Evaluation of safety of *Azadirachta 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 *Azadirachta 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 indexes. *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\(^2\)≤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— *Azadirachta indica*, response surface methodology, rotatable central composite design, haematological indices, enzymes.

1. 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 zintaldehyde, 4-Allyl-anisol, linalool, terpinol in the control of confused flour beetle, *Tribolium confusum*. Okpereke and Bunmi, (2006), Okpereke, (2007) reported effectiveness of *Xylopia aethiop*ia products against the common maize weevil, *Huang and Ho*, (1998) demonstrated effectiveness of *Piper guneense* powder against *Tribolium castaneum* (Herbst), *Sitophilus*...
zeamais Motsch. Mikhail, (2011) showed that some volatile oils of plants could protect packages of irradiated wheat flour against Ephesia kuehniella 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 (Schnutterer, 1990). Igboke (2017) opined that it is the presence of some biologically active principles like azadirachtin meliaicin, gedunin, salalin, nimbin, valasin and their derivatives that confers the anti-inflammatory, anti-hyper glycemic, anti-ulcers, anti-malarial, anti-fungal, anti-bacterial, anti-oxidant, anti-mutagenic, 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 Udom 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°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 OkiRob, (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 Laboratoriums 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
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 analyzer (ERMA) (Model PCE 210 Japan). Catalase was assayed spectrophotometrically according to the method of Aebi et al., (1983), estimation of superoxide dismutase 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.

\[
Y_i = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{j=1}^{3} \beta_{ij} X_i X_j + \varepsilon
\]

where \(Y_i\) is any response, \(\beta_0 = \) intercept, \(\beta_i = \) first order model coefficients, \(\beta_{ij} = \) quadratic coefficient for the \(i^{th}\) variable, \(\beta_{ij} = \) 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 independent variables**

| Variable        | Units     | -3 | -2 | 0 | 1 | +3 |
|-----------------|-----------|----|----|---|---|----|
| 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 |

\(\alpha = 1.4142 for\) RCCD

**III. RESULTS**

Levels of the independent variables, age (X1) 14-40 days, dose (X2) 10-30 mg, and exposure time (X3) 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 from 7.92 to 9.92x10^6/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 2.35-3.90 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 |
|-----|-----|------|------|---------|----------|------|-----|---|-----|----------|------|----------|----|--------|-----|---------|-----|--------|-----|--------|
| 1   | 27  | 10   | 20   | 10      | 39       | 9.52 | 7830 | 13.17 | 394.1 | 5981     | 3.53 |
| 5   | 8   | 10   | 20   | 10      | 38       | 9.21 | 8001 | 12.01 | 593.5 | 6098     | 2.35 |
| 10  | 27  | 24   | 24   | 24      | 37       | 9.51 | 7911 | 13.21 | 394.9 | 6182     | 3.75 |
| 6   | 45  | 10   | 20   | 10      | 35       | 9.22 | 7851 | 12.91 | 374.7 | 5519     | 3.11 |
| 2   | 40  | 10   | 20   | 10      | 37       | 9.11 | 7907 | 13.22 | 392.3 | 6917     | 3.99 |
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 (X₁)  | 0.4299 | 0.9882 | 0.8074 | 0.0684 | N/A | 0.3060 | 0.0198 |
| Dose (X₂) | 1.0000 | 0.7135 | 0.2253 | 0.3545 | N/A | 0.4073 | 0.7687 |
| Exposure time (X₃) | 0.5919 | 0.0992 | 0.6191 | 0.3691 | N/A | 0.8621 | 0.9327 |
| Interaction |      |      |      |      |     |     |      |
| Age x Dose (X₁X₂) | 0.6690 | 0.5341 | 0.3068 | N/A | N/A | 0.9725 | 0.0080 |
| Age x Exposure (X₁X₃) | 0.7863 | 0.2310 | 0.7143 | N/A | N/A | 0.0914 | 0.1968 |
| Dose x Exposure (X₂X₃) | 0.1403 | 0.3674 | 0.3953 | N/A | N/A | 0.0898 | 0.0141 |
| Quadratic |      |      |      |      |     |     |      |
| Age² (X₁²) | 0.4649 | 0.7105 | 0.8688 | N/A | N/A | N/A | 0.0011 |
| Dose² (X₂²) | 0.1427 | 0.9798 | 0.2788 | N/A | N/A | N/A | 0.0120 |
| Exposure² (X₃²) | 0.6318 | 0.5203 | 0.5484 | N/A | N/A | N/A | 0.0755 |
| R²        | 0.6519 | 0.6741 | 0.6317 | 0.3488 | 0.0000 | 0.5030 | 0.9611 |
| Adjusted R² | 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, coefficient 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, R²=0.6519, and the Adjusted coefficient of determination, Adj. R²= 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 positive.
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.

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 34.53x10^6/ml. The model is represented by Fig. 3 and Equation 3.
WCC = 70.88 - 64.15(X1) + 85.47(X2) - 60.80(X1) - 2.55(X1X2) - 0.87(X1X1) + 2.71(X1X2) + 0.16(X1X2) - 1.80(X2X2) + 0.96(X3X3) ... (4)

The model of Hb was not significant (p > 0.05), the coefficient of determination (R² = 0.3488) and Adj. R² = 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 R² = 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.

\[
\text{SOD} = 6363 - 1.56(X_1) - 56.24 + 65.49(X_2) - 6.661(X_3) + 0.0724(X_1X_2) + 3.9053(X_1X_2) - 5.10(X_1X_1)
\]

The model for GSH was not significant (p > 0.05). The coefficient of determination (R² = 0.9611, Adj R² = 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).

\[
\text{GSH} = 1.54 + 0.205 (X_1) - 0.032 (X_2) - 0.145(X_3)
\]

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 officinalis, Agentum conyzales L., Calendula officinalis, 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 food 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 blood cell count (WCC), and haemoglobin content (Hb), very slight variations were noticed with increasing age of the animal 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 remediying 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 oil 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

Azadirachta indica seed oil may be a safer 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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