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Research Article
Essential Oils from the Leaves of *Euphorbia milii* and *Cassia occidentalis* Exert Insecticidal Action Through Gene Toxicity

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Abstract

Background and Objective: *Cassia occidentalis* (Coffee senna) and *Euphorbia milii* (Crown of thorns) are plants which possess some insecticidal properties against insect pests. This study investigated genetic toxicity as the possible route of insecticidal action of botanical oils extracted via soxhlet apparatus from the leaves of these two plants. **Materials and Methods:** This study was carried out at the Molecular Biology Laboratory of Covenant University Otta, Ogun State, Nigeria. It lasted for 12 months from October, 2015 to September, 2016. High quality deoxyribonucleic acids (DNAs) were extracted from *Periplaneta americana* (American cockroach) and *Tettigonia viridissima* (Great green bush cricket) exposed for 24 h to 600 mg of oils extracted from the leaves of *C. occidentalis* and *E. milii.* Purity and concentration of extracted deoxyribonucleic acids was ascertained using Thermo Scientific Nanodrop 2000 Spectrophotometer. The extracted DNAs were then amplified using Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) technique to determine the level of genotoxicity induced by the oils. **Results:** A total of 68 and 31 polymorphic bands were observed for chi-15 and EZ primers, respectively accruing to 100% polymorphism with no monomorphic bands present. The analysis of molecular variance (AMOVA) result for group analysis also revealed 31.8% variation among population and 68.19% variation within population with a PhiST (Φ_{ST}) value of 0.3181. This indicates that the plant oils induced strong polymorphic effects on the DNAs of test insects. **Conclusion:** The plant oils may carry out their insecticidal effects on insect pests through mutation in the DNA of test insects causing gene toxicity and eventual death in their target. Results from this research postulate another class of insecticides "Genetic disruptor insecticides".

Key words: Essential oils, *Euphorbia milii*, *Cassia occidentalis*, gene toxicity, insect pests, insecticidal

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Insecticides are important tools used for the control of insect pests, reducing the damages they cause and thus increasing agricultural crop yield and improved quality of life for humans, livestock and plants[1]. Insects are chief pathogenic agents that cause many human, animals and plant diseases[2]. They also constitute economic damages that can lead to starvation particularly in underdeveloped countries like Nigeria. The synthetic pesticides currently in use have been associated with various forms of cancer, neurological disorders and lung irritations in humans, as most of them are made from toxic heavy metals[3]. There is, therefore, need for a credible alternative preferably of natural origin. Influential scientific papers have proposed a higher level of sustainability using natural products[4]. This has made it necessary to produce safer, more environment friendly and effective alternatives. Botanicals are presently at the fore-front being that secondary metabolites from various plants are popular for their insecticidal efficacies and are sometimes used domestically to kill or minimize the impact of insect pests[5].

The discovery of bioactive secondary metabolites from plants which are toxins to herbivores that attack them opened the vista for their assessment as insecticides. Also, diversity in mode of action is a very important tool for sustaining the ability of an insecticide to control insect pests. By rotating pest control agents that work through different modes of action, insecticide resistance can be reduced[1]. These secondary compounds represent a large reservoir of chemical structures with biological activity[6]. Natural compounds with complex chemistry and structure should effectively combat and overcome this resistance, coupled with the additional advantage of rapid environmental degradation and low toxicity to non-target organisms. Again, the use of natural pesticides is likely to result in healthier agricultural soils with more microbial diversity[1].

Euphorbia milii and *C. occidentalis* are plants which have been reported to possess reasonable levels of insecticidal efficacy, the active ingredients likely responsible for their insecticidal action has also been reported[7]. No study however, has reported the mechanism of insecticidal action of oils from these plants.

Whereas *E. milii* is a low-growing evergreen shrub with very thorny grooved stems and branches which is not indigenous to Nigeria, but is believed to have been imported to Nigeria from India[8], *C. occidentalis* is a shrub which grows 5-8 m high and is found majorly in tropical areas. *C. occidentalis* is also known as Coffee senna in English, Akidi ogbara in Igbo, Dora rai in Hausa and Aboo rere in Yoruba[9] Nigeria. It is claimed to scare away insects and reptiles from its environs by indigenes of Akwa Ibom state in Nigeria, thus making it a popular domestic plant in that part of the world. The aim of this research was to evaluate the insecticidal actions of these oils via gene-toxicity.

MATERIALS AND METHODS

Equipments: Some of the materials used for this research include; Bio-RAD.C.1000 Touch Thermal Cycler Upland C.A USA, Benchtop Variable Transilluminator Cambridge UK, Digital Monochrome Printer Mitsubishi Electric Malaysia, Biorad DNA Electrophoretic system Singapore, Thermo Scientific Nanodrop 2000 Spectrophotometer UV-Vis Spec USA, Thermo Scientific Sorvall Legend Micro 21 Centrifuge, Germany.

Collection and identification of plant samples: Plants were identified by a botanist in the Department of Biological Sciences (Botany), College of Natural Sciences, Michael Okpara University of Agriculture Umudike Abia State Nigeria. Oil was extracted from healthy leaves and test insects were exposed to 600 mg of each oil.

Test insects: Test insects were identified by an Entomologist at the Department of Zoology and Environmental Biology, Faculty of Sciences, University of Calabar. He also assisted with the extraction of fat body and haemolymph from insects. All insects were healthy and of adult stage (except for mosquito larvae).

Biochemicals and chemicals: Some of the reagents and chemicals used include; n-hexane, AST, ALP, ALT, albumin, urea and creatinine assay kits (ELITech Clinical Systems SAS-Zone Industrielle-61500 SEES FRANCE. 1, chloro,2,4-dinitro benzene-BDH laboratory reagents Poole England, Reduced Glutathione-BDH chemicals limited Poole England, DNA extraction and PCR master mix kit; Qiagen Hilden, Germany.

DNA extraction procedure from insect tissues: Cells were lysed during a short incubation with proteinase K in the presence of guanidine-HCl. Bound nucleic acids were purified to remove contaminating cellular components. Finally, low salt elution was used to release the Nucleic Acid from the silicon membrane. The nano-drop was blanked using 2 µL of the elution buffer and was used to determine quality and concentration of DNA at 260 nm. DNA purity was calculated by dividing the absorbance at 260 nm by the absorbance at 280 nm:

$$\text{Purity of sample} = \frac{A_{260}}{A_{280}}$$

All values (ratios) between 1.7 and 2.0 were considered pure and used for the PCR. DNA integrity was determined using 1.5% agarose gel with 1×TAE buffer run for 1 h at 80 V.

Amplification of RAPD markers: Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was carried out as describe by Seufi *et al.*[10] using the Qiagen PCR master mix kit. RAPD-PCR analysis based on two primers chi-15 and EZ gave results in terms of amplification and polymorphism. To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative controls (blank) were carried out for each primer[11]. The reaction products were separated according to size by 1.5% agarose gel in 1×TAE buffer with ethidium bromide and photographed.

Statistical analysis: Analysis of molecular variance (AMOVA) was carried out using PyElph package software version[12] 1.4 with visual aid was used to match and detect bands. The molecular weights of bands were estimated based on the standard bands from 1 kb DNA marker. Only reproducible and clear bands were scored as present (1), absent (0) or missing (?) at a distance migrated on the gel. The data matrix of 1's, 0's and ?'s was prepared from the scored bands and entered into fingerprint analysis with missing data Software version[13] 1.31. The software was used to access genetic diversity among and between populations based on:

- Percentage of polymorphic bands/Loci (PPL)
- Analysis of molecular variance among and between populations

Genetic similarity was also determined based on the Jaccard similarity coefficient as described by Lamboy[14].

RESULTS

Lane M was the molecular weight marker (1 kb) from which the weight of the other bands were estimated. Note the lack of uniformity in the binding patterns of the different groups which connotes, genetic variation (polymorphism) in the DNA of the various groups as compared to 5 and 6 (control for cockroach) 10 and 11(control for Cricket). No amplification was detected in lane B (control) (Fig. 1).

Amplification with prime EZ gave poor results in some groups and no amplification in others. This was likely due to the length of the primer (24 bases) as RAPD-PCR give better results with shorter oligonucleotide primers[15] (Fig. 2).

A total of 68 and 31 polymorphic bands were observed for chi-15 and EZ primers, respectively, which accrued to 100% polymorphism for both primers (Table 1). AMOVA result revealed a 31.81% variation among populations and 68.19% within populations, PhiST value 0.318 indicated significant differentiation between populations (Table 2).

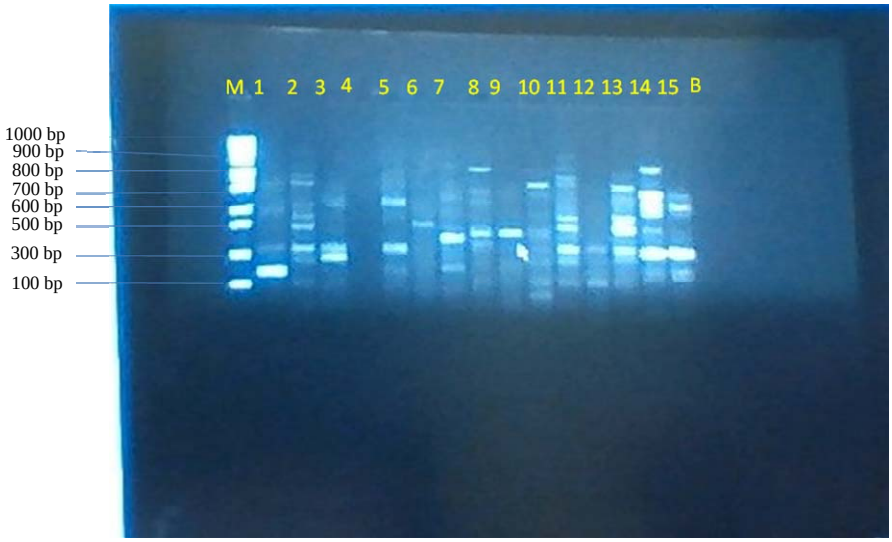

Fig. 1: Agarose gel electropherogram of RAPD-PCR of DNA samples using chi-15 primer (17 bases length)
PCR lanes: M: Molecular weight marker, 1 and 2: Co-SWAN, 3 and 4: Co-EM, 5 and 6: Co-Control, 7, 8 and 9: CR-SWAN,10 and 11: CR-Control, 12 and 13: CR-EM, 14 and 15: CR-Co, B: Blank

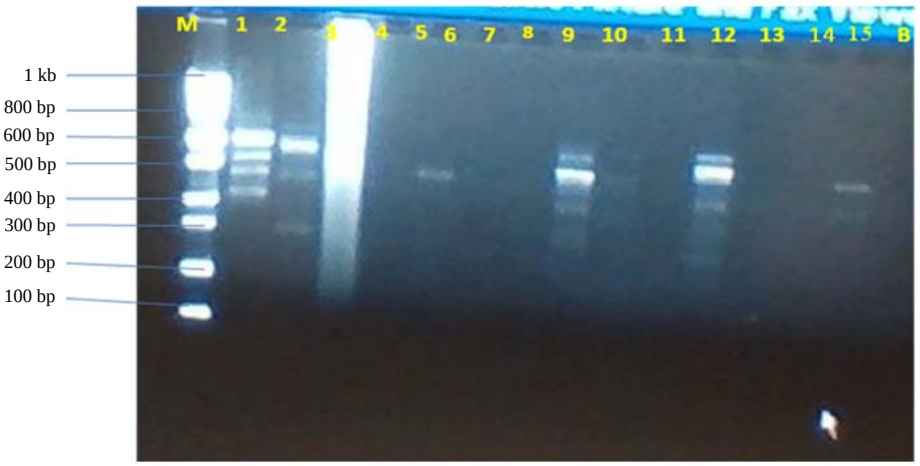

Fig. 2: Agarose gel electropherogram of RAPD-PCR of DNA samples using EZ primer (24 bases length)

PCR lanes: M-Molecular weight marker, 1 and 2-Co-SWAN, 3 and 4 -Co-EM, 5 and 6-Co-Control, 7, 8 and 9-CR-SWAN,10 and 11-CR-Control,12 and 13 CR-EM, 14 and 15-CR-Co, B-Blank. (Co-SWAN)-Cockroach exposed to SWAN insecticide, (Co-EM) -cockroach exposed to *E. milii* oil, (Co-Control)-cockroach not exposed to any insecticidal agent, (CR-SWAN)-Cricket exposed to SWAN insecticide, (CR-control)-cricket not exposed to any insecticide, (CR-EM)-cricket exposed to *E. milii* oil, (CR-Co) -cricket exposed to *C. occiden* talis oil

Table 1: Number of polymorphic and fixed bands for each primer

			No. of bands			
Primers (IDT)	Primer sequence (5'-3')	Tem. (°C)	NPB	NMB	Total	PPB (%)
Chi-15	GGYGGYTGGAATGARGG	49.6	68	0	68	100
EZ	GCATCACAGACCTGTTATTGCCTC	54.6	31	0	31	100
Total primers (2)			99	0	99	100

NPB: Number of polymorphic bands, NMB: Number of monomorphic bands and PPB: Percentage polymorphic bands

Table 2: AMOVA result for group analysis (coefficient:standard jaccard distance transformation)

Marker	Source of variation	df	Sum of square	Variance component	Variation (%)	PhiST (Φ_{ST}) value
RAPD	Among populations	6	2.4818	0.1037	31.81	0.3181
	Within populations	6	1.3333	0.2222	68.19	
	Total	12	3.8151	0.3259	100.00	

DISCUSSION

The extraction of genomic DNA from test insects produced good quality and high purity DNA which was used in the RAPD-PCR analysis. Eight of the ten randomly selected primers gave no amplified products, the reason for the failure of the other primers to amplify genome DNA may be the absence of suitable binding sites for these primers on template DNA[16]. The size of the amplified DNAs were between 100 and 1000 bp (Fig. 1). The differences observed in the pattern of DNA bands of the different groups as compared to the control (Fig. 2) may be due to mutations resulting from exposure to oils as oxidative damage to DNA can induce specific mutations[17].

PCR results of primer chi-15 amplified genomic DNA of test insect showed a total of 68 polymorphic bands. The AMOVA result (see appendix) also showed that the group consisting of crickets exposed to *E. milii* oil possessed the highest no of polymorphic bands 16, followed closely by the group consisting of cockroaches exposed to SWAN (the synthetic insecticide). The cricket group which received no insecticidal treatment (control) also showed the least polymorphism. A polymorphic variant of a gene may lead to the abnormal expression or to the production of an abnormal form of the gene[18]. High levels of polymorphism as compared to the control implied a high level of genetic variation of treated insects from the control. Being from the same population (environment) the insects should possess little or no genetic variability, the increase in genetic variability between the treated groups and the control indicated that the plant extracts and SWAN may exhibited some level of geno-toxicity in carrying out their insecticidal effects.

Appendix

AMOVA results

Coefficient: Standard Jaccard. Distance Transformation: d = 1-s
SSD (AP) = 2.481792396, df (AP) = 6
SSD (WP) = 1.333336069, df (WP) = 6
SSD (T) = 3.815128465, df (T) = 12
Va = 0.103680085, Var (%) = 31.813196069 (Among populations)
Vb = 0.222222678, Var (%) = 68.186803931 (Within populations)
Vt = 0.325902763
PhiST = 0.318131961
Distances d = 1-s (Standard Jaccard coefficients)

1	2	3	4	5	6	7	8	9	10	11	12	13
0.0000000000												
0.7333333333	0.0000000000											
0.8000000000	0.6250000000	0.0000000000										
0.8333333333	0.8571428571	0.8000000000	0.0000000000									
0.8571428571	0.8571428571	1.0000000000	1.0000000000	0.0000000000								
1.0000000000	1.0000000000	0.8571428571	1.0000000000	1.0000000000	0.0000000000							
1.0000000000	0.8235294118	0.7777777778	0.8750000000	1.0000000000	0.3333333333	0.0000000000						
0.6666666667	0.6842105263	0.8888888889	0.9166666667	0.9166666667	0.7272727273	0.6153846154	0.0000000000					
0.6428571429	0.5263157895	0.7272727273	0.8571428571	0.9333333333	0.7857142857	0.6875000000	0.3333333333	0.0000000000				
0.8750000000	0.6111111111	0.6363636364	0.9230769231	0.9230769231	0.8333333333	0.6923076923	0.8333333333	0.5882352941	0.0000000000			
0.6666666667	0.6666666667	0.6666666667	0.9090909091	1.0000000000	1.0000000000	1.0000000000	0.6875000000	0.6666666667	0.8421052632	0.0000000000		
0.9230769231	0.7647058824	0.6666666667	0.8888888889	0.8888888889	0.5000000000	0.6363636364	0.8125000000	0.6250000000	0.5000000000	0.8750000000	0.0000000000	
0.9090909091	0.8823529412	0.7500000000	0.8571428571	1.0000000000	0.3333333333	0.3750000000	0.7857142857	0.7500000000	0.6666666667	0.9333333333	0.4444444444	0.0000000000

Number of polymorphic bands found in group AllData: 24
Number of polymorphic bands found in group AllData/CoSwan: 11
Number of polymorphic bands found in group AllData/CoEM: 8
Number of polymorphic bands found in group AllData/Cocontrol: 4
Number of polymorphic bands found in group AllData/CRswan: 2
Number of polymorphic bands found in group AllData/CRcontrol: 5
Number of polymorphic bands found in group AllData/CREM: 16
Number of polymorphic bands found in group AllData/CRco: 4
Number of fixed bands found in group AllData: 0
Number of fixed bands found in group AllData/CoSwan: 5
Number of fixed bands found in group AllData/CoEM: 4
Number of fixed bands found in group AllData/Cocontrol: 0
Number of fixed bands found in group AllData/CRswan: 5
Number of fixed bands found in group AllData/CRcontrol: 10
Number of fixed bands found in group AllData/CREM: 3
Number of fixed bands found in group AllData/CRco: 5

The term genetic polymorphism is used in molecular biology to describe certain mutations in the genotype, such as single nucleotide polymorphism (SNP) that may not always correspond to a phenotype but always corresponds to a branch in the genetic tree[19]. Polymorphism also refers to the occurrence of structurally and functionally more than two different types of individuals within the same organism. It is distinguishable from mono-morphism (having only one form)[20]. In simple words, the term poly-morphism was originally used to describe variations in shape and form that distinguish normal individuals within a species from each other[19].

Increased genetic poly-morphism connotes a high level of genetic variation, on the other hand decreased poly-morphism or monomorphism explain the stability of the organs and functions of a living organism, thus the viability of the organism depends on this stability[15]. The reason behind DNA poly-morphism among samples may be due to a single base change in genomic DNA, deletions of a priming site, insertions that render priming sites too distant to support amplification, or insertions that change the size of a DNA segment without preventing its amplification[15].

It was observed from this study, that the chi-15 primer (with 17 bases) resulted in clearer and better amplification in DNA than the EZ primer (24 bases). This further strengthens the fact that RAPD-PCR is usually performed with oligonucleotide primers that are shorter in length for better amplification results[15].

Insecticidal efficacy of *E. milii* has been reported by Bakry and Mohamed[21], Shehzadi *et al.*[22] and Okonkwo and Ohaeri[7]. A moderate level of adulticidal activity of crude leaf extracts against the urban malaria vector; *Anopheles stephensi Liston* had also been reported for *C. occidentalis*[23]. *C. occidentalis* has also been reported to suppressed wood damage by termites causing mortality of worker termites within the shortest duration of application[22]. Also, the leaf extract of *E. milii* had been reported to exhibit certain levels of insecticidal action against Diamond back moth (*Plutella xylostella*)[24]. However, the possible route of insecticidal action of these plants is yet to be studied and not much had been reported in this direction.

Even though no studies so far had reported the geno-toxicities of *E. milii* and *C. occidentalis* oils, many scientific papers had reported the geno-toxicity of essential oils extracted from different plants on their target insect pests. In a study by Afify and Negm[25], a significant increase in DNA damage was observed on different stages of fruit fly; *Bactrocera zonata* exposed to the insect growth regulators (pyriproxyfen and novaluron).

In another study, *Rhyzopertha dominica* exposed to different concentrations of volatile oils from selected plants resulted in a significant alteration in DNA concentration; the oils had clear significant effect on the DNA damage compared with the control[26]. Franzios *et al.*[27] also reported the geno-toxicity and insecticidal activities of essential oils from mint on *Drosophilia melanogaster*, the oil of *Mentha spicata* exhibited mutagenic activity on the insect pest. Geno-toxicity of newly developed phytopesticidal formulations from pongam and neem oils against *Helicorerpa armigera* (Hubna) (Lepidoptera:Noctuidae) had been reported. The study showed that the geno-toxicity effect of PONNEEM could be applied as phytopesticide for controlling the lepidopteran insect pests[28]. Geno-toxicity and mutagenic effects of diflubenzuron (DFB), an insect growth regulator on mice has also been reported. The DFB exerted geno-toxic and mutagenic effects in a dose-dependent manner[29].

These studies showed that geno-toxicity of essential oils from botanicals on insect pests is a possible mechanism of insecticidal action of these oils on their target pest. By disrupting the DNA sequence and causing mutations, gene toxicity and eventual death of the insect is in most cases guaranteed. From this study, another class of insecticides may arise; "genetic disruptor insecticides".

CONCLUSION

Essential oils from the leaves of *Euphorbia milii* and *Cassia occidentalis* exert insecticidal effects through gene toxicity in target pests. Though not currently among the classes of insecticides recognised; they may be classified as "Genetic disruptor insecticides".

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SIGNIFICANCE STATEMENT

This study discovered the insecticidal gene toxicity of essential oils from the leaves of *C. occidentalis* and *E. milii*

that can be beneficial for the development of novel insecticidal formulations of botanical origin. This study will help the researcher to uncover the critical areas of genetic toxicity of botanical insecticides that many researchers were not able to explore. Thus a new theory on "Genetic disruptor insecticides" may be arrived at.

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