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Phytochemical Characterisation And Toxicity Effect Of *Tithonia diversifolia* (Hemls.) A. Gray Leaf Extract On Fall Armyworm *Spodoptera frugiperda* (JE Smith) Larvae.

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ABSTRACT

To mitigate the losses due to Fall armyworm (FAW) infestation in maize, chemical pesticides had been the first choice and widely used as an emergency response. However, it comes with attendant health effect. This necessitates the development of effective and safer pesticides, where plant origin is the most explored. One such plant, *Tithonia diversifolia* has traditionally been used to manage storage pests. Hence the need to investigate its dose dependent toxicity assay on FAW at different growth stages under controlled environment in laboratory, followed by its effect under phytotron against control and azadirachtin, a well-known biopesticide from neem on FAW by leaf dip and diet incorporation laboratory bioassays and pot culture trial in a phytotron to verify its efficacy as botanical pesticide in maize. The butanol eluent and crude extract caused 96% mortality at neonate and first instar FAW larvae, whereas it affected the growth of older larvae suggesting its negative effect on the physiology and growth of FAW. The toxic and repellant effects revealed by diet bioassay and phytotron experiment respectively suggest that butanol eluent of *T. diversifolia* leaf extract could be a good and effective target for biopesticide production against FAW.

Keywords: Biopesticide, Fall armyworm, Maize, GC-MS, phytocompound.

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INTRODUCTION

Fall armyworm (FAW), *Spodoptera frugiperda* (JE Smith), is native to tropical and subtropical Americas and is known as a pest for over three centuries. It has great potential to spread quickly as FAW moth was reported to be able to fly up to 100 km in 12 hours [1,2]. It invaded Africa during 2016, where it was first observed in South-Western Nigeria and shortly thereafter from many parts of Nigeria, Sao Tome, Benin and Togo [3]. By 2018 it spread to sub-Saharan Africa invading 44 countries (www.cimmyt.org) and also spread to Asia in the same year. Without any control strategy, FAW can cause up to 53% loss in annual maize production in Africa [4]. In 2018, the presence of FAW was confirmed in Asia [5]. Since then, it had been unstoppable reaching so far up to New Zealand by March 2022 [6], attaining global pest status. Since its first detection, several efforts have been devoted to creating awareness of the pest damage and implementation of control measures, with emphasis on the use of synthetic pesticides to get a quick solution [7], hitherto not used in maize cultivation by many small holder farmers in Africa. These awareness have lessened the yield impact of FAW compared to the level of damage during the initial period of its introduction into Nigeria [8].

Unlike the intensively farmed commercial scale fields, maize farms owned by subsistence farmers have little or no input of synthetic insecticide due to affordability. The worldwide spread of FAW and its link to low grain yield especially maize has motivated a thorough search for more available ecofriendly and more potent natural pesticide.

Moreover, botanicals are well-suited in organic farming. Potent botanicals like Neem *Azadirachta indica*; *Peumus boldus* commonly called Boldo, directly kill insects and interfere with their physiology and biology by acting as growth regulators, antifeedants, repellents, oviposition deterrents and sterilization agents [9].

Numerous literatures have shown that *Tithonia diversifolia* (Hemls.) A. Gray has varied contributions in the field of medicine [10] and agriculture [11]. In the study of Gitahi *et al*. [12] it was found that *T. diversifolia* contains sesquiterpene lactones and diterpenoids, some of which have biological activities against insects. In Nigeria and Uganda, farmers use the fresh leaves in field and storage pest management [8].

Thus, the present research is designed to explore the effect of crude and partially purified *T. diversifolia* leaf extract on fall armyworm *Spodoptera frugiperda* (JE Smith) larvae.

MATERIALS AND METHODS

Plant Materials

Tithonia diversifolia leaves were harvested at Igodan (6° 31' 0" N, 4º 45' 0" E) in Ondo state, Nigeria.The plant samples were provided to an acknowledged taxonomist for botanical authentication and voucher specimens were deposited. The leaf samples were washed with tap water followed by distilled water and air dried at room temperature. The dried samples were ground and stored in airtight container until used.

Extraction Processes

Extractions of active compounds from *T. diversifolia* leaf were done as described by Ejelonu et al. [10] with slight modifications. Hundred grams (100 g) of ground sample was extracted with 2000 ml of methanol for 72 hr. The methanolic extract was concentrated using a rotary evaporator and freeze drier, then partitioned with hexane and water (1:2, v/v) using separating funnel. After a thorough shaking, the mixture was allowed to stand overnight and the water layer was concentrated and partitioned between ethyl acetate and n-butanol (1:3, v/v). The Butanol fractions were concentrated separately using freeze drier and used for this experiment.

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Structural Characterization

Characterization Of Fractions Of The Extract Of *T. Diversifolia* Leaves (TDL) By GC-MS Analysis

GC-Mass was performed on a Shimadzu GCMS-QP2010 Ultra Gas Chromatography Mass Spectrophotometer equipped with a carbowax (30 mm × 0.25 mm ID; 0.25 µm film thickness) capillary column (intercut DB5Ms. Japan). 1 µl of the sample was injected into the capillary column. Helium was used as the carrier gas with a flow rate 1.0 ml/min. Injector and detector temperatures were set at 260°C. Injection was performed in split mode (1:30), the column temperature was programmed initially at 60°C for 3 min and then increase at a rate of 10° per min at final temperature of 300°C. Component molecules were separated at constant pressure (73.2 Kpa) 6 split ratio 30.0, column flow 1.21 ml/min and peaks were identified by comparing the mass spectra with mass spectral database. The chemical constituent was identified using NIST08.LIB library spectra provided by the software on a GC / MS system

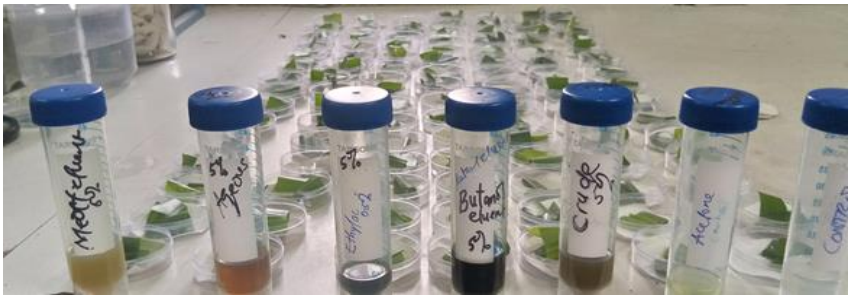
Fourier Transform Infrared (FT-IR) Analysis

The sample was ground in a mortar to reduce the average particle size to 1 to 2 microns. Exactly 0.1 mg of finely pulverized sample was mixed with ground KBr. This mixture was then placed onto the face of a KBr plate with the second window on top. With a gentle circular and back-and-forth rubbing motion of the two windows, the mixture was evenly distributed between the plates until it became slightly translucent. The sandwiched plates were placed in the spectrometer to obtain a spectrum. The Fourier transform infrared spectrum was recorded using Bruker Tensor 27 spectrometer in the wavelength range 400 to 4000 cm-1 by KBr pellet technique with a resolution and scanning speed of 4 cm-1 and 2 mm/s, respectively. The FTIR spectrum was used to identify the functional groups of the active components present in plant sample based on the peak values in the region of IR radiation.

Rearing Of S*podoptera Frugiperda*

Field population of FAW larvae were sampled from maize fields of ICAR-IARI, New Delhi (28°38'39"N+77°09'09"E) and reared in insect mass rearing facility (27±2°C, 65±5% RH and 16/8 h photoperiod) on baby corn plant pieces with husks at ICAR-IIMR, Delhi Unit. Second generation onwards larvae were reared on a chickpea-based protein enriched semi-synthetic diet (unpublished) and used for experiments.

Leaf-Dip Bioassay



A

B

C

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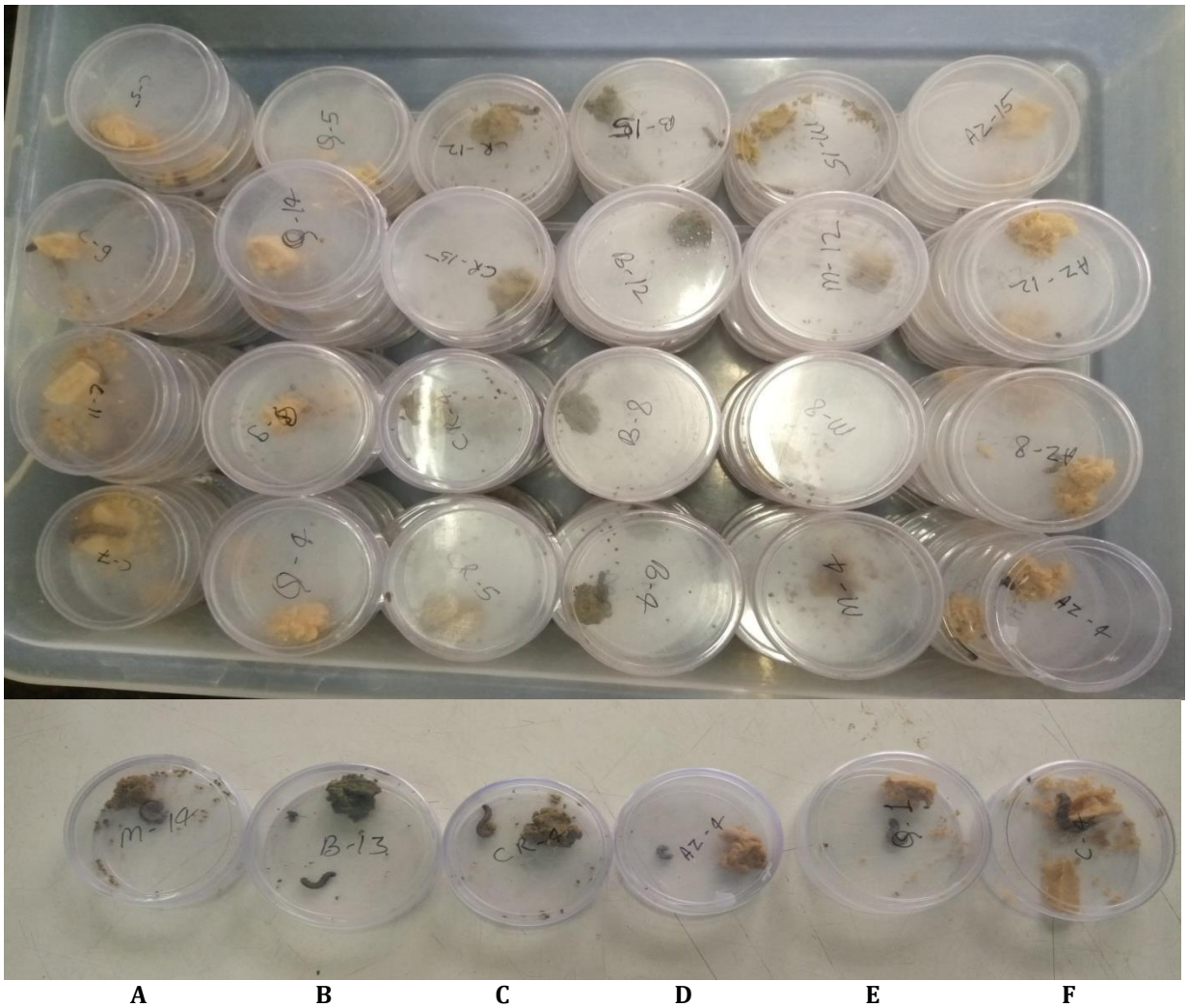
Figure 1: Top panel: Leaf-dip bioassay. Maize leaves cut into 4.5cm pieces were dipped into 5% treatment solutions of *T.diversifolia* viz. methanol extract, Aqueous extract, ethyl acetate extract, Butanol eluent, crude extract and control (water).

Bottom panel: Extent of leaf feeding and larval growth in A (Control) B (Butanol eluent) and C (Methanol extract) 72 hours after leaf dip assay.

Toxicity Bioassay

Laboratory diet incorporation

Dry powder of five different botanical treatments viz., Azadirachtin 29% from neem seeds at 0.625mg/g, *T. diversifolia* leaf extract butanol fraction at 2.5mg/g, *T. diversifolia* crude extract at 2.5mg/g, *T. diversifolia* leaf extract methanol fraction at 2.5mg/g and, Quillaja Saponin 30% from soapwort tree bark at 0.625mg/g incorporated into FAW diet were fed to second instar FAW larvae weighing 6.79±1.31 mg. Untreated diet served as control. The experiment was laid-out in CRD, where each treatment was replicated thrice with 15 larvae/ replication, each placed singly along with ~0.5g diet in containers (5cm dia). The larvae were observed after 72 hours of feeding.



A **B** **C** **D** **E** **F**

Figure 2: Top panel: Laboratory diet incorporation toxicity bioassay. Diet incorporated with 2.5g (Crude, methanol extract, butanol eluent)/100g of diet and 0.625g (Azadiractin and Quillaja saponin)/100g diet was fed to FAW respectively for 72 hours.

Bottom panel: Extent of diet feeding and larval growth in **A** (Methanol extract) **B** (Butanol eluent) **C** (Crude extract*)* **D** (Azadirachtin), **E** (Quillaja saponin) and, **F** (Control).

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Pot Culture Assay

Under controlled atmospheric conditions of (28° C, 65% RH and 12:12 light:dark phase) phytotron chamber in ICAR-IARI, New Delhi, plants of the popular hybrid IMH 1308 was raised by sowing five seeds in 9'' pots filled with sterilized mixture of soil:sand:cocopeat:FYM in 2:1:1:l ratio and three plants were maintained per pot. The plants were raised for assigning seven treatments (table 1) in triplicates, where four pots served a replication. Fifteen days after germination, individual pots were covered with transparency sheets of 52 cm height and two larvae of second instar FAW were released into the whorl of each plant except for treatment no.1. After 48 hours, treatments solutions prepared in 0.1% xanthan gum (as dispersant) in water and applied 0.5 ml into the whorl of each plant with a micropipette. Control treatments (T 1&2) only had 0.1% xanthan gum in water. After 72h, damage symptoms were rated for whole plant (WPD) and central leaf (CLD) in 1-9 scale (1 no damage, 9 completely damaged) and per plant weight (PPW) was recorded.

NB (1, 2, 3); AZ(1, 2, 3, 4); B(1, 2, 3, 4); CR(1,2,3); I; H; ST(1,2,3, 4)

Figure 3: Relative growth of fall armyworm infested plants to botanical treatments viz., Neem Baan (containing Azadirachtin 1500) @ 5ml/l (NB1, NB2, NB3); Azadirachtin 29% @ 6.25g/l (AZ1, AZ2, AZ3, AZ4); *T. diversifolia* butanol fraction @ 50g/l (B1, B2, B3, B4); *T. diversifolia* crude extract @ 50g/l (CR1, CR2, CR3); Control (infested), Control (healthy), *S. trilobata* methanol extract @ 50g/l (ST1, ST2, ST3, ST4) under controlled conditions in phytotron.

Data Analysis

Data collected were subjected to Analysis of Variance (ANOVA) and means of treatments and replicates were compared using Student-Neuman-Keul sand Least Significant differences (LSD) at 5% for laboratory bioassays and Tukey HSD for pot culture assays.

RESULTS

A total of twenty (20) compounds were identified from the butanol eluent of the methanolic leaf extract of T*. diversifolia*. The identification of the phytochemical compounds was confirmed based on the peak area; retention time and molecular formula were presented in Table 1. The GC-MS analysis of the butanol eluent of the leaf extract of *T. diversifolia* showed the presence of the following phytochemicals (Table 1)

The FT-IR spectroscopic analysis of the butanoleluent of *Tithonia diversifolia*leaf (Hemsl.) A. Gray extract revealed the presence of alcohols, phenols, aldehydes, ketones, alkanes and primary amines (Table 2).

Laboratory Diet Incorporation Toxicity Bioassay On Second Instar FAW Larvae

Mortality, larval weight, frass weight and RGR of 2nd instars when exposed to extracts of *T. diversifolia* 2.5mg/g, 30% Quillaja saponin 0.625mg/g and 29% Azadiractin 0.625mg/g for 72 hours in laboratory diet incorporation toxicity bioassay are presented in (Table 3). After 72 hours of feeding, where 57.77±3.84% of the larvae died in Azadirachtin treated diet, which was followed by *T. diversifolia* butanol

fraction (22.22±3.84%) while no mortality was observed in control and *T. diversifolia* methanol fraction and only 2.22±3.84% mortality in Quillaja Saponin (table 3). The significant differences in toxicity observed in terms of mortality was well reflected in post feeding weight, weight gain, and relative growth rate.

Infact, a weight reduction was observed in Azadiractin (2.92±0.54 mg), while weight of the larvae fed TD butanol fraction (6.98±0.60 mg) remained more or less the same of initial weight compared to growth in control (130.57±1.59 mg).

To better explain the effect of treatments in growth, the RGR calculated for the treatments (Azadirachtin 0.625mg/g of diet, *T. diversifolia* leaf extract butanol fraction at 2.5mg/g of diet, T. diversifolia Crude extract at 2.5mg/g of diet, *T. diversifolia* leaf extract methanol fraction at 2.5mg/g of diet, Quillaja Saponin 30% from soapwort tree back at 0.625mg/g of diet) in 72 hours were 0.90, -0.40,-0.02, 0.37, 0.65, 0.59 mg/mg of body weight/day respectively.

Response Of Fall Armyworm Infested Plant To Botanical Treatments Under Controlled Conditions

No-insect control plants recorded no damage (score 1 for WPD and CLD) and highest PPW (5.8g), whereas highest damage (score 9) and lowest PPW (1.57g) was recorded in FAW infested control plants. Among botanical treated plants, least WPD (2.58) and CLD (1.58) were recorded in Azadirachtin treated plants, which are followed by treatments of NeemBaan 1500 and by *S. trilobata* extract. PPW recorded in NeemBaan was at par with control which was followed by statistically similar results in all other botanicals treatments except butanol fraction of *T. diversifolia*, which recorded significantly lower plant weight (2.8g) (Table 4).

DISCUSSION

In the present work, twenty (20) compounds were isolated from the butanol eluent of the methanolic leaf extract of *T. diversifolia* (Hemsl.) A. Gray. The identified compounds belong to class of; alkaloids, terpenoids, fatty acids, phytosterols, and benzaldehyde, among others. These botanical constituents could be responsible for the observed mortality and antifeedant action of the insects in the diet bioassay and the phytotron experiment. Although the compounds identified has been reported to possess many biological properties. Among which are, nHexadecanoic acid which has been identified to possess lubricant, anti-androgenic, hypocholesterolemic, hemolytic, antioxidant, pesticide, 5-alpha reductase inhibitor activities [13]. Hexadecanoic acid, methyl ester, a derivative of palmitic acid which has been indicated to have antibacterial, antifungal, antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha reductase inhibitors [10,14]. Octadecanoic acid has antibacterial, soap lubricant and cosmetic potentials. These biochemicals could be responsible for the in-vivo activities of the extract of *T. diversifolia* against the proliferation of microorganisms as reported by Ogundare [15], Oyewole *et al*. [16] and Omotuyi *et al*. [17]. The concentration of the major bioactive components Glucopyranoside, methyl (15.23%), and Palmitic Acid, TMS Derivative (10.28%) is observed to be consistent with the demonstrated properties of this plant as a pesticide. Since previous study by (Gitahi *et al*. [12] Yang *et al*. [18] indicated that repellent activities of *T. diversifolia* and *V. lasiopus* extracts could have been due to presence of monoterpenes. Monoterpenes such as eugenol, limonene, camphor, and thymol commonly found in basil have strong repellent activities against storage insects [19]. Arthropods are known to release oleic and linoleic acids upon death, smell of these compounds is believed to repel other insects, thereby keeping them away from approaching their death zone [12]. The repellent activity observed in this present study could be because of presence of oleic acid and linoleic acid as shown in the GC-MS analysis.

The ability of the butanol eluent of *T.diversifolia* to cause mortality on the neonate of FAW could also be as a result of the Glucopyranoside and Palmitic Acid, present which has been identified as the insecticidal principle in plant [19].

The FT-IR analysis is an effective analytical technique for the identification of the functional groups resident in biological sample. The presence of Alcohols, phenols, aldehydes, ketones, alkanes and primary amines as the functional group could be accountable for the already established medicinal properties of *T. diversifolia* leaves (Hemsl.) A. Gray.

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Table 1: GC-MS (Gas chromatography-mass spectrometry) results for butanol eluent of *Tithonia diversifolia* leaf Extract.

S/N	Retention Time	Name of Compound	% Composition	Molecular formula	Molecular weight
1	10.70	Beta.-D-Glucopyranoside, methyl	15.22	C7H14O6	194
2	14.00	6-epi-shyobunol	6.24	C15H26O	222
3	5.72	1-Dimethyl(isopropyl)silyloxypropanr	2.17	C8H20O	160
4	6.23	1-Butanol, 4-(ethenyloxy)-	7.50	C6H12O2	116
5	7.18	1,4-Dioxan-2-ol, TMS derivative	2.62	C7H16O3	176
6	12.22	2-Methyltetracosane	2.17	C25H52	352
7	12.45	1-Heptadec-1-ynyl-cyclopentanol	2.69	C22H40O	320
8	14.54	Hexadecanoic acid, Trimethylsilyl ester	1.58	C19H40O2Si	328
9	17.79	Pregn-4-ene-3,20-dione,6-hydroxyl-, (6.beta.)-	2.40	C21H30O3	330
10	19.61	1-(3-Isobutyryl-bicyclo[1.1.1]pent-1-yl)-2-2methylpropan-1-	4.46	C30H60O6Si3	600
11	19.79	(9Z,12Z,15Z)-(E)-3,7-Dimethylocta-2,6-dien-1-yl octadeca	2.96	C28H46O2	414
12	10.99	Myristic acid, TMS DERIVATIVE	7.03	C17H36O2Si	300
13	13.97	6-epi-shyobunol	3.95	C15H26O	222
14	14.49	Palmitic Acid, TMS Derivative	10.29	C19H40O2	328
15	15.79	Androst-5-en-17-one, 3,16-bis[(trimethylsilicilate)	1.79	C25H44O3Si2	448
16	16.09	Alpha,-Linolenic acid, TMS Derivative	1.66	C21H38O2Si	350
17	19.07	Methyl (13E)-6-Oxo-9,11,15-Tris[(Trimethylsilyl)oxy	6.39	C30H60O6Si3	600
18	19.58	Undeca-3,4-diene-2,10-dione,5,6,6-trimethyl-	5.25	C14H22O2	222
19	25.26	Hexadecanoic acid, 2-[(Trimethylsily)….	8.75	C19H40O2Si	328
20	30.29	Arabinofuranose, 1,2,3,5-tetrakis-o-(trimethylsilyl)-	4.91	C17H42O5Si4	438

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Table 2: The FT-IR spectroscopic analysis of the butanol eluent of *Tithonia diversifolia* leaf extract

Peak values	Functional groups
3896.9	C-H lipid region
3436.91	C-H lipid region
2936.42	C-H lipid region
1726.17	Acid
1608.52	Alkane
1426.26	Aromatic
1057.88	Carboxylic acid

Table 3: Laboratory diet incorporation toxicity bioassay on second instar FAW larvae for 72hours treatment in mean± standard deviation values with different superscript shows statistical significant different

Treatment number	Treatment (mg/g) diet	Mortality	Larva Post wt (mg)	Frasswt (mg)	Larva wt gain (mg)	RGR
T1	Control	0 (0.13)^{b}	130.57±1.59^{f}	182.92±8.67^{d}	123.9±1.54^{f}	0.90±0.01^{f}
T2	Azadirachtin 29% @0.625mg/g	57.77(0.86) ±3.84^{d}	2.92±0.54^{a}	1.14±0.06^{a}	-3.87±0.18^{a}	-0.40±0.05^{a}
T3	*T.diversifolia* butanol fraction @2.5mg/g	22.22(0.49) ±3.84^{c}	6.98±0.60^{b}	6.15±1.05^{a}	-0.22±0.23^{b}	-0.02±0.02^{b}
T4	*T.diversifolia*Crude extract @2.5mg/g	13.33(0.37) ± 6.66^{b}	15.72±1.33^{c}	19.98±3.58^{b}	8.55±1.21^{c}	0.37±0.03^{c}
T5	*T.diversifolia* methanol fraction @2.5mg/g	0 (0.13)^{a}	35.83±0.31^{e}	41.58±10.18^{c}	28.45±0.57^{e}	0.65±0.02^{e}
T6	QuillajaSaponin 30% @0.625mg/g	2.22 (0.17) ±3.84^{a}	29.98±2.84^{d}	22.216±5.26^{b}	22.37±2.87^{d}	0.59±0.03^{d}
	General Mean	15.92	37.00	45.66	29.87	0.35
	C.V. (%)	24.16	4.00	13.28	4.84	9.18
	DF	12	12	12	12	12
	F (5,12)	75.3	3099.9	385.18	3274	666.73
	S.E.M	0.03	0.85	3.50	0.83	1.87
	S.E.D	4.67	1.21	4.95	1.18	2.64

Table 4: Response of maize plants infested with fall armyworm to botanical treatments- in terms of whole plant and central leaf damage score, and plant weight.

Treatment No.	Treatments	Whole Plant Damage Score	Central Leaf Damage score	Plant Weight (g)
T1	Control healthy	1.00^{d}	1.00^{d}	5.80^{a}
T2	Control infested	9.00^{a}	9.00^{a}	1.57^{c}
T3	NeemBaan(containing Azadirachtin 1500) @ 0.5ml/100ml	3.33^{bc}	3.92^{b}	4.98^{a}
T4	Azadirachtin 29% @ 0.625g/100ml	2.58^{cd}	1.58^{cd}	4.53^{ab}
T5	*T.diversifolia*butanol fraction @ 5g/100ml	4.58^{b}	3.58^{bc}	2.80^{bc}
T6	*T.diversifolia*methanolic extract @ 5g/100ml	4.42^{b}	3.33^{bc}	4.57^{ab}
T7	*S.trilobata*methanolic extract @ 5g/100ml	3.50^{bc}	3.00^{bcd}	4.52^{ab}

Treatment No.	Treatments	Whole Plant Damage Score	Central Leaf Damage score	Plant Weight (g)
	General Mean	4.06	3.63	4.11
	p-Value	<.0001	<.0001	<.0001
	F (6, 27)	65.77	47.15	14.05
	CV(%)	15.12	20.84	18.62
	SE(d)	0.434	0.535	0.541
	Tukey HSD at 1%	1.7331	2.1363	2.1599

The results suggest strong antifeedant and repellent effect of Azadiractin that is well known in insects and our results first time reveal antifeedant action in TD butanol fraction. The poor growth observed in TD crude and methanol fraction and QuillajaSaponin reveal their anti-nutritional effect, even though inadequate in pest management point of view. Interestingly, the amount of frass produced is a direct indication of weight gain and a better indication of antifeedancy, where Azadirachtin recorded the least (1.14±0.06 mg), which was followed by TD butanol fraction (6.15±1.05 mg), while control recorded the highest (182.92±8.67 mg).

There was almost perfect correlation between the mortality, growth rate and other related parameters which suggest that all parameters are indicators of relative toxicity of the test botanicals and their usefulness in toxicity bioassays. These results indicate *T. diversifolia* leaf extract butanol fraction as a potential botanical pesticide for the management of fall FAW.

The least CLD after 72h in Azadirachtin treated plants reveals the longest antifeedant action of the same (Table 4). NeemBaan which was effective next to Azadirachtin treatment is a formulation containing neem seed kernel methanolic extract containing minimum 1500 ppm Azadirachtin as mentioned in the label. The effective concentration of Azadirachtin was 0.2% and 0.02% respectively considering 29% purity of the lyophilized powder of the former and 1500ppm in formulation of the later suggest its bioactivity against FAW, which is also an established fact in many insect pests.

The better performance of butanol fraction in bioassay and its similar performance in plant damage control with methanolic extract, whereas its marked reduction in plant weight suggests the bioactive compound would be affecting plant growth. Similarly, it is interesting to note that weight of NeemBaan treated plants were like control even though plants were damaged more than Azadirachtin treated plants suggesting that crude extract could have better efficacy with less side-effect in plants.

Observed interferes with the physiology and biology functions of the insect FAW in the present study could partly be attributed to the presence of butanol eluent of the plant volatile bioactive components, which are well-known to be toxicant and insect repellents acting in the vapour form on the olfactory receptors [12]. However, the mechanism of interaction of the olfactory receptors and the photochemical are still not clear.

CONCLUSION

The main active ingredients in the butanol eluent of *T. diversifolia* are beta. -D-Glucopyranoside, methyl and Palmitic acid. The toxic and repellant effects revealed by diet bioassay and phytotron experiment respectively suggest that butanol eluent of *T. diversifolia* leaf extract can be a good and effective target for biopesticide production against FAW, especially when applied to maize crop with the onset of symptoms of FAW damage. The FAW may have found a new home in Africa and Asia, thus: we must not destroy our ecosystem in the name of its management and control, we must manage it eco-friendly. *T. diversifolia* may be one of the answers to the long awaited botanical pesticides. There is need to further the investigation on the field.

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