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Investigation of Larvicidal Activity of Leaf Extracts Of *Alstonia Boonei De Wild* against *Spodoptera Frugiperda* and Isolation of Insecticidal Compound

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Abstract

This study examined the larvicidal activity of different fractions of leaf extracts of the medicinal plant *Alstonia boonei De Wild* (Apocynaceae) against the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). It also encompassed investigation of the phytochemicals and the isolation of an insecticidal compound from the leaf extract. The crude extract was obtained through maceration and subsequently partitioned using the Kupchan scheme with three solvents: hexane, dichloromethane, and ethyl acetate. Each fraction was subjected to phytochemical screening and later evaluated for contact toxicity against fall armyworm larvae at varying concentrations, with the experiments conducted in duplicate. The phytochemical analysis revealed the presence of alkaloids, terpenoids, flavonoids, steroids, tannins, glycosides, and saponins. Bioassay-guided isolation from the fraction with the highest larvicidal activity was then performed. Results indicated that both the hexane and dichloromethane fractions significantly reduced larval survival in a dose-dependent manner. The median lethal concentrations (LC_{50}) for hexane, dichloromethane, and ethyl acetate fractions were 0.02, 0.14, and 0.3 mg/µL (20, 140, and 300 mg/mL), respectively, while the LC_{90} values were 0.1, 0.6, and 0.7 mg/µL (100, 600, and 700 mg/mL), respectively. In conclusion, the leaf extracts of *A. boonei* exhibit significant toxicity against *S. frugiperda* by reducing larval survival rates, and the isolated compound, citronellic acid, demonstrates potential as a natural crop protectant against fall armyworm infestations.

Key words: *Alstonia boonei de wild*, crude, extract, larvae, phytochemicals, *Spodoptera frugiperda*.

Introduction

Nature is a rich source of products with interesting and useful biological activity. In the field of crop protection, natural products and analogues have successfully been introduced to the market to control insect pests, plant diseases and weeds [1]. Plants belonging to the families *of Acanthaceae, Acoraceae, Amaranthaceae, Anacardiaceae, Annonaceae, Apiaceae, Apocynaceae* among others, have been reported as promising sources of botanical insecticides against maize storage and field insects [2] [3].

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Alstonia boonei de wild is a large evergreen tree belonging to the family *Apocynaceae*. It is distributed throughout the tropics and rain forests of West and Central Africa where the roots, leaves, stem bark, latex, flowers and fruits are used extensively for medicinal purposes [4]. *Alstonia boonei de wild* is among the many plants reported to have remarkable pharmacological properties. It is a common, large deciduous medicinal tree found in the lowlands and rain-forest areas of Nigeria as well as in various parts of Angola, Central African Republic, Ghana, Democratic Republic of Congo, Cote d'Ivoire [5]. Despite its popularity as an aromatic plant, the insecticidal potential of A. boonei has not been investigated as has been done for other medicinal plants like neem, *Azadirachta indica A. Juss* [6] [7] [8]. The objective of this study is to determine the insecticidal properties of the extracts of the leaf of *A. boonei* against the *Spodoptera frugiperda* (Lepidoptera: Noctuidae), a recent invasive pest species that has successfully established across sub-Saharan Africa where it continues to disrupt agriculture, particularly smallholder cereal production [9].

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a noctuid moth indigenous to the Western Hemisphere where it has long been a major agricultural problem for both continents 10]. It is primarily a pest of maize but has a wide host range and is capable of feeding on over 80 plant species, periodically causing significant economic damage to rice, sorghum, and millet, soybean, wheat, alfalfa, cotton, turf, and fodder crops. Fall armyworm has recently become established in Africa, the first major introgression into the Eastern Hemisphere. The initial detection was on the island nation of São Tomé and Príncipe in April 2016, followed by outbreaks in the western African countries of Benin, Nigeria, Ghana, and Togo reported in June 2016 [11], [12]. As of October 2017, fall armyworm is present throughout most of sub-Saharan Africa [13]. The infested region is predominated by smallholder farming with maize typically the most important staple food and a major component of livestock feed [14]. The damage from fall armyworm to African maize production could total $3 billion (USD) over the next year, with the potential for losses in other crops still to be determined [15]

Materials and Methods

Sample Collection and Identification Plant Material

Fresh leaves of *Alstonia boonei de wild* were collected from Ondo west, Ondo Local Government area of Ondo state, Nigeria. The sample was taxonomically identified and authenticated by a Botanist from herbarium section of Biology Department, Umaru Musa Yar'adua University Katsina, and given a voucher no. UMYUH 2387.

Extract preparation and fractionation

The leaves were thoroughly rinsed with clean water and dried under shade at room temperature for three weeks. The dried leaves were ground into a fine powder using an electric blender (Silver Crest ZQ-G0120) to ensure homogeneity, and the resulting powder was stored in a clean, airtight container until extraction. A total of 400 g of the powdered plant material was macerated at room temperature in 2 L of 98% methanol with occasional shaking for 72 hours. The suspension was filtered through a Buchner funnel fitted with Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure at 40 °C using a rotary evaporator to obtain the methanolic crude extract, which was subsequently dried, weighed, and stored in a clean, airtight container. The crude methanolic extract was subjected to solvent partitioning using the Kupchan scheme of fractionation with n-hexane, dichloromethane, and ethyl acetate. Each fraction obtained was dried, weighed, and stored appropriately prior to bioassay

Phytochemical Screening

Qualitative phytochemical screening was performed to detect the presence of major secondary metabolites, including alkaloids, flavonoids, terpenoids, steroids, glycosides, tannins, and saponins in the fractions. Standard phytochemical procedures were employed: the Salkowski test for steroids and terpenoids, the froth test for saponins, Mayer's reagent for alkaloids, ferric chloride test for phenols and tannins, and the Keller–Killiani test for glycosides**.**

Larvae Collection

Second Instar Fall Armyworm larvae were collected from insect rearing unit of Biology department, Umaru Musa Yar'adua University, Katsina state. A total of 100 larvae were collected. 45 larvae were tested per treatment and the experiment was carried out twice for result authentication. After the experiment, mean percentage mortality data for the two experiments conducted separately was recorded as the % mortality.

Bioassay

The bioassay was carried out in the insect rearing unit of the department of Biology, Umaru Musa Yar'adua University, Katsina. Serial dilutions of the extract fractions were prepared and administered topically to the larvae using a micropipette. The fractions were evaluated at concentrations of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 mg/µL. A 10 µL aliquot of each test solution was applied directly to the larval integument using a 20 µL pipette. Subsequently, the treated larvae were individually transferred into sterile plastic Petri dishes containing maize grains, maize leaves, and a moistened filter paper at the base, and the dishes were sealed with aluminum foil.

Control experiments were performed using both positive and negative controls. The positive control consisted of larvae treated with chlorpyrifos at the manufacturer's recommended field concentration (0.5% w/v), while the negative control consisted of larvae treated with a DMSO/water solution only. Mortality was assessed at 24, 48, and 72 hours post-treatment under constant temperature and humidity conditions. Larvae were classified as dead when they exhibited no observable response to gentle tactile stimulation, defined as brief pressure applied with a spatula.

Thin-Layer Chromatography (TLC)

The hexane fraction of *Alstonia boonei* De Wild was selected for isolation of bioactive constituents based on its superior activity compared to the dichloromethane and ethyl acetate fractions. Analytical thin-layer chromatography (TLC) was performed on aluminum-backed plates using graded mixtures of n-hexane and ethyl acetate (9.9:0.1, 9.8:0.2, 9.7:0.3, 9.6:0.4, 9.5:0.5, 9.4:0.6, 9.3:0.7, 9.2:0.8, 9.1:0.9, and 9.0:1) as mobile phases to identify the system with optimum separation efficiency. After every ten separations, TLC plates were examined under UV illumination and further visualized by staining with ferric chloride solution. The solvent system affording the best resolution (*n*-hexane: ethyl acetate, 9:1) was subsequently employed for column chromatography.

Column chromatography

The separation of the hexane fraction was performed using a glass column with an internal diameter of 8 cm and a length of 100 cm. Column-grade silica gel G (70 g, 60–120 mesh size) was wet-packed into the column employing *n*-hexane: ethyl acetate (9:1) as the packing solvent system. Once the silica had settled firmly, 5 g of the crude hexane fraction extract was dry-loaded onto the pre-packed column and subsequently eluted continuously with graded mixtures of n-hexane and ethyl acetate in the following ratios: 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, and 90:10. A total of 50 eluates (F1–F50) were collected, and the progress of separation was monitored by examining the migration of samples along the column as well as by thin-layer chromatography (TLC). The TLC analysis involved monitoring bands corresponding to identical sample spots, with different chromatographic spots visualized under UV illumination and by staining with potassium permanganate ($KMnO_4$) and ferric chloride solution.

Micro column

Five fractions from the initial column were pooled together and re-chromatographed for isolation of a pure compound, using a micro glass column of internal diameter 5 cm and length 30 cm over a silica gel eluting with the solvent system 90:10 respectively. After running TLC, the isolates yielded a compound (S1), and was

weighed to be 42 mg and found to be a dark green oily compound. The solvent was evaporated at room temperature (25 ^{o} C) and then transferred into sterile bottle prior to NMR and IR analysis.

Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectrum of the isolated compound was recorded on an Agilent Technology's 630 FTIR spectrophotometer for studying the presence of functional groups. The sample was fixed in potassium bromide (KBr) disc and the sample was scanned in the range of 4000-650 cm-^{1} wavenumber.

Nuclear Magnetic Resonance Spectroscopy

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR) analyses were conducted using deuterated chloroform ($CDCl_3$) as solvent on a Bruker NMR DBX-400 MHz spectrometer. The chemical shifts expressed in ppm were recorded.

Results

Extraction and Fractionation

The solvents used for extraction are hexane, dichloromethane and ethyl acetate. Among them, dichloromethane showed the highest fractionation yield (16g) followed by Hexane (10g), then Ethyl Acetate (8g) using 50.1 g of the crude extract. This indicated that the extraction efficiency favored both the polar and non-polar solvents.

%Mortality and probit analysis

The results of the larvicidal activities of the different fractions of the leaves of *Alstonia boonei de wild* on spodoptera frugiperda larvae are presented in this paper. The larval mortality was presented as a percentage of the total larval population. The lethal concentration $\mathbf{LC_{50}}$ (concentration at which 50% of the larvae died) and lethal concentration $\mathbf{LC_{90}}$ (concentration at which 90% of the larvae died) was also analysed using the Probit Analysis method. The software used in the analysis is Microsoft Excel 201

Table 1: Weight, color and texture of crude extract and fractions of *Alstonia boonei de Wild*

S/N	Fraction	Weight (g)	Yield (%)	color	Texture
1.	Hexane Fraction	10g	19.96	Deep green	Sticky
2.	Dichloromethane fraction	16g	31.93	Deep green	Nonsticky
3.	Ethyl Acetate fraction	8g	15.96	Light brown	Nonsticky
4.	Crude extract	50.1g	27.56	Deep green	Oily&sticky

Table 2 Phytochemical Screening of Fractions of Alstonia boonei de Wild

S/N	Phytochemical	Hexane frac.	Dichloromethane frac.	Ethyl Acetate frac.
1.	Alkaloids	--	+	+
2.	Terpenoids	+	+	+
3.	Flavonoids	--	+	+
4.	Steroids	+	+	--
5.	Tannins	--	+	+
6.	Glycosides	--	+	+
7.	Saponins	+	+	+

Key: (+) =present, (--) =absent

Table 3: Hexane fraction %mortality and probit values

Conc (mg/**µ**L)	$\log_{10}$(conc)	%mortality(mean)	Probit
0.005	-2.301029996	40	4.75
0.01	-2	40	4.75
0.02	-1.698970004	60	5.25
0.05	-1.301029996	80	5.84
0.1	-1	80	5.84
0.2	-0.698970004	100	8.9
0.5	-0.301029996	100	8.9

LC50=0.02, LC90=0.1

Table 4: Dichloromethane fraction %mortality and probit values

Conc (mg/**µ**L)	log_{10}(conc)	%mortality(mean)	Probit
0.005	-2.301029996	0	0
0.01	-2	20	4.16
0.02	-1.698970004	20	4.16
0.05	-1.301029996	40	4.74
0.1	-1	40	4.75
0.2	-0.698970004	60	5.25
0.5	-0.301029996	60	5.25

LC50=0.14, LC90=0.6

Table 5: Ethyl Acetate fraction %mortality and Probit values

Conc (mg/**µ**L)	log10(conc)	%mortality(mean)	Probit
0.005	-2.301029996	0	0
0.01	-2	0	0
0.02	-1.698970004	0	0
0.05	-1.301029996	20	4.16
0.1	-1	20	4.16
0.2	-0.698970004	40	4.75
0.5	-0.301029996	40	4.75

LC50=0.3, LC50=0.7

Table 6: ^{1}H and ^{13}C NMR Spectroscopic Data of Compound C1

δH (ppm)	C-number	δC (ppm)	Type of C-atom
1.58	C1	14.92	CH_3
1.66	C2	14.76	CH_3
Not assigned	C3	130.74	C (Quaternary)
5.12	C4	125.64	CH
2.04	C5	32.59	CH_2
1.27	C6	27.47	CH_2
2.21	C7	26.06	CH
0.89	C8	25.34	CH_3
2.81	C9	34.52	CH_2
11.97	C10	175.25	C (Quaternary)

Table 7: FTIR Spectroscopic Data of Compound S1

Wavenumber (cm^{-1})	**Functional Group**	**Assignment**
3393	Hydroxyl (Alcohol)	O-H Stretch
2922	Methyl (Alkane)	C-H Stretch
1714	Carbonyl	C=O Stretch
969	Alkene	=C-H

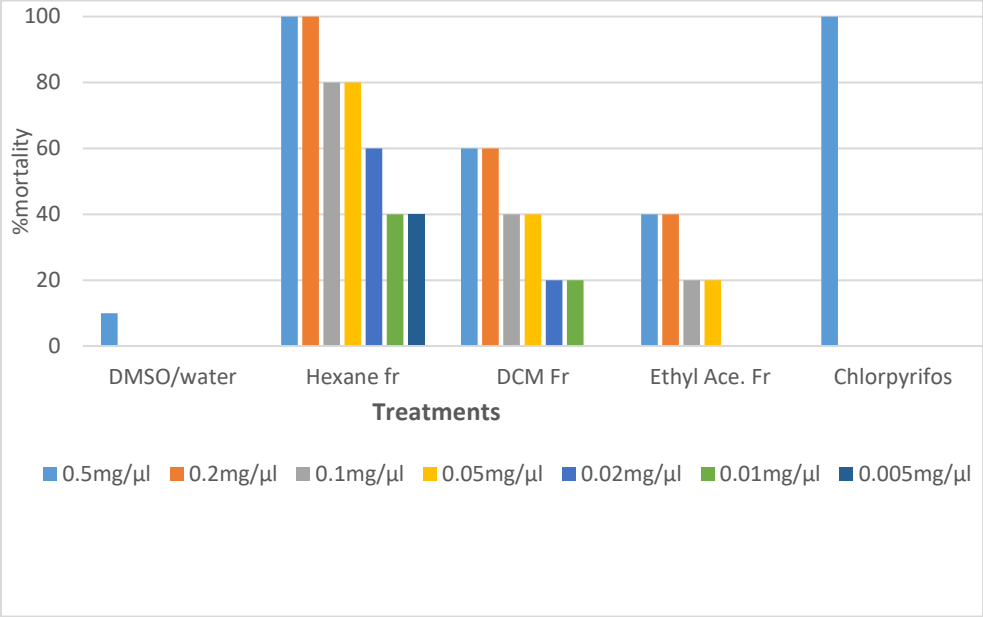


Figure 1: Comparison of the effects of individual concentrations of leaves fractions of *Alstonia boonei de Wild* and controls on Fall Armyworm Larvae

Discussion

Extraction and Fractionation

Hexane fraction yielded a significant amount of crude extracts which was further fractionated to yield three different fractions. Among the three solvents, Dichloromethane showed the highest fractionation yield (16 g) followed by Hexane fraction (10 g), then Ethyl Acetate (8 g) using 50.1 g of the crude extract. This indicated that the extraction efficiency favored both the polar and non-polar solvents. Some works on *Alstonia boonei de wild* as insecticidal plants also reported the fractionation of extracts with various organic solvents [9]. Low polar solvent extracts such as *n*-hexane, petroleum ether, chloroform and dichloromethane are capable of extracting non-polar compounds mainly of terpenoids or highly methoxylated phenolics.

Phytochemical screening

The phytochemical analysis of *Alstonia boonei de wild* fractions showed evidence of the presence of many secondary metabolites. The qualitative test carried out in this study indicated that, the fractions contain Alkaloids, Terpenoids, Flavonoids, Glycosides, Saponins, Tannins and Steroids. This has been reported by [16]. Flavonoids, alkaloids, glycosides, saponins, terpenoids, steroids, and tannins are well known to possess biological and pharmacological activity against various chronic diseases and

insects pests. Therefore, through phytochemical screening, one could detect the various important compounds which may be used as the basis of modern pesticide development.

Bioassay

The results of this experiment showed that the leaf extract of *Alstonia boonei de wild* is larvicidal against *Spodoptera Frugiperda* larvae. Our study showed that the Hexane fraction of the leaf extract of *Alstonia boonei de wild* adversely affected survival of Fall Armyworm larvae, leading up to 100% mortality. Since larvae treated with Hexane extract suffered 100% mortality and other doses of the crude extract caused higher larval survival rate than Hexane fraction, *Alstonia boonei* Hexane fraction likely contains more potent compounds than the other fractions of the leaves. That the LC_{50} of the Hexane fraction is 0.02 mg/**µ**L tells a lot about the potent insecticidal compounds in the fraction. Dichloromethane fraction has also showed activity, but less than the hexane fraction, with LC_{50} of 0.14 mg/**µ**L and LC_{90} of 0.6 mg/**µ**L. The least active fraction is the ethyl acetate fraction which showed activity only at the highest concentrations. The positive control, chloropyriphos, a synthetic pesticide prepared at 0.5% w/v and applied topically on the larvae has also showed 100% larval mortality whereas the negative control of the experiment (20 % DMSO) did not show activity on the larvae. This agrees with the study conducted by [20] where he demonstrated the use of DMSO as a chemical solvent in concentration not exceeding 80 % as non-inhibitor against growth of larvae. Therefore, it was found that DMSO was a safe solvent and offered no interference.

NMR and FTIR spectroscopic analyses

The interpretation of the ^{13}C and ^{1}H NMR spectra (Table 6) was performed with the aid of Wiley spectral database. The chemical shift values in both carbon and proton analysis come in close proximity to the chemical shift signals of Citronellic acid, a carboxylic monoterpenoid that occurs in *P. Papillonaceum and P. Vitifolium* [17].

Citronellic Acid is reported to have certain degree of insecticidal activity according to a study reported by [21]. Several studies also revealed that citronella extract, the extract of the plant from which Citronellic acid was first reported and isolated, was effective in being used as *Aedes Aegypti* repellent [19]. The FTIR data (Table 7) of compound S1 displayed absorption band at 3393 cm^{-1} characteristics of O-H stretching, another band appeared at 2922 and 2855 cm^{-1} which was due to aliphatic C-H stretching bands respectively. The band around 1714 cm^{-1} corresponds to C=O stretching of the compound, 1453 and 1379 cm^{-1} for C-H (methylene and methyl) bending vibrations. The FT-IR spectrum of compound C1 presented absorption band in 969 cm-^{1} region, indicating O-H deformation.

These absorption frequencies in compound S1 corresponds with the absorption frequencies observed for citronellic acid.

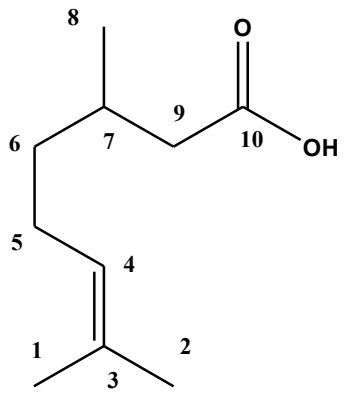


Figure 2: Proposed structure of compound S1 citronellic acid

Conclusion

In conclusion, the larvicidal activity demonstrated by the leaf extracts of *Alstonia boonei* against fall armyworm larvae can be strongly linked to the diverse array of phytochemicals detected during preliminary screening as well as the bioactive compound successfully isolated. These secondary metabolites, known for their ecological roles in plant defense, are likely the key contributors to the mortality and growth-inhibitory effects observed in the treated larvae. The findings therefore not only highlight the pesticidal potential of *A. boonei* as a promising source of natural larvicides, but also provide scientific support for its traditional use in pest management. By establishing a connection between specific phytochemical constituents and larvicidal activity, this study offers valuable insights into the development of eco-friendly and sustainable alternatives to synthetic insecticides in the management of fall armyworm infestations.

References

[1]. Pachlatko, J.P. (1998). Natural products in crop protection. *JOURNAL OF CHIMIA*, 52(1-2),29. https://doi.org/10.2533/chimia.29

[2]. Ribeiro L.P, Vendramim J.D, Gonçalves G.L.P, Ansante T.F, Gloria E.M, Lopes J.C, Mello-Silva R, Fernandes J.B. (2016). Searching for promising sources of grain protectors in extracts from *Neotropical Annonaceae. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*. 15(4): 215-232

[3]. Soujanya, P., Sekhar, J., Kumar, P., Sunil, N. and Prasad, C. (2016). Mallavadhani UV.Potentiality of botanical agents for the management of postharvest insects of maize: a review. *J Food Sci Technol*.; 53(5): 2169-2184.

[4]. Oze, G.; Nwanjo, H. and Onyeze, G. (2007) Nephrotoxicity Caused by the Extract of Alstonia boonei (De Wild) Stem Bark in Guinea Pigs. *The Internet J of Nutr and Wellness*. 3(2).

[5]. Adotey, J. P. K., Adukpo, G., Boahen, Y. O., and Armah, F. A. (2012). A review of the ethnobotany and pharmacological importance of *Alstonia boonei De Wild* (Apocynaceae). *International Scholarly Research Notices,* 2012, 1–9.

[6]. Bruce, Y.A., Gounou, S., Chabi-Olaye, A., Smith, H., Schulthess, F., (2004). The effect of neem (Azadirachta indica A Juss) oil on

oviposition, development and reproductive potentials of Sesamia calamistis Hampson (Lepidoptera: Noctuidae) and Eldana saccharina Walker (Lepidoptera: Pyralidae). *Agricultural and Forest Entomology*, 6(3): 223-232. [doi:10.1111/j.1461-9555.2004.00218.x].

[7]. James, B., Caulibaly, O., Gbaguidi, B., 2003. New solutions for cowpea production in Africa. Pesticide News, 61: 12-13.

[8]. Maala, E., Kiiya, W., Omollo, P., and Omondi, P. (2000). Participatory Evaluation of the Effectiveness of Locally Known Plant Extracts and Insecticides in Controlling Maize Stalk Borer.KARI/KEFRI/ICRAFTechnicalReport,44.http://www.kari.org/Legume_Project/Legume2 Conf_2000/44.pdf.

[9]. Oigiangbe ON, Igbinosa IB, Tamo M. (2007). Insecticidal activity of the medicinal plant, Alstonia boonei De Wild, against Sesamia calamistis Hampson. *Journal of Zhejiang University Science*, 8. 752-5. 10.1631/jzus.2007.B0752

[10]. Nagoshi, R. N., Meagher, R. L. & Hay-Roe, M. (2012). Inferring the annual migration patterns of fall armyworm (Lepidoptera: Noctuidae) in the United States from mitochondrial haplotypes. *Ecology and Evolution* 2, 1458–1467.

[11]. Goergen, G., Kumar, P. L., Sankung, S. B., Togola, A. & Tamo, M. (2016). First report of outbreaks of the fall armyworm Spodoptera frugiperda (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. Plos One 11

[12]. Cock, M. J. W., Beseh, P. K., Buddie, A. G., Cafa, G. & Crozier, J. (2017). Molecular methods to detect Spodoptera frugiperda in Ghana, and implications for monitoring the spread of invasive species in developing countries. *Sci Rep-Uk* 7.

[13]. FAO (2017). Briefng Note on FAO Actions on Fall Armyworm in Africa, Oct. 1, 2017, http://www.fao.org/3/a-bt415e.pdf.

[14]. Ranum, P., Pena-Rosas, J. P. & Garcia-Casal, M. N. (2014). Global maize production, utilization, and consumption. *Ann Ny Acad Sci* 1312, 105–112.

[15]. Stokstad, E. (2017). FOOD SECURITY New crop pest takes Africa at lightning speed. *Science* 356, 473–474.

[16]. Okoye, N.N. and Okoye, C. O. B. (2016). Antioxidant and antimicrobial flavonoid glycosides from Alstonia boonei de wild leaves. *Journal of pharmaceutical research international,* 10(6), 1-9

[17]. Lis-Balchin, M., and Roth, G. (1999). Citronellic Acid: A Major Component in Two *Pelargonium* Species (Geraniaceae). *Journal of Essential Oil Research*, 11(1), 83–85. https://doi.org/10.1080/10412905.1999.9701079.

[19]. Aditama, W., Yosep Sitepu, F., and Zulfikar, C. (2019). The effect of lemongrass (Cymbopogon nardus) extract as insecticide against *Aedes aegypti. International Journal of Mosquito Research,* 6(1), 101–103.

[20]. Brito, R., Silva, G., Farias, T., Ferreira, P., and Ferreira, S. (2017). Standardization of the safety level of the use of DMSO in viability assays in bacterial cells. *Journal of Toxicology and Environmental Health*, Part A, 80(23), 1269-1275.

[21]. Peterson, C. J., Tsao, R., Eggler, A. L., & Coats, J. R. (2000). Insecticidal Activity of Cyanohydrin and Monoterpenoid Compounds. *Molecules*, **5**(4), 648-654. https://doi.org/10.3390/50400648.

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