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Evaluation of safety of *Azdirachta indica* seed oil on Albino rat through haematological and some antioxidants by the rotatable central composite design (RCCD) of the response surface methodology (RSM)

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Abstract**—** *Raw Azdirachta indica seed oil is gradually adopted by local farmers as a protector of stored grains and legumes against the common insects, but with some reservations on its safety. The study investigated the safety A. indica on mammalian objects using haematological and antioxidant indixes. A. indica seed was obtained from the trees in the same location in Utu Ikot Ukpong, Essien Udim Local Government Area, Nigeria. The Albino rats were obtained from the Animal House of Biochemistry Department of the University of Calabar, Nigeria. The animals were treated with varied A. indica seed oil at varied concentrations, periods of exposure and age. Haematological analyses revealed that models of packed cell volume (PCV), red blood cell (RBC), white cell count (WCC) and hemoglobin content (Hb) were not significant. Catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) were also not significant ($p>0.05$). Linearity coefficients of the models however appeared to be significant ($R^2 \leq 0.8000$). The mathematical and pictorial models showed slight influence of the test substance on the parameters. The investigation revealed that A. indica seed oil showed no adverse effect on the haematological and antioxidant profile of the animal models at the level of administration, the slight statistical significance could be attributed to other experimental values. A. indica oil seems to be safe on mammalian subjects. More work is recommended on the topic using higher levels of the plant materials at a longer period of exposure.*

Keywords**—** ***Azdirachta indica, response surface methodology, rotatable central composite design, haematological indices, enzymes.***

I. INTRODUCTION

Insecticides are organic and inorganic substances which are used to kill or inactivate insects to check damaging effects of their activities, application is useful in agriculture, medicine, industry and in the homes (Coats, 1999). Deployment of synthetic chemical insecticides in agriculture is claimed to be a major factor behind the increase in the 21st-Century's agricultural productivity (Gerald, 2006). Long-term applications of synthetic insecticides have resulted in residues accumulating in different environmental components of water, food, air and soil (Lorendo-Pino *et al*., 2014). In order to circumvent their ill effects, ideal insecticides which are not toxic and more environmentally friendly are sought from the plant kingdom (Ojimelukwe, 2008; Lorendo-Pino *et al*., 2014).

. Botanical insecticides in integrated insect management program have greatly reduced environmental pollution, toxicity to users, reduce cost of medical treatment of insecticide related diseases. Safety to farmers and consumers of treated foods has also been demonstrated. Efforts to develop and apply botanicals as alternatives to the synthetic chemical insecticides have been reported by many researchers, for instance, (Ojimelukwe and Adler, (1999) reported on the potential of zimtaldehyde, 4-Allyl-anisol, linalool, terpinol in the control of confused flour beetle, *Tribolium confusum*. Okpereke and Bunmi, (2006), Okpereke, (2007) reported effectiveness of *Xylopia aethopia* products against the common maize weevil, Huang and Ho, (1998) demonstrated effectiveness of *Piper guneense* powder against *Tribolium castaneum* (Herbst), *Sitophilus*

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zeamais Motsch. Mikhaiel, (2011) showed that some volatile oils of plants could protect packages of irradiated wheat flour against *Ephestia kuheniella* and *Tribolium castaneum*. Insecticidal activities of aromatic plant extracts and essential oils against *Sitophilus oryzae* and *Callosobruchus chinensis* had been demonstrated by (El-Wakil, 2013). Commercial *neem* seed oil is currently in use in the integrated insect management programme (Chaudhary, 2017). *A. indica* is native to Asia, but has now naturalized in Nigeria. *A. indica* is an important item in the traditional medicinal practice in the area of study, according to Pallav *et al*., (2014) every part of the plant is claimed to posses some medicinal and insecticidal properties, but the seed is grossly underutilized (Schmutterer, 1990). Igbokwe (2017) opined that it is the presence of some biologically active principles like azdirachtin meliacin, gedunin, salanin, nimbin, valasin and their derivatives that confers the anti-inflammatory, anti-hyper glycemic, anti-ulcers, anti-malarial, anti-fungal, anti-bacterial, anti-oxidant, anti-mutargenic, and immunomodulatory properties of *neem* seed oil in mammalian models including anti-feedant, repellent, effect in insects (Ndodo, 2013).

Oil of *A. indica* seed is sprayed on grains/legumes for protection against the damaging effect of the common insects. Protected products are either used for food or feed or replanting. Oily nature of *A. indica* seed oil prevents its total removal from the stored product and therefore may contain residue at levels high enough to elicit health problems in susceptible individuals of the population, the oil may become rancid and develop mycotoxin (Yin *et al*., 2003), which is a known carcinogen especially at high level of accumulation. As a new product, *A. indica* seed oil deserves safety evaluation on mammalian models before injected into the market for human consumption.

In this study, the Rotatable Central Composite Design (RCCD) of the Response Surface Methodology (RSM) was employed to test the linear, combined and quadratic effects of graded levels of age, dose and exposure time on haematological indices and antioxidants of blood of Albino rats. Response surface methodology tests effect of many variables on parameters of interest at the same time, therefore the method is robust, reliable and predictable, the design saves time and cost (Kwak *et al*., 2015). According to Kanu *et al*., (2016), chronic diseases affect blood cells adversely, therefore haematological data provides the most important information in the determination of biochemical and physiological state of animal models (Jorum *et al*, 2016). Damage mediated by free radicals results in high rate of disruption of membrane fluidity, protein denaturation, lipid peroxidation, oxidative deoxyribonucleic acid (DNA) and alteration of platelet functions, the molecular damages have been linked with diabetes, cancers, inflammation, aging and atherosclerosis (Kanu *et al*., 2016).

From literature, many works have been done on the safety or toxicity of *A. indica* seed oil products but to the best of our knowledge, few data is available on the effect of *A. indica* seed oil on haematological and antioxidant levels using the rotatable central composite design of the response surface methodology.

Therefore the work evaluated the safety of *A. indica* seed oil through levels of haematological and enzyme indices of Albino rats using the RCCD of the RSM. Data from the work is expected to enhance food security and low cost of insect pest management, cultivation and utilization of *neem* seed can provide employment.

II. MATERIALS AND METHODS

Forty-five (45) Albino rats of 14 - 40 days were obtained from the animal house of the Department of Biochemistry, University of Calabar, Cross River State, Nigeria. The commercial rodent meal was bought from Pfizer, Lagos. *A. indica* seed was obtained from Essien Udim Local Government Area in Akwa Ibom State. All reagents used for the study were of analar grade, double distilled water was used for their preparation.

Preparation of *A. indica* seed powder

Preparation of *A. indica* seed was sorted, decorted, washed and dried in the sun for 24 hours. It was then dried in hot air oven at 50^{o}C for 24 hours to a moisture content of about 12%. The dried seed was milled to pass through 0.2-0.5 mm sieve (Fritsch GMBH, Germany BRD-6580), packaged and stored in moisture proof container for subsequent use.

Preparation of *A. indica* seed oil

Preparation of *A. indica* seed oil was carried out according to the method adopted by Okigbo, (2008). Five hundred (500) grams of *A. indica* seed powder was soaked in 1000ml of 95% ethanol in a round-bottomed flask and allowed to stand at room temperature for 24 hours. The filtrate was evaporated with a rotary evaporator (Buch Laboratorriums Technic, AG CH - 9230), to free sample from the solvent leaving a dark slurry as the oil.

Animal models

The animal models were handled according to CLSI (2000). They were stabilized for 5 days with water and fed *ad lib*. A dose of ampicillin was administered through the drinking water to check possible infection, they were housed in separate cages, the housing guaranteed adequate lighting, ventilation and standard humidity.

Administration of *A. indica* seed oil

Treatment of Albino rats was done according to the method of (Adewale *et al*., (2014) with slight

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modification to fit the experimental design. Animals with similar blood and antioxidants parameters were randomly assigned to the experimental units; (15 laboratory animal cages) (Table 1, 2), watered with commercial table water (ARSAN Water) and fed standard rodent diet (Pfitzer, Lagos) *ad lib* between 0 to 40 days. Graded levels of *A. indica* oil was administered orally at concentrations according to the experimental design in Table 1. At the end of each experimental run, animals in the experimental unit were anesthetized with diethyl ether, blood samples were taken from the heart and preserved with EDTA to prevent clotting. Samples were centrifuged at 3,000 rpm for 10 min to obtain the plasma.

Estimation of haemoglobin and antioxidant assay of blood of treated Albino rats

Pack cell volume (PVC), red blood cell (RBC), white cell count (WCC), haemoglobin content (HB) according to the method of Adebayo *et al*. (2005) using automated haematology analizer (ERMA) (Model PCE 210 Japan). Catalase was assayed spectrophotometrically according to the method of Aebi *et al*., (1983), estimation of superoxide dismutse was done according to the method of Aebi *et al*., (1983), estimation of reduced glutathione was determined by the method of Kwak *et al*., (2016).

Background of experimental design and statistical analysis

RSM enables evaluation of effects of multiple independent variables singly or in combination simultaneously on dependent variables (Meyers *et al*., 2002). Design Expert Version 10 (Statease Inc., Minneapolis, MN, USA) was deployed for the design and analysis of the experimental data. Age of animal model, dose of the test substance, and exposure time of the animal model to the test substance were chosen from experimental experience (Table 1). The Rotatable Central Composite Design assumes equation 1.

$$Y_i = \boldsymbol{\beta}_\theta = \sum_{l=1}^{3} \beta i Xi + \sum_{l=1}^{3} \beta ii \; x^2 i + \sum_{j=i+1}^{3} \beta ij \; Xi \; Xj + e \quad \ldots \quad (1)$$

where Y_i is any response, β_θ = intercept, β_i = first order model coefficients, β_{ii} = quadratic coefficient for the i^{th} variable, β_{ij} = interaction coefficients for the interaction variables i and j, X_i = any independent variable. Second order coefficient was generated by regression analysis with backward elimination. Responses were first fitted for the factors by the coefficients of determination, R^2.

Table.1: Experimental design and levels of independents variables

Variable	Units	-α	-1	0	1	+ α
Age	Days	8.6152	14	13.5	40	45.3848
Dose	mg/100g	5.8578	10	20	30	34.1421
Time of exposure	Days	-4.1421	0	10	20	24.1421

α = 1.4142 for RCCD

III. RESULTS

Levels of the independent variables, age (X_1) 14-40 days, dose (X_2) 10-30 mg, and exposure time (X_3) 0-20 days were assigned to the experiment by experience and done according to RCCD in Table 1. Effect of the variables on packed cell volume (PCV), red blood cell (RBC), white cell count (WCC), haemoglobin content (Hb), catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) of the blood samples of the animal models are presented in Table 2. From the table, PCV ranged from 20-39%, RBC ranged fron 7.92 to 9.92x106/ml, WCC ranged from 7110-8221x10^6/ml, Hb ranged from 12.01-13.17g/100g and means of 33.38%, 8.86x10^6/ml, 7745.23 x10^6/ml, 12.69 g/100ml respectively. CAT, SOD and GSH ranged from 328.9-954.90 IU/gHb, 5478-6917 IU/gHb and 2.35-3.99 IU/gHb and mean of 441 IU/gHb, 6164 IU/gHb, and 3.50 IU/gHb respectively. Standard deviations of the parameters were 4.59%, 0.52%, 454.20%, 12.69%, 166.90%, 416.07% and 0.493% respectively. The low standard deviation of the parameters indicated low variability in the observation as can be seen in Table 2 except SOD and GSH which showed higher values than the control. Run 14 was not included in the calculation due the extraordinarily low values.

Table.2: Experimental runs, results and standard values of the parameters

Run	Age Days	Dose mg/100g	Exposure Days	PCV %	RBC x10^6/ml	WCC x10^6/ml	Hb g/100g	CAT IU/gHb	SOD IU/gHb	GSH µM/gHb
12	27	20	10	39	9.52	7830	13.17	394.1	5981	3.53
5	8	20	10	38	9.21	8001	12.01	593.5	6098	2.35
10	27	24	24	37	9.51	7911	13.21	394.9	6182	3.75
6	45	20	10	35	9.22	7851	12.91	374.7	5519	3.11
2	40	10	20	37	9.11	7907	13.22	392.3	6917	3.99

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4	14	10	0	30	9.25	8110	12.10	389.9	6391	3.34
8	27	34	10	32	9.22	7110	13.00	389.6	6551	3.91
13	27	34	10	35	9.17	8113	12.21	954.9	6276	3.56
7	27	6	10	32	8.97	7917	13.11	377.8	6088	3.98
3	14	30	30	20	9.04	8211	12.01	328.9	5617	3.90
14	**27**	**20**	**34**	**9**	**8.90**	**8221**	**12.01**	**328.9**	**5617**	**3.21**
1	40	30	0	35	7.92	7117	12.22	389.7	6315	2.66
9	27	20	-4	35	8.21	8220	13.00	399.2	6277	3.77
11	27	20	10	34	8.22	7110	13.00	349.9	6616	3.65
15	27	20	10	34	8.22	7110	13.00	398.3	5478	3.55

PVC = packed cell volume, RBC = red blood count, WCC = white cell count, Hb = haemoglobin, CAT = catalase value, GSH = superoxide dismutase, GSH = glutathione level.

Table.3: Analysis of variance (ANOVA) of the experimental data

	PVC	RBC	WCC	Hb	CAT	SOD	GSH
Model	0.5112	0.4634	0.5540	0.1780	N/A	0.3382	0.0050
Linear effect							
Age (X_1)	04299	0.9882	0.8074	0.0684	N/A	0.3060	0.0198
Dose (X_2)	1.0000	0.7135	0.2253	0.3545	N/A	0.4073	0.7687
Exposure time (X_3)	0.5919	0.0992	0.6191	0.3691	N/A	0.8621	0.9327
Interaction							
Age x Dose (X_1X_2)	0.6690	0.5341	0.3068	N/A	N/A	0.9725	0.0080
Age x Exposure (X_1X_3)	0.7863	0.2310	0.7143	N/A	N/A	0.0914	0.1968
Dose x Exposre (X_2X_3)	0.1403	0.3674	0.3953	N/A	N/A	0.0898	0.0141
Quadratic							
Age^2 (X_{11}^2)	0.4649	0.7105	0.8688	N/A	N/A	N/A	0.0011
Dose^2 (X_{22}^2)	0.1427	0.9798	0.2788	N/A	N/A	N/A	0.0120
Exposure^2 (X_{33}^2)	0.6318	0.5203	0.5484	N/A	N/A	N/A	0.0755
R^2	0.6519	0.6741	0.6317	0.3488	0.0000	0.5030	0.9611
Adjusted R^2	0.0254	0.0874	-0.0313	0.1712	0.0000	0.1302	0.8911
Mean	33.38	8.86	7745.23	12.69	441.04	6164	3.50
Lack of Fit	0.6907	0.5066	0.9441	0.6786	0.9996	0.6797	0.9539

Table 2 shows random order of the experimental runs, coeficient of estimate, coefficient of determination, and control values of the parameters. Table 3 shows statistic of analysis of variance (ANOVA) of the experimental data. The model for PCV was not significant ($p>0.05$). The coefficient of determination, R^2=0.6519, and the Adjusted coefficient of determination, Adj. R^2= 0.0524 confirmed a weak statistical confidence of the model. Linear, interaction and quadratic terms of age, dose, and exposure time were not significant ($p>0.05$). Variation of the parameter is represented by Fig. 1 and equation 1. According to Fig. 1 and Equation 2, the value of the parameters deviated very slightly with age, dose and exposure time. According to the equation, terms of age, dose and exposure time were synergistic, while interactive terms showed antagonistic effects, quadratic terms were very low but postive.

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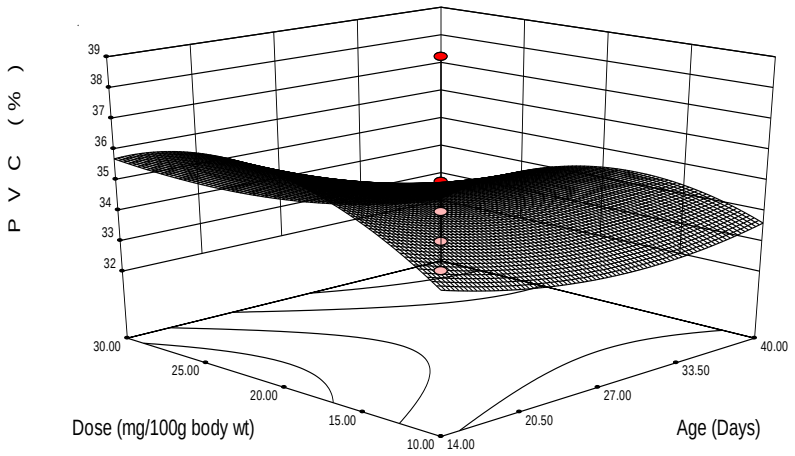

Fig.1: Response surface-contour plot of dose of test substance and age of model animal on pack cell volume of model animal blood

$$\mathbf{PVC} = 23.10 - 0.146(X_1) + 1.09(X_2) + 0.70(X_3) - 6.099(X_1 X_2) - 0.03(X_2 X_3) \quad \ldots \quad (2)$$

The model of RBC was not significant ($p>0.05$), the coefficient of determination, R^2 =0.6741 indicated weak confidence on the model. The linear, interaction, and quadratic terms of the model were not significant ($p>0.05$) except time (X_3) with which appeared to be significant p=0.0992. The model is represented by Fig. 2 which shows that red blood cells increased with age of the animal model and not as a function of dose and time (not shown here) and was corroborated by Equation 3.

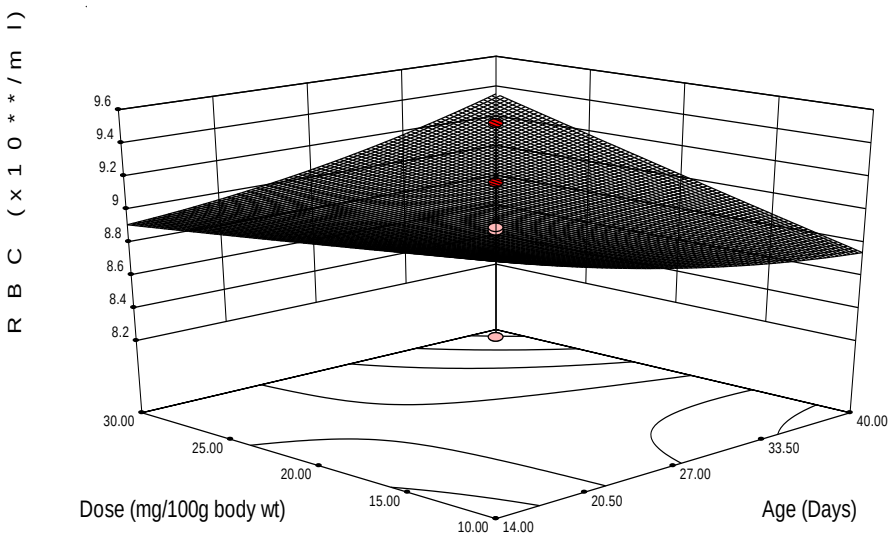

Fig.2: Response surface-contour plot of dose of test substance and age of model animals on red blood cell content

$$\mathbf{RBC} = 10.10 - 0.09(X_1) - 0.07(X_2) - 0.07(X_3) \quad \ldots \quad (3)$$

The model for WCC was not significant ($p>0.05$). The coefficient of determination (R^2 =0.6317), the linear, interaction, and quadratic terms of the model were not significant ($p>0.05$), with mean value of 34.53×10^6m/l. The model is represented by Fig. 3 and Equation 3.

$$WCC = 7088 + 47.44(X_1) + 85.47(X_2) - 60.79(X_3) - 2.55(X_1X_2) - 0.87(X_1X_3) + 2.71(X_2X_3) + 0.15(X_{11}^2) - 1.80(X_{22}^2) + 0.95(X_{33}^2) \quad .. \quad (4)$$

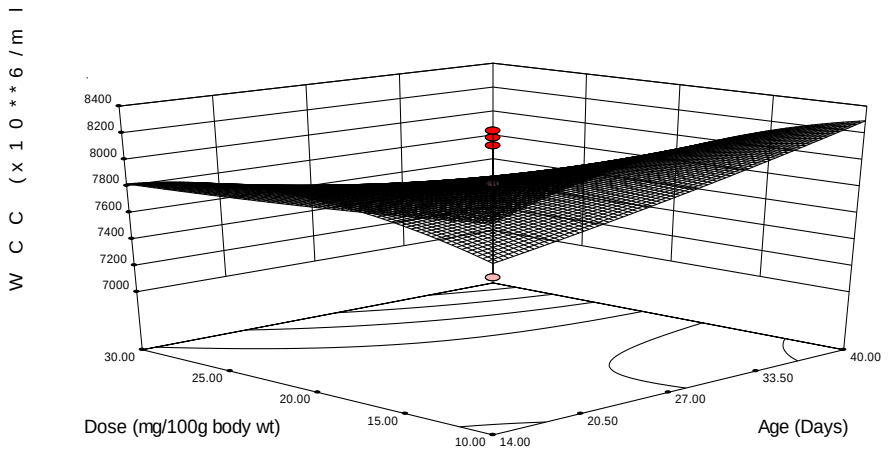

Fig.3: Response surface-contour plot of dose of test substance and age of model animals on white cell count.

WCC $=70.88 - 64.150(X_1) + 85.47(X_2) - 60.80(X_3) - 2.55(X_1X_2) - 0.87(X_1X_3) + 2.71(X_2X_3) + 0.16(X_{11}^2) - 1.80(X_{22}^2) + 0.96\ (X_{33}^2)$ ... (4)

The model of Hb was not significant ($p>0.05$), the coefficient of determination (R^2 =0.3488) and Adj. R^2= 0.1712 confirmed weakness of prediction of the model. The linear, interaction, and quadratic terms of the model were not significant ($p>0.05$), except age which appeared to be significant, p=0.0684. The model for Hb estimation is given in Equation 5. The model of CAT was not significant and could not be calculated, the linear, interaction, and quadratic terms of the model could not be calculated due to low range of the data (N/A), while the mean value was (420.37 IU/gHb). The model is represented by Equation 5. The model for SOD was not significant ($p>0.05$), the coefficient of determination R^2= 0.5030 confirmed lack of confidence of the model. The linear, interaction and quadratic terms of the model were not significant ($p>0.05$). The model was represented by Equation 6.

SOD $= 6363 - 1.56(X_1) - 56.24 + 65.49(X_2) - 6.661(X_3) 0.0724(X_1X_2) + 3.9053(X_1X_2) -5.10\ (X_1X_3)$

The model for GSH was not significant ($p>0.05$). The coefficient of determination (R^2= 0.9611, Adj R^2= 0.8911) showed some confidence on the model. The linear, interaction and quadratic terms of age, dose and exposure time were not significant ($p>0.05$). The model is represented by equation 7 and a response surface plot (not shown).

$GSH = 1.54 + 0.205\ (X_1) - 0.032\ (X_2) - 0.145(X_3)$ ...

IV. DISCUSSION

Many people in the local community where the study was undertaken believed that the oil of *A. indica* seed at the level they apply to stored grains and legumes could retain the oil residues at a levels high enough to induce toxicity in mammalian system especially if consumed at high doses at an extended period of time. It is well known that the oil contains some bioactive components like alkaloids, polyphenols and flavonoids which have convinced proofs of influence on good biochemical processes in mammals. This is why they are used for as 'drugs' and nutrients (Wararut *et al*., 2012). In spite of proven assurances of safety of some botanical oils in nutrition and health, information on every plant material to be introduced into the food chain must be made available (Ojimelukwe *et al*., 2008) to the consumer population. Fear of toxicity of *A. indica* seed oil could originate from previous reports of toxicity of *Galega officialis, Agentum conyzoles* L., *Calendula officicinalis, Cedrus deodara* (Adebayo, *et al*., 2010). In our work, the major haematological parameters and organic enzymes were provided on the health status of the animal models, this method is used as a preliminary or decisive step in medical diagnosis before treatment (Wasser *et al*., 2004). Mean percentage PCV of the blood samples of 15 Albino rats treated with oil of *A. indica* seed was within the control value. The value obtained was in agreement with that reported by Wasser *et al*., (2004) on the effect of *Gonderma spp* on haematological parameter of mice model, but different from that reported by Shamaki *et al*., (2014) but using the same plant material on Albino rat. The variation in the values of the parameter reported could be attributed to the high dosage of the plant material which was administered, time of exposure, sex, age of the animal models and other experimental conditions. In real life situation, high residues of the *A. indica* seed oil are rare in processed foods after washing and boiling, therefore treated foods may not contain residual concentration high enough to alter blood parameters above the control values. Therefore results of our study

showed no significant variations in pack cell volume (PCV), red blood cell (RBC), white cell count (WCC), and haemoglobin content (Hb), very slight variations were noticed with increasing age of the animals except in run 14 which did not show reasonable values, this was in agreement with the observation of Adel *et al*., (2015). The observation suggest that the *A. indica* seed oil failed to alter most biochemical processes in the animal models to detectable level at the levels and time administered. Catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) are some of the important endogenous antioxidants (Geta *et al*., 2002) which counteract the damaging effects of free radicals in living system, the marginal variation among the animal models did not suggest toxic effect of the plant material. This is not surprising because *A. indica* plant has a good history of remedying liver cirrhosis, inflammation and cancer (el-Shazyl *et al*., 2000), and is expected to control oxidative stress at the level of administration (Tables 2, 3).

In the study, it could be concluded that the of *A. indica* seed oil which was administered orally to the animal models did not alter haematological indices and antioxidant values significantly from the control. The slight changes observed did not mean that the animals were sick but could be attributed to experimental errors. Therefore, the use of *A. indica* oil as an alternative to some chemical synthetic insecticides should be considered.

V. CONCLUSION

Azdirachta indica seed oil may be a safe alternative to the synthetic chemical insecticides. Because the plant material failed to elicit appreciable alteration on the haematological indices and antioxidant levels in Albino rats at the conditions of administration. The use of the plant material as an alternative to the synthetic chemical insecticides should be encouraged, but more work should be carried out on higher levels of other independent variables.

Conflicts of interest: The authors declare no conflict of interest.

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