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ORIGINAL ARTICLE

Chemical screening of *Azadirachta indica* seed kernel extract and its *in-vitro* selected enzyme responses in *Zonocerus variegatus* (Grasshopper)

[1]Gbadebo E. Adeleke,[1]Olaniyi T. Adedosu, [1]Peter I. Adegbola, [2]Adebola O. Akintola, [1]Itunuoluwa A. Ogunmola, [1]Opeyemi K. Omidiran
[1]Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.
[2]Department of Science Laboratory Technology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.
Corresponding Author's Email: geadeleke@lautech.edu.ng

ABSTRACT
Azadirachta indica (Neem) is a plant belonging to the Meliaceae family, with insecticidal potential applicable in management and control of insect pests of Agricultural interest. Azadirachta indica seed kernel extract (AISKE) obtained from the seed kernel, using Soxhlet extraction, was subjected to Fourier-transform (FT-IR) and ultraviolet (UV) spectroscopy as well as High performance liquid chromatography (HPLC) and Gas chromatography- flame ionization detector (GC-FID) for chemical elucidation. The in-vitro effects of AISKE on the activities of catalase, superoxide dismutase (SOD), carboxylesterase (CE) and acetylcholinesterase (AChE) in nymph and adult grasshopper, Zonocerus variegatus, were determined spectrophotometrically. The FT-IR data of AISKE reveals seven peaks having wavenumbers 1457.4 cm^{-1}, 1541.3 cm^{-1}, 1647.5 cm^{-1}, 1742.5 cm^{-1}, 2853.3 cm^{-1}, 2922.2 cm^{-1} and 3285.6 cm^{-1}, while the UV Spectrum shows maximum absorption between 207nm and 281nm wavelengths. The HPLC analysis of AISKE reveals fourteen peaks, with retention times 0.413, 1.080, 1.226, 1.554, 1.657, 1.834, 2.182, 2.424, 3.838, 4.065, 5.069, 6.261, 6.634, 7.797 minutes. The GC-FID chromatogram of AISKE shows the presence of maliacin, nimbin, nimbidin, nimbolide, quercetin, salannin, beta-sitosterol, saladucin, azadirachtin, azadiradione and valassin. The activities of catalase and SOD in nymph Z. variegatus were significantly ($p < 0.05$) reduced by AISKE and CPF, while the activities were elevated by AISKE in adult at most concentrations. The CE activity was significantly reduced by AISKE, CYP and CPF in the nymph, while the activity in adult was increased by AISKE at most concentrations. The AISKE significantly ($p < 0.05$) reduced the AChE activity in nymph and adult Z. variegatus as against CYP. The present study has revealed that Azadirachta indica seed kernel extract is rich in several chemicals which could induce insecticidal activities in nymph and adult Z. variegatus via mechanisms involving oxidative stress, reduced carboxylesterase activity and cholinergic stress.
Keywords: *Azadirachta indica, Zonocerus variegatus, chemical constituents, antioxidant enzymes, esterases*

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INTRODUCTION

Azadirachta indica (Neem) belongs to the Meliaceae family, originating from India, Bangladesh, Pakistan and Nepal, and have been widely distributed throughout the tropical and subtropical regions of the world [1-2].Neem plant has been documented to contain several chemical agents such as azadirachtin, nimbin, nimbolide, nimbiol, nimbandiol, nimbanene, n-hexacosanol, 17-hydroxyazadiradione and 7-desacetyl-7-benzylazadiradione [3-4]. Several plants have been documented to synthesize secondary metabolites with potentials against Agricultural insect pests [5-6]. Proximate and micronutrient analysis of ethanol extract of neem leaves by Amadi et al. [7] noticed the presence of carbohydrates, fibre, manganese, zinc, copper, calcium, lead, and vitamins including A, D, C, B_2, B_3. and K. The use of automated online solid-phase extraction coupled with liquid chromatography/quadrupole-time-of-flight mass spectrometry (SPE-LC-Q-TOF-MS) enhanced the isolation of five different azadirachtins (A,B, D, H and I) from leaf

extracts of Azadirachta indica [8]. A phytochemical analysis done by Nkechinyere [9] revealed the presence of alkaloids, tannins, phenols, terpenes, saponins, cardiac glycoside and sterol in an aqueous extract of *A. indica*. The presence of the several chemical constituents in neem has been responsible for its several biological properties, making the plant useful in control and management of many diseases [2]. Such properties include anti-inflammatory [10-11], antimalarial [12-13], antibacterial [14], antifungal [15-16]. Recently, hyperoside identified from neem leaf extract was reported to be potential against influenza viral strains [17]. In addition, activities of neem as a pesticide against many insect pests have been reported [18-19]. For instance, neem-based pesticides have shown efficacies against mites and nematodes [20], Cotton aphid [21] and *Oxya chinensis*[22]. The use of neem has also been reported useful for boosting the productivity of rice (*Oryza sativa*) [23]. The various pesticidal agents present in neem have been reported to interfere with certain physiologic aspects of insect pests, such as feeding ability [24] and hormone synthesis, leading to reduced pupation, adult growth, reproduction and increased mortality [9, 18, 25].

In the West and Central African regions, *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae) is an insect pest which commonly causes devastation to many food crops, resulting in serious economic losses to farmers[26].Studies have revealed that plant community structure is an important selection factor influencing distribution of grasshopper [27-29]. For instance, *O. asiaticus* has been reported to show low outbreak in areas with large plant density and height, with limited sunshine [30-31]. However, habitats with sparse and highly grazed grass vegetation experience serious outbreaks of grasshoppers with larger body size [32-33].This insect has been reported to have nutritional importance among the peoplein certain parts of Ondo[34]and Borno[35]states in Nigeria. Several studies have revealed that grasshopper is rich in nutritional entities including crude fiber, lipids, protein, essential amino acids, calcium, sodium, potassium and phosphorus [36]. This array of nutrients is the underlying factors for the nutritional significance of the insect. Furthermore, Chatsuwan et al. [37] characterized some water-soluble proteins, with antioxidant activities, from two grasshopper species, *Chondracris roseapbrunner* and *Patanga succincta*. Due to high contents of protein, insects have been incorporated as part of feed formulation used for mono-gastric animals as documented by Al-Qazzaz and Ismail [38].High activities of enzymes including glutathione-S-transferase, carboxylesterase and cyt-P450s in grasshoppers and some other insects have been suggested to be the possible underlying mechanisms of resistance of these insects against toxic secondary metabolites of plant origin [39]. The purpose of this study was to determine the various chemical compounds of *Azadirachta indica* seed kernel, and evaluate the in-vitro effects on selected antioxidant and esterase enzymes of nymph and adult grasshopper, *Zonocerus variegatus*.

MATERIAL AND METHODS

Collection of Azadirachta Indica (Neem) fruits

The fruits of *Azardiracta indica* (Neem) were collected from the premises of Ladoke Akintola University of technology Ogbomoso, Oyo State, Nigeria, in March 2018. The fruits were air-dried at the room temperature for about 3 weeks and then depulped to obtain brownish oily seeds (368.97g). The seeds were pulverized using an electric grinder, defatted with n-hexane using Soxhlet apparatus [40]. The defatted residue was further subjected to Soxhlet extraction with dichloromethane and then concentrated using a rotary evaporator45^{0}C, and later dried inside an electric Oven at37^{0}C to obtain a brownish yellow powder, used as *Azardiracta indica* seed kernel extract (AISKE) for this study.

Ultraviolet (UV) and Fourier transform Infrared (FT-IR) spectroscopic analyses

The UV spectral data of AISKE was obtained using a UV-1800 series machine (Shimadzu) at a wavelength of 340nm. The FT-IR spectral analysis was done using Agilent machine (Cary 630 FTIR), expressing the wavelength in reciprocal centimetre (cm^{-1}). The spectral data obtained was compared with literature.

High performance liquid chromatography (HPLC) analysis

An HPLC machine (Agilent) was used to analyze the AISKE at a flow rate of 0.5 mL/min with an injection volume of 20µL. The isocratic mobile phase contained a mixture of acetonitrile and methanol (80:20, v/v). The C18 (4.5 x 250 mm, 5µm) column was run at the room temperature for 8 minutes and the eluent was detected at 210nm.

Gas Chromatography-Flame Ionization Detection (GC-FID)

GC-FID identification of compounds in AISKE was performed on HP SERIES II (5890)coupled to Flame Ionization Detector (FID). Nitrogen was the carrier gas at a flow rate of 20ml/min and Hydrogen/Compressed Air as combustion gas at a flow rate of 45ml/min. The initial, injector and detector temperatures were 50^{o}C, 220^{o}C and 270^{o}C, respectively, while the oven temperature was programmed to 240^{0}C at a rate of 10^{o}C/min with a holding time of 2 min**.** Chemical constituents were identified by comparing the mass spectra with the standard available in the NIST 11 library. The area

under peak of the chromatogram was used to estimate the percentage composition of the constituents of AISKE.

Collection and homogenization of insects

Thirty each of nymph and adult grasshopper (*Zonocerus variegatus*)were collected using a sweeping net on a maize farmland in Ogbomoso, Oyo State, Nigeria. The insects were confirmed by Dr. Olayioye A. (Department of Crop and Environmental Protection, Faculty of Agricultural Science, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria). Each of the nymph and adult groups was divided into two portions (fifteen insects each) and then de-winged. One portion each of the insect groups was homogenized in phosphate buffer (pH 7.4) and centrifuged at 10000 x g for 10 minutes. The supernatant was kept at 4^0c until use. The second portion of the insects was used for evaluation of Carboxylesterase activity.

Protein determination

The protein levels of the insect homogenates were determined according to the method of Lowry et al. [41] using bovine serum albumin as standard.

Catalase activity

The method described by Aebi[42] was used to determine the Catalase activity in the insect homogenates. The assay mixture contained 4 ml of hydrogen peroxide solution (0.2 M) and 5 ml of Phosphate buffer (0.01 M, pH 7.0) in a 10ml flat bottom flask. An aliquot of 1.0 ml of properly diluted crude enzyme preparation (homogenate) was rapidly mixed with the reaction mixture by a gentle swirling motion and 0.3 ml of the extract AISKE (10, 20, 30, 40, 50, 60 and 70µg/ml) was added in separate test tubes at the room temperature. Similar varying concentrations of commercial Chlorpyrifos and Cypermethrin were separately used for comparison with the extract. The control mixture contained neither AISKE nor the commercial pesticides. An aliquot of the reaction mixture (1 ml) was blown into 2 ml of dichromate acetic acid reagent at 60 seconds intervals, monitoring the change in absorbance at 240nm for 180 seconds at an interval of 60 seconds. The enzyme activity was expressed as units/mg protein.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity in insect homogenates was determined by the method of Misra and Fridovich[43]. Briefly, an aliquot of 0.2 ml of the diluted insect homogenate was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. Then, 0.2 ml of the extract AISKE (10, 20, 30, 40, 50, 60 and 70µg/ml) followed by 0.3ml of freshly prepared 0.3M adrenaline (substrate) were added to the mixture. Similar concentrations of commercial Chlorpyrifos and Cypermethrin were used in place of the extract. The control mixture contained neither AISKE nor the commercial pesticides. The absorbance was measured spectrophotometrically at 480nm at an interval of 30 seconds for 150 seconds, and enzyme activity was expressed as Units/mg protein.

Carboxylesterase Activity

Carboxylesterase (CES) enzyme activity in insect homogenate was determined using the method of Clement and Erhardt [44] with paranitrophenyl acetate as a substrate for the enzyme. Fifteen each of nymph and adult grasshoppers were de-winged and homogenized in ice-cold Tris-HCl buffer (0.1 M Tris-HCl, pH 7.8 with 1 % Triton X-100 at 25^0C) using a tissue homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at 10,000 x g for 10mins at 4 ^{0}C. The supernatant was diluted with distilled water in ratio 1:10. The reaction mixture contained 0.5 ml of diluted supernatant and 2 ml of the working buffer (0.1 M Tris-HCl, pH 7.8, containing 2 mM EDTA at 25 ^{0}C), followed by 0.3 ml of AISKE (10, 20, 30, 40, 50, 60 and 70µg/ml). The mixture was incubated at 37 ^{0}C for 10minutes and the reaction was initiated by adding 0.2 ml of 50 mM paranitrophenyl acetate (in acetone) as a substrate. Similar concentrations each of Chlorpyrifos and Cypermethrin were used in place of extract. The control contained neither AISKE nor the commercial pesticides. Change in absorbance was measured spectrophotometrically at an interval of 60 seconds for 5 minutes at 405nm wavelength. The blank reagent contained 2 ml working buffer and 0.2 ml paranitrophenyl acetate. A paranitrophenol standard curve was prepared to evaluate the Carboxylesterase activity, which was expressed as nmol/min/ml protein.

Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity in insect homogenates was determined following the methods described by Ellman [45], and Nachmansohn and Neumann [46] with modification. The reaction mixture contained 2.6 ml phosphate buffer (0.1M, pH 7.4), 0.1 ml Ellman's reagent (DTNB), 0.4 ml insect homogenate and 0.3 ml of the extract AISKE (10, 20, 30, 40, 50, 60 and 70µg/ml). This was followed by addition of 0.1 ml of acetylthiocholine iodide solution (substrate) to the reaction mixture, to initiate the reaction. Similar concentrations of commercial Chlorpyrifos and Cypermethrin were used in place of extract. The control mixture contained neither AISKE nor the commercial pesticides. The rate of acetylcholinesterase activity was monitored spectrophotometrically by measuring the absorbance of the

product at 412nm at an interval of 2 minutes for 10 minutes. Acetylcholinesterase activity was calculated using the formula below, taking the molar extinction to be 1.361x $mmol^{-1}$ xmm^{-1}:

$$\text{AChE activity} = \frac{\text{Change in absorbance x Total reaction volume}}{\text{Time x sample volume x molar extinction}}$$

AChE activity = U/mg protein

RESULTS

Spectroscopy

The result of FT-IR analysis of AISKE (Figure 1) shows seven peaks outside the fingerprint region, with different wavenumbers including 1457.4 cm^{-1}, 1541.3 cm^{-1}, 1647.5 cm^{-1}, 1742.5 cm^{-1}, 2853.3 cm^{-1}, 2922.2 cm^{-1} and 3285.6 cm^{-1}. Figure 2 shows the UV Spectrum of AISKE with strong absorption between 207nm and 281nm wavelengths.

Chromatography

The HPLC analysis of AISKE (Figure 3) reveals fourteen peaks, with their retention times(and % peak areas) as 0.413, (1.6 %), 1.080 (37.8 %), 1.226 (34 %), 1.554 (4.2 %), 1.657 (0.7 %), 1.834 (3.0 %), 2.182 (0.1 %), 2.424 (0.8 %), 3.838 (2.6 %), 4.065 (2.1 %), 5.069 (0.1%), 6.261 (1.5 %), 6.634 (0.3 %), 7.797 (11.2 %) minutes. The GC-FID chromatogram (Figure 4) shows eleven spectral peaks, indicating the presence of 11 compounds in AISKE. The indicated compounds with their respective retention times and percentage compositions include maliacin (5.010mins, 13.9%), nimbin (7.150mins, 2.7 %), nimbidin (7.533 mins, 9.5 %), nimbolide (7.816mins, 8.6 %), quercetin (8.600mins, 12.5 %), salannin (9.200mins, 47.0 %), beta-sitosterol (11.016 mins, 1.2 %), saladucin (11.483 mins, 3.2 %), azadirachtin (11.916 mins, 0.4 %), azadiradione (12.550 mins, 0.6%) and valassin (14.566 mins, 0.4 %).

Enzyme activities

The result in table 1 shows that AISKE, CYP and CPF significantly ($p < 0.05$) reduced the in-vitro activity of catalase in the nymph grasshopper, while the activity was elevated in the adult insects, compared with the control. The in-vitro activity of superoxide dismutase in the nymph grasshopper was significantly ($p < 0.05$) lowered by both AISKE and CPF, while the activity was increased by CYP at most of the concentrations (Table 2). However, the SOD activity was significantly elevated by AISKE and CYP, while CPF has no significant effect in the adult insects, as shown in table 2. The in-vitro activity of carboxylesterase enzyme was significantly ($p < 0.05$) reduced by AISKE, CYP and CPF in nymph *Z. variegatus* at all the concentrations, while the activity was only reduced at most concentrations (Table 3). The in-vitro activity of acetylcholinesterase in the nymph grasshopper was significantly ($p < 0.05$) reduced by AISKE and CPF at all the concentrations, whereas CYP has no significant ($p > 0.05$) effect (Figure 5). However, the AChE activity was significantly ($p < 0.05$) reduced by AISKE alone, whereas both CYP and CPF have no significant ($P > 0.05$) effect in the adult grasshopper (Figure 6).

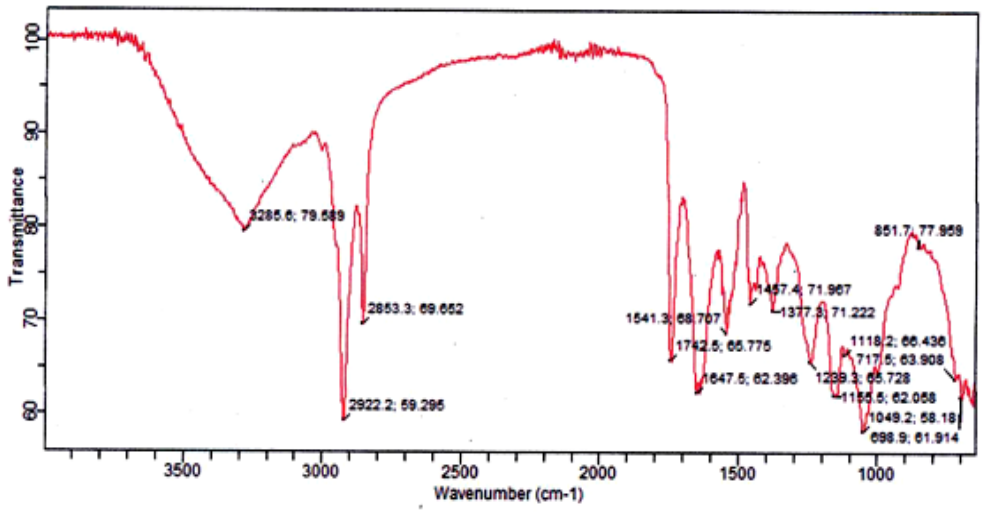

Fig. 1:Fourier Transform-Infrared (FT-IR) Spectrum of *Azardiracta indica* seed kernel extract

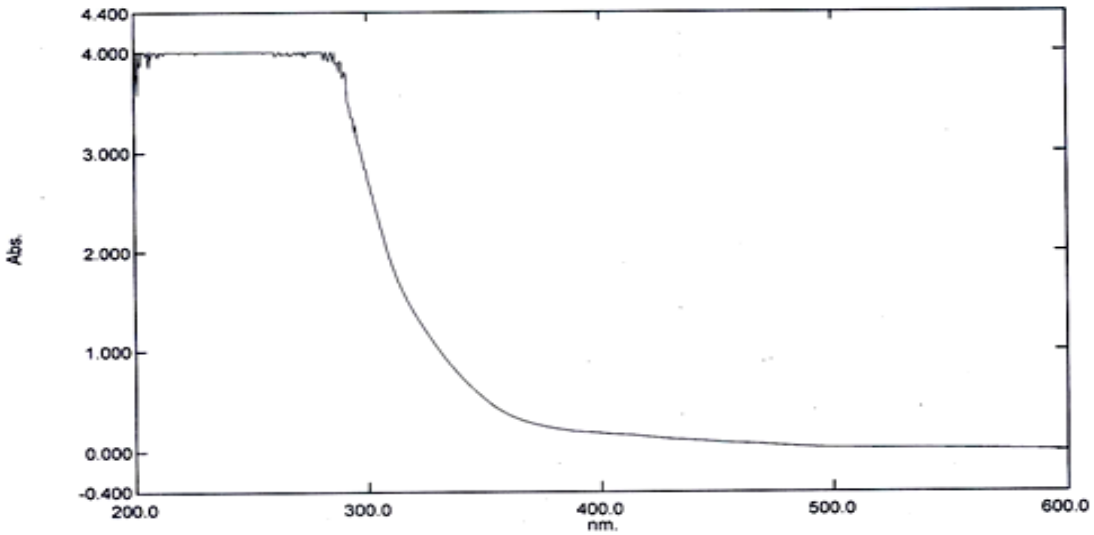

Fig. 2. Ultraviolet (UV) Spectrum of *Azardiracta indica* seed kernel extract

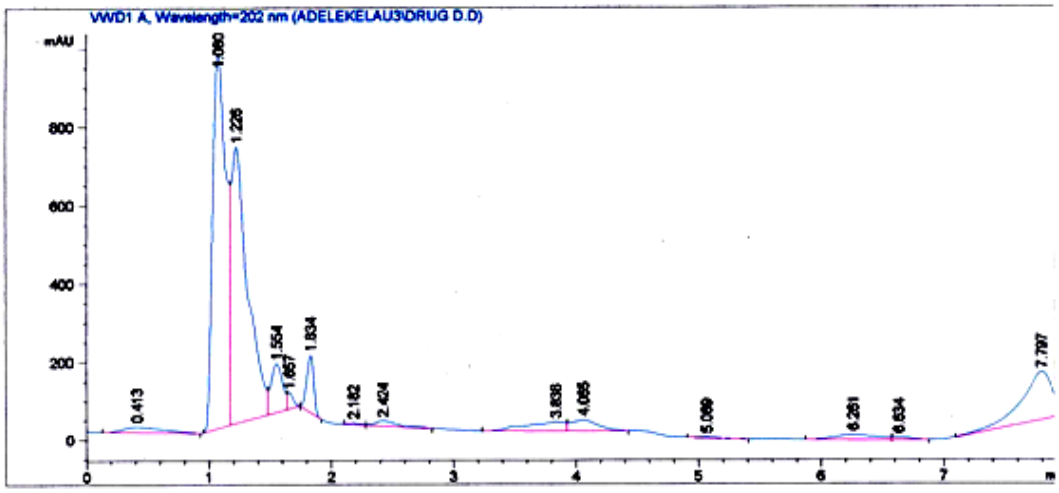

Fig.3. High Performance Liquid Chromatographic Chromatogram of *Azardiracta indica* seed kernel extract

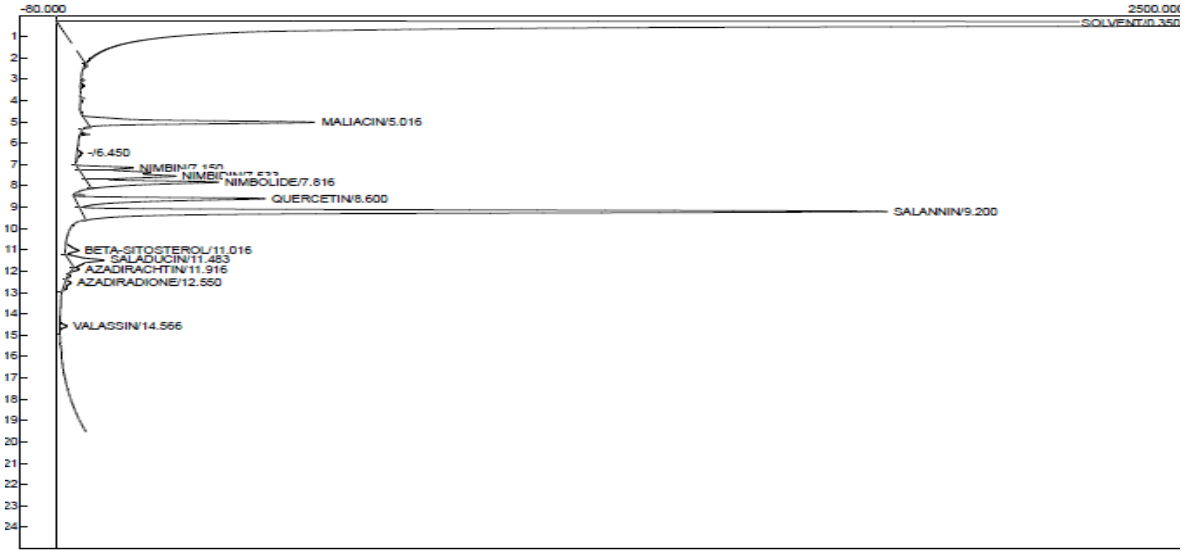

Fig. 4. Gas Chromatography – Flame Ionization Detection (GC-FID) Chromatogram of *Azardiracta indica* seed kernel extract

Table 1: Effects of *Azardiracta indica* seed kernel extract, chlorpyrifos and cypermethrin on Catalase activities in nymph and adult *Zonocerus variegatus*

Concentration (µg/ml)	Catalase activity ×10^{-3} (U/mg protein)					
	Nymph *Zonocerus variegatus*			Adult *Zonocerus variegatus*		
	AISKE	CYP	CPF	AISKE	CYP	CPF
10	0.075 ± 0.01*	0.258 ± 0.02*	0.505± 0.03*	0.288± 0.03**	0.730± 0.05**	1.086 ± 0.01**
20	0.127± 0.05*	0.679± 0.02*	0.394± 0.01*	0.679 ± 0.05**	1.144± 0.04**	0.899± 0.06**
30	0.062± 0.00*	0.242± 0.01*	0.120± 0.02*	0.655 ± 0.02**	0.561± 0.07**	0.272± 0.03**
40	0.345± 0.02*	0.854 ± 0.06	0.223± 0.02*	0.276 ± 0.01**	1.290± 0.03**	0.651± 0.07
50	0.691± 0.01*	0.095± 0.00*	0.125± 0.01*	0.425± 0.07**	1.012± 0.05**	1.102 ± 0.08**
60	0.453± 0.05*	0.855± 0.04	0.586± 0.03*	0.543±0.00**	0.516 ± 0.09**	0.816 ± 0.04**
70	0.180± 0.01*	0.430± 0.02*	0.475± 0.02*	0.619± 0.04**	0.523± 0.03**	1.108 ± 0.06**
Control	0.906 ± 0.05			0.195 ± 0.02		

Data expressed as Mean ± Standard deviation
AISKE- *Azardiracta indica* seed kernel extract, CYP- Cypermethrin, CPF- Chlorpyrifos
*- Significantly low compared with control, **- Significantly high compared with control

Table 2: Effects of *Azardiracta indica* seed kernel extract, chlorpyrifos and cypermethrin on Superoxide dismutase activities in nymph and adult *Zonocerus variegatus*

Concentration (µg/ml)	SOD activity ×10^{-5} (U/mg protein)					
	Nymph *Zonocerus variegatus*			Adult *Zonocerus variegatus*		
	AISKE	CYP	CPF	AISKE	CYP	CPF
10	1.50± 0.04*	1.50± 0.07*	3.52± 0.51**	6.42± 0.89**	1.10± 0.09	1.37± 0.01
20	1.54± 0.12*	3.80± 0.51**	1.32± 0.08*	3.20± 0.53**	1.56 ± 0.60**	1.46 ± 0.03
30	1.98± 0.20*	2.81± 0.09	2.42±0.06	3.58 ± 0.09**	2.38± 0.41**	1.28 ± 0.10
40	1.54± 0.03*	2.86± 0.06	1.76± 0.32*	5.32± 0.70**	1.38± 0.03	1.38± 0.01
50	2.20± 0.18	3.08± 0.21**	1.10± 0.05*	4.95± 0.77**	1.65± 0.05**	1.28± 0.00
60	4.40± 0.41**	2.86± 0.30	1.98± 0.09*	6.88 ± 0.93**	1.28± 0.09	1.28± 0.05
70	4.21± 0.66**	3.08± 0.07**	3.74± 0.33**	7.88 ± 1.02**	1.28± 0.01	2.21 ± 0.09**
Control	2.42± 0.10			1.22 ± 0.08		

Data expressed as Mean ± Standard deviation
AISKE- *Azardiracta indica* seed kernel extract, CYP- Cypermethrin, CPF- Chlorpyrifos
*- Significantly low compared with control, **- Significantly high compared with control

Table 3: Effects of *Azardiracta indica* seed kernel extract, chlorpyrifos and cypermethrin on Carboxylesterase activities in nymph and adult *Zonocerus variegatus*

Concs. (µg/ml)	**Carboxylesterase activity ×10^{-5} (nmol/min/ml protein)**					
	Nymph *Zonocerus variegatus*			**Adult *Zonocerus variegatus***		
	AISKE	CYP	CPF	AISKE	CYP	CPF
10	0.317± 0.03*	0.304± 0.01*	0.289± 0.04*	2.594± 0.41**	0.069± 0.00*	0.356± 0.02**
20	0.166± 0.02*	0.166± 0.05*	0.355± 0.01*	1.504± 0.43**	0.166 ± 0.01*	0.483± 0.08**
30	0.289± 0.00*	0.138± 0.03*	0.207± 0.03*	2.539 ± 0.04**	0.262± 0.00	0.289 ± 0.04
40	0.314± 0.04*	0.372± 0.02*	0.289± 0.03*	1.698± 0.09*	0.368 ± 0.02**	0.283 ± 0.03
50	0.317± 0.01*	0.289± 0.04*	0.255± 0.05*	1.836± 0.04*	0.110 ± 0.00	0.140 ± 0.01*
60	0.138± 0.02*	0.221± 0.00*	0.151± 0.03*	1.711± 0.11**	0.179 ± 0.02*	0.276 ± 0.03
70	0.483± 0.01*	0.289± 0.03*	0.255± 0.01*	1.532± 0..04**	0.772 ± 0.20**	0.345 ± 0.01**
Control	0.648 ± 0.05			0.289± 0.03		

Data expressed as Mean ± Standard deviation
AISKE- *Azardiracta indica* seed kernel extract, CYP- Cypermethrin, CPF- Chlorpyrifos
*- Significantly low compared with control, **- Significantly high compared with control

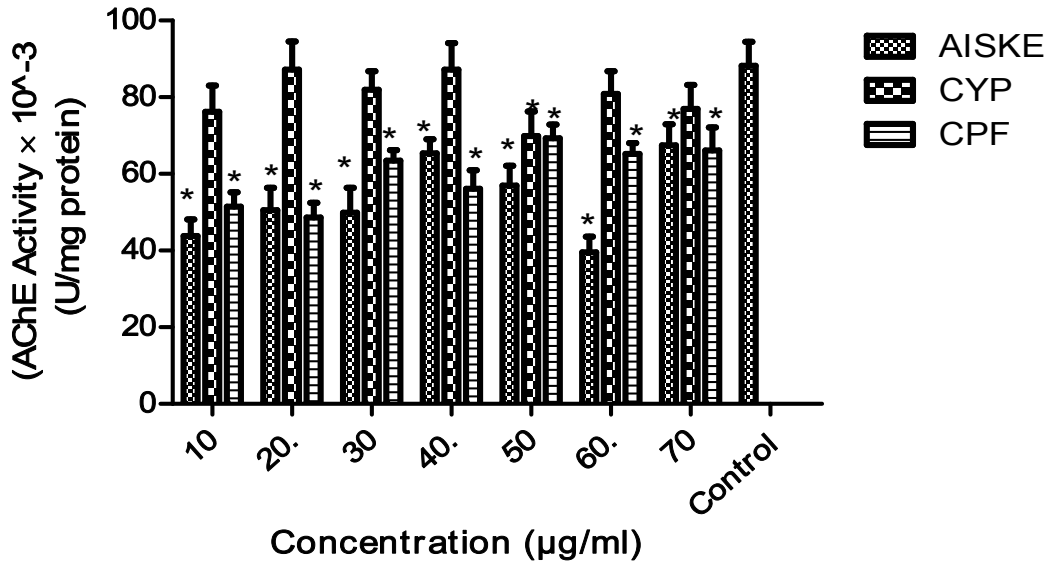

Fig. 5: Effects of *Azardiracta indica* seed kernel extract, chlorpyrifos and cypermethrin on Acetylcholinesterase activity in nymph *Zonocerus variegatus*
Data expressed as Mean ± Standard deviation (values obtained in duplicates)
AISKE- *Azardiracta indica* seed kernel extract, CYP- Cypermethrin, CPF- Chlorpyrifos
*- Significantly low compared with control

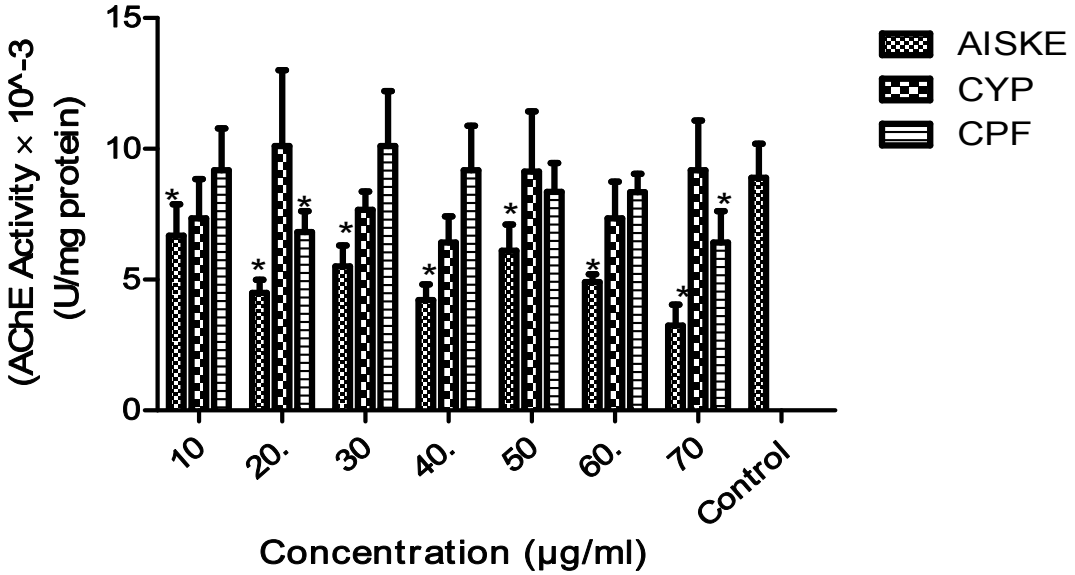

Fig. 6: Effects of *Azardiracta indica* seed kernel extract, chlorpyrifos and cypermethrin on Acetylcholinesterase activity in adult *Zonocerus variegatus*
Data expressed as Mean ± Standard deviation (values obtained in duplicates)
AISKE- *Azardiracta indica* seed kernel extract, CYP- Cypermethrin, CPF- Chlorpyrifos
*- Significantly low compared with control ($p < 0.05$)

DISCUSSION

Azadirachta indica(neem) is known to contain more than 300 chemical agents most of which are terpenoid compounds present in the leaves, stem, fruits, seeds and root bark of the plant [47]. This plant has been well documented to possess substantial pesticidal and human medicinal potentials [48]. The present study examined the chemical constituents of *Azadirachta indica* seed kernel extract (AISKE), and the in-vitro enzymatic responses in nymph and adult grasshopper, Zonocerus variegatus. Two commercial pesticides, cypermethrin (CYP) and chlorpyrifos (CPF) were used as reference compounds.

The present study investigated the possible functional groups in the AISKE using FT-IR and UV spectroscopic techniques. The FT-IR profile shows the presence of functional groups including C-C stretch in aromatic ring (1457.4 cm^{-1} and 1541.3 cm^{-1}), ethylenic double bond (1647.5 cm^{-1}), aliphatic carbonyl stretch of carboxylic acid or aromatic C-H bond (1742.5 cm^{-1}),aliphatic C-H bond (2853.3 cm^{-1}), methyl bending (2922.2 cm^{-1}) and aliphatic hydroxyl group of carboxylic acid or alcohol (3285.6 cm-1) [49-51]. The datum from the UV Spectroscopy of AISKE shows maximum absorption between 207nm and 281nm wavelengths. This finding is in agreement to that of a study by Soni et al. [52]who reported that a UV analysis of neem leaf extract showed a maximum absorption at around 200.00nm.

The analysis of AISKE using HPLC technique showed fourteen peaks, with the retention times as 0.413, 1.080, 1.226, 1.554, 1.657, 1.834, 2.182, 2.424, 3.838, 4.065, 5.069, 6.261, 6.634 and 7.797minutes. A quantitative HPLC study by Soni et al. [52] has indicated that azadirachtin content was up to 73.62% in a methanol extract of neem leaves. The GC-FID chromatogram of the AISKE shows the presence of eleven compounds with their percentage compositions including maliacin (13.9 %), nimbin (2.7 %), nimbidin (9.5 %), nimbolide (8.6 %), quercetin (12.5 %), salannin (47.0 %), beta-sitosterol (1.2 %), saladucin (3.2 %), azadirachtin (0.4 %), azadiradione (0.6%) and valassin (0.4 %). Different analyses of neem leaf extracts showed the presence of hydrocarbons, phenolics, terpenoids, alkaloids and glycosides, saponins, phenolics and steroids [9, 54]. HPLC technique has been explored in monitoring the quality of commercial neem products [53]. An HPLC study by Vergallo et al. [55] revealed the presence of flavonoids including rutin, quercitrin and isoquercitrin, while a GC-FID analysis of neem extract showed the presence of epicatechin, tannins, phytate, rutin, oxalate, anthocyanin and sparteine [7]. The insecticidal and pharmacological activities of neem have been suggested to be due to the presence of nimbin and azadirachtin [56].

Investigation of the effects of AISKE, CYP and CPF on the in-vitro activity of catalase enzyme showed that the three agents significantly reduced the activity in the nymph grasshopper, whereas the activity was increased in adult grasshopper compared with control treatment. The in-vitro activity of SOD enzyme was significantly reduced by AISKE and CPF, but increased by CYP in the nymph grasshopper, at most of the concentrations. The reduction in the activities of both catalase and SOD in the nymph suggests accumulation of superoxide anion in the system, constituting oxidative stress. However, in the adult Z. variegatus, in-vitro SOD activity was increased by AISKE and CYP, while CPF has no significant effect. The increased activities of both SOD and catalase in the adult grasshopper is an indication that superoxide anion, and subsequently oxidative stress, was generated by the extract. An investigation by Adeleke et al. [57] noticed a high in-vitro activity of catalase in adult *Zonocerus variegatus* grasshopper on treatment with castor seed kernel extract. Extracts of neem kernel and leaves were reported to have a protective potential on a cassava field against grasshopper, *Zonocerus variegatus* [58]. The possible generation of oxidative stress as noticed in the present study may be responsible for such protective ability of the neem extract. Compounds such as rutin, quercetin and azadirachtin, which have been reported to be present in neem, have been documented to induce oxidative stress in a grasshopper, *Calliptamus abbreviatus*, by increasing the activities of antioxidant enzymes such as, superoxide dismutase and catalase [59]. Furthermore, a study by Nkechinyere [9] revealed the insecticidal potential of both powdered and water extract of neem against bean weevils.

Treatment with AISKE, CYP and CPF was observed to significantly lower the in-vitro activity of carboxylesterase enzyme in nymph *Z. variegatus* at all the concentrations, while the activity in adult grasshopper was significantly reduced by ASIKE alone at most of the concentrations used in the investigation. An investigation by Wang et al. [59] revealed that rutin, quercetin and azadirachtin, compounds reported to be present in *Azadirachta indica*, could substantially increase the activity of carboxylesterase enzyme in grasshopper, *Calliptamus abbreviates*.One of our recent investigations revealed an induced in-vitro activity of carboxylesterase enzyme in adult *Z. variegatus* grasshopper when exposed to an extract of castor seed kernel [57]. Several studies have shown that induction of resistance in insects is associated with activation of enzymes including carboxylesterase, glutathione-S-transferase, superoxide dismutase, catalase and peroxidase [60-62]. The reduction in activity of CE enzyme being observed in the present study has indicated that neem extract could potentially induce susceptibility of *Z. variegatus* to insecticides.

Acetylcholinesterase is an enzyme involved in the decomposition of acetylcholine to acetate and choline in the synaptic cleft or neuromuscular junctions [63-64]. The in-vitro activity of AChE was reduced by AISKE and CPF at all the concentrations, whereas no significant effect was noticed with CYP in the nymph grasshopper. However, in the adult *Z. variegatus*, in-vitro activity of AChE was significantly reduced by AISKE, while both CYP and CPF have no significant effect. The effect of azadirachtin on the AChE activity in *Nilaparvata lungens* has been studied by Senthil-Nathan et al. [65]. Studies by Sengottayan et al. [66]

and Sami et al. [67] examined also the effect of crude neem powder and azadirachtin on the activity of acetylcholinesterase in red flour beetle, *Tribolium castaneum*, and noticed a decrease in the enzyme activity. A study by Wang et al. [68] reported that neem could potentially induce some insecticidal action in *Spodoptera frugiperda*. Natural insecticides have been reported to inhibit acetylcholinesterase and antioxidant enzymes in insects as documented by Adeleke et al. [57] and Singh et al [69]. Azadirachtin has been suggested to bind at various sites on AChE molecule instead of binding to the active site of the enzyme [67, 70]. The reduction in the activity of AChE by the neem extract in the present study may have resulted from alteration of the genes or pathways responsible for the synthesis the enzyme. The reduced activity of AChE in both nymph and adult grasshopper by the extract suggests possible accumulation of acetylcholine at the neuromuscular junctions, leading to cholinergic stress, detrimental to the insect [71].

CONCLUSION

The findings from the present study have indicated that *Azadirachta indica* seed kernel extract is rich in several chemical compounds which could induce damage in grasshopper, *Zonocerus variegatus* by pathways involving antioxidant imbalance, impaired carboxylesterase activity and cholinergic stress.

CONFLICT OF INTEREST

The authors declare that there was no conflict of interest.

ABBREVIATIONS

AISKE: Azadirachta indica seed kernel extract, CYP: cypermethrin, CPF: chlorpyrifos, CE: carboxylesterase, AChE: acetylcholinesterase.

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