromatographic procedures from the leaf of *V. glaberrima* were reported to have analgesic and anti-inflammatory effects validating the folkloric use of the plant in the management of pain (Yusuf *et al*., 2020).

The larvicidal assay showed that all the solvent fractions exhibited good larvicidal activity on fourth instar Aedes *aegypti* mosquito larvae. Aqueous methanol extract gave the highest activity with $LD_{50}$ of 2.807 mg/ml while n-hexane fraction gave the lowest activity with $LD_{50}$ of 11.137 mg/ml. N-hexane, methanol and chloroform fractions have been reported to have anti-cytotoxic, antibacterial, antidiabetic and anti-inflammatory activity (Yusuf *et al*., 2020; Gangas *et al*., 2021, Odoh *et al*., 2022). However, there is no report on the larvicidal property of this plant. This appears to be the first report on the larvicidal activity of aqueous methanol fraction of *Vernonia glaberrima.*

The results of Gas chromatography-mass spectrometry analysis identified some chemical compounds present in the leaf extract of *Vernonia glaberrima* (Table 6). The compounds in abundance as revealed by the peak areas were 2, 4-Di-tert-butylphenol, Gamma-terpinene and 14-Octadecenoic acid. The results are consistent with the previous studies that have reported the larvicidal activity of 2, 4-Di-tert-butylphenol from other plant sources. 2, 4-Di-tert-butylphenol, a phenolic compound isolated from the seeds of *Magnolia*

*denudate* has been demonstrated to have potent larvicidal activity on Aedes mosquito larvae.(Wang et al, 2016). *Vernonia glaberrima* has been reported to contain lupeol and 5-methylcoumarin-4-β-glucoside with analgesic, anti-inflammatory, antidiabetic, antiparasitic and anticancer activities (Abubakar *et al*., 2022; Alhassan *et al*., 2018; Yusuf *et al*., 2020). The presence of these compounds shows that *Vernonia glaberrima* has good biological activities including larvicidal activity. In conclusion, our study has shown that the leaves of *Vernonia glaberrima* have a potent larvicidal activity. The study identified 2, 4-Di-tert-butylphenol, Gamma-terpinene and 14-Octadecenoic acid as the phytochemical richly present in the plant sample and might be responsible for the larvicidal effect.

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