

**Extraction, Microbial Load and Acute Toxicity Determinations of the Exudate Gum of *Anacardium occidentale* - A Potential Excipient in Drug Delivery.**

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**ABSTRACT**

Natural polymers are increasingly used as excipients in formulation systems over the synthetics due to their comparative advantages. Exudate bark gum of *Anacardium occidentale* (family: Anacardiaceae) has been suggested for its potential usefulness in drug delivery systems. Characterizations of material substances are an integral part of excipient development. This study sought to extract *Anacardium occidentale* gum (AoG) from the crude exudate and determine its microbial load and acute toxicity using established methods and official guidelines. Data were expressed as mean Standard Error of the Mean ( $\pm$  SEM) and were statistically analyzed using one-way Analysis of Variance (ANOVA); level of significance was set at ( $P < 0.05$ ). High yield, 72% w/w of the extracted gum was achieved. Microbial evaluation revealed the absence of objectionable organisms such as *Salmonella species*, *Shigella species*, *Proteus species*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella species*, *Pseudomonas aeruginosa*, *Vibrio cholera* and *Bile Tolerant Gram-Negative bacteria*. Total aerobic microbial, yeast and mould counts were  $1.70 \times 10^2$  and  $6 \times 10^1$  respectively. These values did not exceed the microbial limits specified by the United States Pharmacopoeia. LD<sub>50</sub> value greater than 5000 mg/kg was obtained. There was no observance in the experimental animals of any morbidity, mortality or signs of acute toxicity, treatment related abnormalities on the body weights, haematological parameters and organs histopathology at all the administered doses. There was no significance on these parameters between the control and AoG tested groups. AoG microbial and acute toxicity profiles have suggested its safety and potential excipient usefulness in formulation systems such as drug delivery.

**Key Words:** Natural Polymers, *Anacardium occidentale* gum, Microbial Load, Toxicological Safety, Excipient Potential.

**INTRODUCTION**

Drug dosage forms contain in addition to the active Pharmaceutical substance(s) some formulation components which are referred to as excipients. Pharmaceutical excipients are components of dosage forms that enable the formulations to acquire some characteristics which will establish the basic features of the formulated product. These excipients control physicochemical properties as well as the release profiles and availability of the drug in the system (Ram, 2004). It has been reported that 83% of the excipients used to manufacture drugs in Nigeria are imported (Mahmud *et al*, 2008). This great dependency brings about great use up of the Nigeria foreign exchange reserves with a resultant increase in production costs and prices of drugs.

It has become necessary therefore to source for excipients that are cheap, readily available and meet the quality of pharmaceutical grade excipients. Plant products serve as an alternative to synthetic products because of local accessibility, eco-friendliness and

lower prices compared to the imported synthetic products. One of the commonly used groups of compounds as excipients is the natural polymers. Natural polymers are polysaccharides composed of a large group of polymers with varying chemical composition, large derivatizable groups and a wide range of molecular weights. They are characterized by inertness, low toxicity, high stability, biocompatibility, and biodegradability and economically cheaper than the synthetics. These properties make them appealing as pharmaceutical excipients (Anekant *et al.*, 2007). Natural polymers especially gums hydrate in aqueous environment to form viscous dispersions or gels (hydrogels).

Hydrogels are mesh like structures of gum dispersions that result in increased diffusional path length of fluids which slow down capillary flow of fluids in the systems. Gelation formation and rheological characteristics/properties of hydrogels are affected by environmental factors such as polymer concentration, metallic ions, pH and temperature.

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These environmental factors can thus be used singly or combined to obtain hydrogel systems that could be tailored for different drug delivery systems. Natural polymers gums thus have the potential to be used as matrix formers in modified, sustained release dosage forms or as carriers in drug delivery technologies due to their environmental responsive gelation characteristics which can be tailored to specific therapeutic needs.

*Anacardium occidentale* (Cashew) gum has been evaluated on its binder properties in different tablet systems with indication of potential usefulness in sustained release tablet formulation systems (Ofori-Kwakye *et al.*, 2010). This conventional use of cashew is dependent on its thickening and stabilizing properties however, *Anacardium occidentale* is a hydrocolloid which on aqueous hydration, gels to form dispersion (hydrogel) whose viscosity is concentration dependent. This implies its usefulness as a matrix former or carrier agent in sustained and novel drug delivery systems; however, there is paucity of data on the evaluation of AoG excipient potential as a matrix former or carrier agent in drug delivery. The ability of excipients to provide their intended function and performance throughout the shelf life of a product must be established such that the information obtained would justify the choice, concentration and characteristics that may influence the final product (EMA, 2004). Material characteristics such as solid state, physicochemical, pharmacognostical, rheological, microbial load and toxicological safety are critical attributes and integral to excipient development and utilization. These aforementioned represent preformulation parameters which should be characterized and evaluated for the elucidation of material properties and potentials in formulation systems.

Microbial load and acute toxicity profile determinations are two critical material attributes used in the elucidation and evaluation of the safety of material substances. The microbial load determination gives an estimation of the measurable quantity of bacteria in a biomaterial. The microbial limit test is therefore designed to perform the qualitative and quantitative estimations of specific viable microorganisms present in biomaterial samples. It includes total viable count (bacterial and fungi) and *E. coli*.

Acute toxicity is usually defined as the adverse changes occurring immediately or within a short time following a single or short period of exposure to a substance or substances or as adverse effects occurring within a short time administration of a single dose of a substance or multiple doses given within 24 hours. An adverse effect is "any effect that

results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ's ability to respond to an additional challenge (Rhodes *et al.*, 1993).

AoG being a bark exudate gum of a plant material could be contaminated with both bacterial and fungal micro-organisms and /or could also encourage their growth. There is scarcity of data on the microbiological profile evaluation of AoG which informed one of the objectives of this study.

In addition, though AoG being a polysaccharide, it is thus assumed to be inert, non-toxic and had also been reported to be and safe by Okoye *et al.*, (2012) there could be variations in this property due to plant species, age, geographical source, environmental conditions such as climatic, soil types etc, it is thus expedient that the toxicity profile of AoG sourced from a different geographical location from previous studies be determined.

This study thus aimed to determine the microbial load and toxicity profile of the extracted AoG.

## **MATERIALS AND METHODS**

### **Collection and Extraction of AoG**

The AoG was obtained by incision as exudate of bark stems and trunks of the *Anacardium occidentale* (Cashew) (Fam: Anacardiaceae) trees grown in Ipe-Ikun area in Akoko South-West Local Government of Ondo State, South West, Nigeria. The gum was collected between September and October, 2012 during the day time. The plant was identified, authenticated by the Curator of Botany Department (B.O. Daramola) and assigned voucher number LUH 5453 in the herbarium of the Department of Botany, Faculty of Science, University of Lagos, Nigeria. The crude exudate gum was dried in an oven at 40°C for 6 hours and blended into shreds and reduced to fine powder using glass mortar and packaged into a wide mouthed air tight transparent plastic container.

A purer form of cashew gum was extracted from the gum exudate by a modified method reported by Okoye *et al.*, (2012) and indicated as follows, the clean pulverized cashew exudate gum was screened through a 600 µm sieve. Thereafter, 100 g of the powder was soaked in 500 mL of distilled water at 30 °C with intermittent stirring for one week. At the end of the one week, the dispersion was strained through a muslin bag and the resulting mucilage was precipitated by mixing it with thrice its volume of 96 % ethanol (Sigma-Aldrich, Germany). The precipitated gum was filtered using a filter cloth (calico linen) to ensure that all debris was removed and air dried. Further purification of the gum was carried out by dissolving it in fresh distilled water to

yield 1.0 % w/v solution. This solution was filtered using a 100 % cotton cloth overlaid with 2-inch-thick surgical cotton wool (Maimed GMBH, Germany) and the resulting filtrate mixed with thrice its volume of 96 % ethanol (Sigma-Aldrich, Germany) to precipitate the gum. The precipitated AoG was harvested and soaked in 96 % ethanol (Sigma-Aldrich, Germany) for 18 hours and finally air dried in order to kill peroxidase. The dried flakes of this purified AoG were finely powdered using a blending machine and the purified powdered drug was stored in an air tight open-mouthed screw capped glass bottle.

### **Microbial Load and Toxicity Study Determinations**

The microbial load determination is critical because it gives an estimation of the number of viable aerobic microorganisms' present indicates the rationale for this determination

#### **Determination of Microbial Load**

This was carried out using different selective media (Biotec products)- Sabouraud Dextrose Agar (specific for fungal organisms-yeast and mould organisms); Tryptone Soya Agar (specific for aerobic count); Mannitol Salt Agar (specific for Staphylococcal count); *Pseudomonas Cetrimide* Agar (specific for *Pseudomonas*); Eosine Methylene Blue Agar (specific for *E.coli*); Thiosulphate Citrate Bile Salt Sucrose Agar (specific for *Vibrio cholerae*); Nutrient Agar (specific for *Proteus swarming*); *Salmonella Shigella* Agar (specific for salmonella/shigella) and Mac Conkey Agar (specific for enteric bacteria- Coliform and Bile Tolerant gram negative organisms) .

Microbial Load Determination was undertaken for this extracted indicates the rationale for this determination exudate polymer gum using established methods (USP, 2015) for Microbial Limit Test. The microbial load determinations included:

Total combined yeast and mould count; Total aerobic count; Total staphylococcal count; *Pseudomonas* count; *E. coli* count; *Vibro cholerae*; *Proteus swarming*; *Salmonella/Shigella* counts; Coliform counts; Bile Tolerant gram-ve organisms.

The dispersion of the AoG extract in sterile 3 % Tween 80 was vortexed with a Vortex Vibrator (JP Selecta s.a. Spain; Serial No: 0514265, Model code: 7001721) and diluted to give two different dilutions (1:10 and 1:100). Petri dishes were labeled appropriately with the dilutions and the media to be populated.

All petri dishes that were labeled 1:10 dilutions had 19 mL of the respective media aseptically poured into them and were correspondingly inoculated with 1 (one) ml of the 1 in 10 dilution (stock solution of the AoG dispersion). This procedure was undertaken inside a sterile cupboard (ESCO class II Biohazard Safety Cabinet, Singapore). This procedure was repeated for the petri dishes of the different media labeled (1: 100) dilution. At the end of this procedure, each microbial specific media had two petri plates of 1 mL of a (1: 10 & 1: 100) dilutions of the gum polymer dispersion and filled with 19 mL of the appropriate agar type. They were thoroughly mixed and allowed to set. For each culture medium, three determinations and one control were prepared. Sampling and inoculation was separately undertaken with these two dilutions.

Sabouraud Dextrose Agar (SDA) plates were incubated up-right at room temperature (ambient temperature) to avoid the spores being dispersed. All other plates were incubated and their controls were incubated at 37± 2°C (Remi Industries Ltd, Mumbai-India; Model 400053; Serial No: I11C-2368 and Astell Hearson, England, Model: JBF042, Nov 86, Serial No: OV10045) for the required specific incubation period (72 hours/one week).

Petri dishes maintained for bacterial studies were observed daily for 72 hours for growth and further identification of the organisms. Sabouraud Dextrose Agar plates were observed daily for one week to allow for full development of fungi for identification.

This microbial load evaluation protocol was undertaken for the powdered sample of AoG that had been packaged in an airtight open-mouthed plastic container and stored at room temperature was subjected to the above enumerated microbial evaluation protocols after 6 and 12 months of storage.

#### **Determination of Acute Toxicity**

It has been indicated that some of the risks that may be associated with the use of herbs and plants can be revealed through toxicity testing (Obidike and Salawu, 2013).

The primary goal of evaluating the safety is to identify the nature and significance of adverse effects as well as to determine the exposure level where the effects are observed.

The study method used is as described by Anigu *et al.*, (2005) and modified by Okoye *et al.*, (2010). Ethical approval for this study was granted by the Research Grants and Experimentation Ethics Committee (RGEEC) of the College of Medicine,

University of Lagos. The approval code is CM/HREC/03/16/008.

The study upheld the principles of Good Laboratory Practices and Animal handling based on the Organization for Economic Co-operation Development (OECD, 2001): Guidelines for the care and use of laboratory animals. The study involved the acute toxicity evaluation (once oral dosing) of AoG extract dispersions using the albino mice. The Study sites were the Departments of Physiology and Biochemistry, Faculty of Basic Medical Sciences, Department of Anatomic and Molecular Pathology and D-4 Laboratory Paediatric Ward, College of Medicine, University of Lagos, Idi-Araba. The acute toxicity study evaluation protocols followed the up and down procedure of the Organization for Economic Co-operation and Development (OECD) "Acute, Oral Toxicity –up and down procedure" Test Guidelines 423 and 407 for testing of chemicals with the use of healthy female (Lipnick, *et al.*, 1995) nulliparous and non-pregnant mice aged between 8-12 weeks old, wherein the limit test dose of 5000 mg/kg is stipulated. This procedure involved the limit and main test which was also used for the selection of a starting dose as well as determination of LD<sub>50</sub> of the test AoG extract.

#### **Experimental Animals**

A total of 55 healthy mice which were of 21.5 grams average body weight were obtained from the animal farm of Priceless Test Farm, Lagos, Nigeria. The animals were housed at the Animal House of the College of Medicine, University of Lagos Nigeria, with the provision of adequate ventilation and room temperature maintenance of 25°C ± 2°C as well as timing of light that simulated light and day for a two-week (14 days) acclimatization period to laboratory conditions before the commencement of the study. The animals were adequately fed with standard animal feed from Animal Care Services<sup>R</sup> and water *ad libitum*. The cages, beddings and water bottles were cleaned on a daily basis. The animals were divided into groups based on the dose of the AoG to be administered.

#### **Acute Toxicity Study Protocol**

A total of 55 mice were randomly divided into 5 groups and each dose group consisted of 5 animals each. The animals were grouped based on the five different treatment doses of AoG dispersions with one control group. The animals were dosed once per oral with distilled water (control) or with the respective concentrations of the gum polymer dispersions. The limit dose of 5000 mg/kg was used (OECD, 2001). The dose levels that were used ranged from 5-5000 mg/kg. After 24 hour and 14 days, the animals were fasted of feed but left with

drinking water *ad libitum*. Following, the overnight fasting period, the body weights of the mice were determined and the administered dose calculated with reference to the body weight. The volume of the gum dispersions that was administered orally to these mice was based on 5 mg/kg for dose concentration ranges of 5 mg/kg, 50 mg/kg and 300 mg/kg whilst for the higher concentrations of 2000 mg/kg and 5000 mg/kg, the volume of the gum dispersions that was administered was based on 20 mg/kg and 40 mg/kg respectively. Body weights of individual animals in each group were assessed. Changes in body weights are sensitive indices of adverse effects of drugs, chemicals and plant materials. (Santos *et al.*, 2009; Wang *et al.*, 2014; Ibrahim *et al.*, 2016). Signs of toxicity (behavioral changes such as drowsiness, gait, tremors, convulsions and other physiological activities) were observed continuously every 15 minutes for the 1st hour; every 30 minutes for the next 4 hours and then at an hourly interval for the next 24 hours; 6 hourly interval for the next 48 hours and once daily for the next 14 days following AoG extract dispersion administration (Ogbonnia, *et al.*, 2010), Ibrahim *et al.*, 2016). The number of deaths in each group within 72 hours was recorded. The acute toxicity LD<sub>50</sub> is calculated as the geometric mean of the dose that resulted in 100% mortality and that which caused no lethality at all. Perhaps, any inconsistencies is observed in the mortality pattern, then an estimation of the LD<sub>50</sub> shall be carried out using the probit log analysis

On the 15<sup>th</sup> day of the study, the mice were subjected to fasting for 16-18 hours and then sacrificed prior to behavioral and physical assessment/examination. The animals were sacrificed by decapitation under inhaled diethyl ether anaesthesia. A deep longitudinal incision was made into the ventral surface of the abdomen and thorax of the sacrificed mice by blunt dissection of the muscles and fasciae and vital organs such as liver, kidney, heart, spleen were exposed and harvested

#### **Measurement of Body and Organ Weight**

Throughout the experimental study, the animals were weighed at four day intervals and the percent (%) weight change for each animal at the end of each study was calculated as indicated below:

$$\% \text{ Weight change} = \frac{BW_F - BW_I}{BW_I} \times 100 \dots \dots \dots$$

Equation 1

BW<sub>I</sub>

Where: BW<sub>I</sub> is Initial body weight  
BW<sub>F</sub> is Final body weight.

Also, the weight of each harvested organ was standardized for 100 g body weight of the individual mice.

#### **Haematological Analysis**

Animals were anaesthetized with diethyl ether/anaesthetic ether just prior to the haematological procedure was to be undertaken. At the appropriate time, venous blood samples (2 mL) in the different marked animal groups were collected from each mouse in the concerned group by retro-orbital puncture using capillary tube into heparinized Ethylene diamine tetra acetic acid (EDTA) centrifuge tubes for haematological parameters. The haematological analysis was undertaken using an automated haematology analyzer (BC-3200). Haematological parameters enumerated below were evaluated as described by Kasim et al., (2015): Red Blood Cells (RBC), White Blood Cell (WBC), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Volume (MCV), Mean Corpuscular Haematocrit Count (MCHC), Haemoglobin (Hb), Haematocrit (HCT) and Platelet.

#### **Determination of PCV**

The Packed Cell Volume (PCV) was determined according to micro method using capillary tubes. Blood samples were collected by cardiac puncture into Ethylene diamine tetra acetic acid (EDTA) treated sample bottles. This was carried out using capillary tubes of 75 mL in length and internal diameter of about 1 mm. The blood was drawn into the tube by capillary action, leaving about 15 mm unfilled and was sealed. After about 5 minutes centrifugation, the PCV was then measured using a reading device (Dacie and Lewis, 1991; Kasim et al., 2015).

#### **Determination of Haemoglobin Concentration**

This was done by using the cyanomethaemoglobin method (Dacie and Lewis, 1991). 20  $\mu$ L of blood was added to 4 mL of diluents. The tube containing the solution was stoppered and inverted severally; it was then allowed to stand at room temperature for about 3-5 minutes to ensure complete reaction. The solution of Hydrogen cyanide (HCN) was then compared with the standard and a reagent blank in a spectrophotometer at 540 nm

#### **Determination of Red Blood Cells**

This was determined by the visual method. 20  $\mu$ L of blood was taken into a micro pipette into 4 mL of diluting fluid contained in a glass tube. The diluted blood was then mixed for at least 2 minutes by tilting the tube. The Neubauer counting chamber was filled with solution with the aid of stout glass capillary and covered with a cover glass. The cells were counted using a 4 mm dry objective and x 6 eye piece determined using the visual method as

described by (Dacie and Lewis., 1991; Kasim et al., 2015).

#### **Determination of White Blood Cells**

A 1 in 20 dilution was made by adding 20  $\mu$ L of blood to 0.38 mL of diluting fluid in a 75 x10 mm plastic tube. The tube was tightly corked and mixed for at least 1 minute then the Neubauer containing chamber was filled. The preparation was viewed using a 4 mm objective and x 6 eye piece determined using the visual method as described by (Dacie and Lewis., 1991; Kasim et al., 2015).

#### **Determination of Platelet**

A 1 in 20 dilution of the blood was made. The suspension was then mixed on a mechanical mixer for 10-15 minutes. Neubauer counting chamber was filled with the suspension with the aid of a stout glass capillary. The counting chamber was closed in a moist petri-dish and left untouched for at least 20 minutes to give time for the platelets to settle. The preparation was examined with the 4 mm objective and x 6 eye piece determined using the visual method as described by (Dacie and Lewis., 1991; Kasim et al., 2015)

#### **Histopathology Evaluation**

Gross pathological features were examined at necropsy (all external surface of the body, all orifices cranial, thoracic and abdominal cavities). The harvested vital organs were weighed and the weight per 100 gram body weight (relative) was calculated based on fasted animals body weight (prior to sacrifice) and absolute body weight. These weighed harvested organs were then fixed in 10 % (v/v) buffered formalin solution in labeled bottles for histopathology examination. Following fixation, the tissues were exposed to routine processing, by embedding in paraffin section at 3-5  $\mu$ m. Tissue sections were stained with haematoxylin and eosin stain using Leica DM500 microscope attached with camera Leica ICC50 HD (Leica Microsystems Ltd, Switzerland)

#### **Data Reporting and Documentation**

All data were summarized in tabular form showing for each test group, the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animal, description and time course of toxic effects and reversibility and necropsy findings

#### **Statistical Analysis**

All the values obtained for respective parameters, (microbial load, body weight, physical appearance, food consumption, water intake, haematological, histopathological, organ weights and relative organ weights were expressed as means  $\pm$  standard error of the mean (SEM). Comparisons between groups were performed using SPSS, version 23 statistical

software. A value of  $<0.05$  was considered significant

## RESULTS

### Extraction

The extraction of AoG was successfully undertaken. High yield (10.63 gram) of the extracted AoG was obtained from the 12.5 gram sample of the crude exudate form. Upon further purification of the extract the AoG yield was (9 gram).

### Microbial Load Determination/Evaluation

#### Microbial Profile

The exudate AoG did not contain enteric bacteria and fungi. It was observed that AoG extract had good and acceptable microbial load profile as indicated in Table 1. The total aerobic microbial count as well as total yeast and mould count fell within the USP acceptable limits for natural plant

material. No enteric or fungal microbe was present in the extract. It was also observed that there was no significant difference in the microbial load result of AoG that was stored for 6 and 12 months with the obtained values of freshly extracted AoG indicated on Table 1. The total aerobic microbial count as well as total yeast and mould count fell within the USP acceptable limits. It was also observed that there was no significant difference in the microbial load result of AoG that was stored for 6 months and one year (12 months) with the obtained values indicated on Table 1 for the freshly extracted AoG. The total microbial counts for the stored samples were within the USP 2015 specified limits. This indicates that AoG, though polysaccharide does not permit microbial growth with storage time and thus microbiologically safe.

Table 1: Microbial Profile of Extracted AoG over a 12 months storage period.

Microbial Assessed	Group	**Observed Microbial Colony Counts/mL in extracted AoG over a 12 month storage period			USP Limit for Colony Count/mL*
		Months of Storage			
		1	6	12	
Total Aerobic Microbial		$1.70 \times 10^2$	$1.73 \times 10^2$	$1.73 \times 10^2$	NMT $10^5$
<i>Shigella species</i>		Absent	Absent	Absent	Absent
<i>Salmonella species</i>		Absent	Absent	Absent	Absent
<i>Escherichia coli</i>		Absent	Absent	Absent	Absent
<i>Pseudomonas aeruginosa</i>		Absent	Absent	Absent	Absent
<i>Proteus species</i>		Absent	Absent	Absent	Absent
<i>Staphylococcus aureus</i>		Absent	Absent	Absent	Absent
<i>Bacillus species</i>		Absent	Absent	Absent	Absent
Total Combined Yeast		$5.9 \times 10^1$	$6.0 \times 10^1$	$6.0 \times 10^1$	NMT $10^3$
Bile Tolerant Gram		Absent	Absent	Absent	NMT $10^3$
<i>Vibrio cholera</i>		Absent	Absent	Absent	Absent

NMT-Not More Than

\*Microbial Load Determination (USP, 2007)

\*\* **Extracted Microbial Load levels of extracted AoG at (a: Freshly extracted); (b: 6months after extraction); (c:12months after extraction).**

Good microbial profile. Compliance with the USP microbial limits for natural plant materials. No enteric or fungal microbe was present in the extracted gum

### Acute Toxicity Study Determination/Evaluation

#### Physical Appearance, Behavioural Patterns and Clinical Manifestations.

It was observed that AoG extract dispersions at all the administered doses which ranged from 5 mg/kg to 5000 mg/kg had no adverse effects on the behavioral responses of the tested mice up to 14 days of observation. Physical observations indicated no signs of changes in the skin, fur, eyes, mucous membranes, behavioral patterns, tremors, salivation

and diarrhea of the mice. The stool and urine outputs were normal and not colored. There was no mortality observed at the tested doses nor was there weight loss in the different mice groups. There was an observed gradual corresponding increase in body weight with increasing study days in the different treatment groups (Table 2, Fig 1). Therefore, there were no treatment related changes in the body weight. There was no significant difference in body

weights of the treated mice and the control group.

**Animals Organ Weights**

There was generally no significant difference in both the organ weights and the relative organ weights. The Relative Organ Weights (ROW) of the treated 14-day animals is shown in Tables 3 and 4. The relative organ weight of each organ recorded at necropsy in the treatment groups did not show a

significant difference ( $P > 0.05$ ) compared to the control (Table 3 and 4).

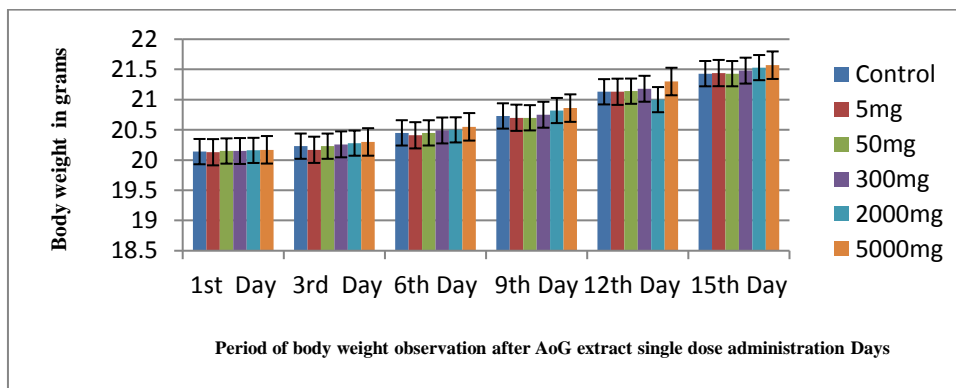
**Haematology Analysis.**

Effects of acute oral administration of AoG extract on haematological parameters at 24 hours and 14 days are presented in Tables 5 and 6 respectively. Most haematology parameters (red blood cells, white blood cells, platelet count, haemoglobin,

**Table 2: Changes in body weight over a 15-day period of mice administered different single doses of AoG extract dispersions**

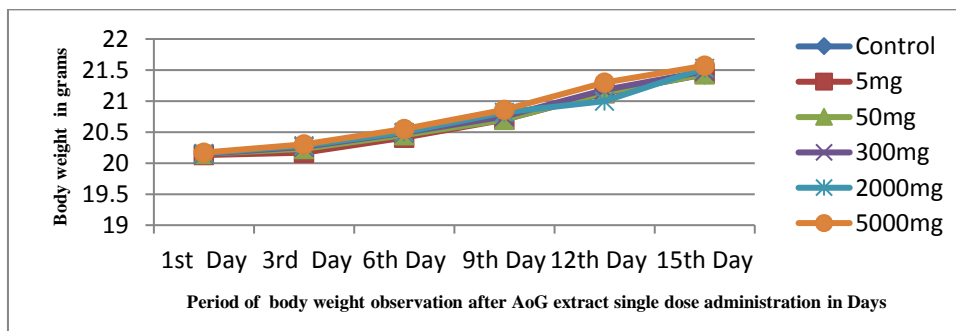
Dose of AoG extract Dispersion	Changes in body weights (g±SEM) of mice in days with different single doses of AoG extract					
	1 <sup>st</sup> Day	3 <sup>rd</sup> Day	6 <sup>th</sup> Day	9 <sup>th</sup> Day	12 <sup>th</sup> Day	15 <sup>th</sup> Day
Control	20.14±0.12	20.23±0.11	20.45±0.10	20.73±0.09	21.13±0.12	21.43±0.11
5 mg	20.13±0.10	20.17±0.10	20.41±0.13	20.70±0.11	21.13±0.10	21.44±0.13
50 mg	20.15±0.11	20.23±0.12	20.45±0.10	20.70±0.09	21.14±0.11	21.43±0.10
300 mg	20.15±0.10	20.26±0.09	20.49±0.11	20.75±0.10	21.18±0.09	21.48±0.10
2000 mg	20.16±0.10	20.28±0.10	20.50±0.10	20.82±0.11	21.23±0.10	21.53±0.09
5000 mg	20.17±0.09	20.30±0.11	20.55±0.12	20.86±0.10	21.3±0.10	21.57±0.12

Results expressed as mean body weight of mice after 14 days of treatment with different concentrations of AoG extract dispersions; and are non-significant changes when compared with control mice ( $p > 0.05$ ). All the values are mean ±SEM n=5



**Fig. 1: Changes in body weight over a 15-day period of mice administered different single doses of AoG extract dispersions.**

Results were expressed as mean ± SEM (n=5) of mice after 14 days of treatment with AoG extract. The treated groups showed no significant changes compared with control mice ( $p > 0.05$ )



**Fig. 2: Changes in body weight over a 15-day period of mice administered different single doses of AoG extract dispersions.**

**Table 3: Organ Weights of mice after 15 days of treatment with different single doses of AoG extract.**

Organ Type	Organ weights (g±SEM) of mice treated with different single doses of AoG extract					
	Control	5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	5000 mg/kg
Heart	0.168±0.01	0.169 ±0.01	0.169±0.01	0.168±0.03	0.169±0.04	0.170±0.03
Liver	1.52±0.02	1.52±0.01	1.52±0.07	1.51±0.04	1.52±0.04	1.53±0.09
Kidney	0.35±0.01	0.35±0.03	0.35±0.02	0.35±0.03	0.36±0.02	0.36±0.03
Spleen	0.16±0.03	0.16±0.01	0.16±0.03	0.17±0.01	0.17±0.04	0.17±0.03

Results were expressed as mean ± SEM (n=5). AoG extract treated groups showed no significant changes compared with control mice (p> 0.05).

**Table 4: Relative Organ Weights of mice after 15 days of treatment with different single doses of AoG extract.**

Organ Type	Relative Organ Weights (g ±SEM) of mice treated with different single doses of AoG extract					
	Control	5mg mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	5000 mg/kg
Heart	0.7839± 0.04	0.7882 ±0.03	0.7886 ±0.04	0.7821±0.04	0.7850 ±0.04	0.7881 ±0.05
Liver	0.7093 ±0.02	7.0895 ±0.02	7.0928 ±0.03	7.0297±0.04	7.0591 ±0.03	7.0931 ±0.03
Kidney	1.6330 ±0.02	1.6324 ±0.03	1.6332 ±0.03	1.6294±0.03	1.6720 ±0.04	1.6689 ±0.04
Spleen	0.7606 ±0.04	0.7466 ±0.03	0.7462 ±0.02	0.7914±0.03	0.7895 ±0.03	0.7881 ±0.04

Results were expressed as mean ± SEM (n=5). AoG extract treated groups showed no significant changes compared with control mice (p> 0.05).

**Table 5: The Effect of different single dose concentrations of AoG extract treatment on haematological parameters of mice after 24 hours**

Haematological Parameters	Control	Different single dose concentrations effects of AoG extract after 24 hours of treatment				
		5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	5000mg/kg
WBC (x10 <sup>9</sup> )	10.0±0.02	9.8000±0.10	9.85.000±0.16	9.9.0±0.10	10.00±0.12	10.200±0.06
RBC (x 10 <sup>12</sup> )	9.60±0.03	9.55±0.02	9.66±0.01	9.69±0.006	9.70±0.006	9.72±0.012
Platelet (x10 <sup>9</sup> /L)	727.0±0.04	718.0 ±1.5	720.000±1.15	725.0±1.15	730.0±1.5	732.000±1.5
HGB (g/dL)	14.90±0.05	14.70± 0.06	14.70±0.06	14.80±0.06	14.90±0.06	14.90±0.06
HCT (%)	47.50±0.04	47.25±0.06	47.400±0.06	47.350±0.06	47.600±0.06	47.50±0.10
MCV (f/L)	49.30±0.06	49.10±0.06	49.20±0.06	49.20±0.06	49.30±0.06	49.400±0.06
MCH (pg)	15.40 ±0.05	15.10±.06	15.20±0.06	15.30±0.06	15.40±0.01	15.50±0.28
MCHC(g/dL)	31.30±0.06	31.20±0.06	31.100±0.06	31.20±0.01	31.30±0.01	31.300±0.06

Doses of AoG extract = mg/kg. b.wt; b.wt =body weight; WBC= White Blood Cell; RBC= Red Blood Cell ; HGB= Haemoglobin ; HCT = Haematocrit ; MCV= Mean Corpusular Volume ; MCH= Mean Corpuscular Haemoglobin ; MCHC= Mean Corpusular Haemoglobin Concentration.

Results were expressed as mean ± SEM (n=5). AoG extract treated groups after 24 hours showed no significant changes as compared with control mice (p> 0.05). Means of 5 mice only

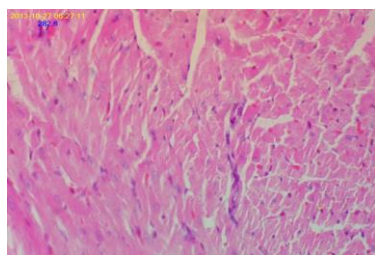
The Effect of different single dose concentrations of AoG extract dispersion on haematological Parameters in mice after 14 days .

Haematological Parameters	Control	Concentration Effect of <i>Anacardium occidentale</i> Gum Polymer @2weeks				
		5 mg/kg	50 mg/kg	300 mg/kg	2000mg/kg	5000mg/kg
WBC (x10 <sup>9</sup> )	<b>10.000±0.02</b>	9.80±0.12	9.90±0.10	10.00±0.10	10.20±0.06	10.180± 0.058
RBC (x 10 <sup>12</sup> )	<b>9.650±0.03</b>	9.50±0.010	9.55±0.01	9.50±0.01	9.72±0.01	9.60±0.015
Platelet(x10 <sup>9</sup> /L)	<b>727.000±0.04</b>	719.0±0.57	722.0± 1.15	730.0±0.57	728.0±0.55	732.000±0.6
HGB (g/dL)	<b>14.90±0.05</b>	14.400± 0.058	14.60±0.06	14.50±0.06	14.80±0.058	14.70±0.06
HCT (%)	<b>47.50±0.04</b>	47.30± 0.058	47.40±0.06	47.45±0.06	47.50±0.06	47.20±0.06
MCV (f/L)	<b>49.30±0.06</b>	49.10±0.03	49.20±0.0	49.25±0.06	49.30±0.06	49.35±0.3
MCH (pg)	<b>15.40 ±0.05</b>	15.20±.06	15.30±0.06	15.50±.00	15.5±.06	15.54±.06
MCHC(g/dL)	<b>31.30±0.06</b>	31.300±0.06	31.200+ 0.058	31.300±0.06	31.300+ 0.058	31.500±0.058

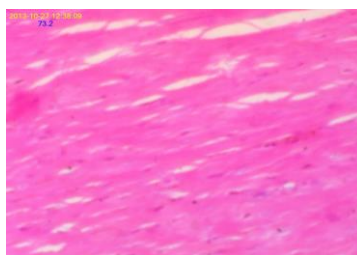
Doses of AoG extract = mg/kg. b.wt ; b.wt =body weight, WBC= White Blood Cell ; RBC= Red Blood Cell;HGB= Haemoglobin ; HCT = Haematocrit; MCV= Mean Corpusular Volume ;MCH= Mean Corpuscular Haemoglobin ; MCHC= Mean Corpusular Haemoglobin Concentration.

Results were expressed as mean ± SEM (n=5). AoG extract treated groups after 14 days showed no significant changes as compared with control mice (p> 0.05). Means of 5 mice only.



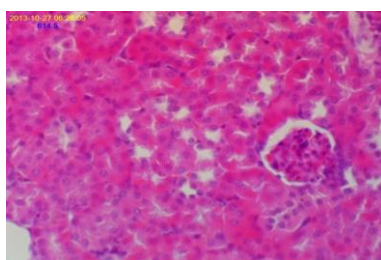


(a) x 282.6

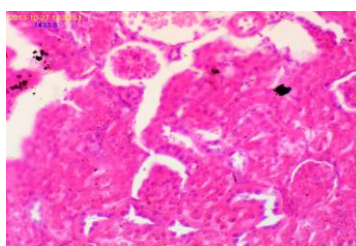


(b) x 151.5

Fig. 3. Showing the heart tissue sections of control and AoG extract treated mice. Photomicrographs of heart H & E stain 400x ; (a) 0 mg/kg body weight: Normal architecture of heart section; A cardiac myocyte appears within the normal limits with nuclei present. A normal intercalation disk. (b) 5000 mg/kg body weight: Normal architecture of heart section, with visible cardiomyocyte and nuclei, observed normal intercalation disk.

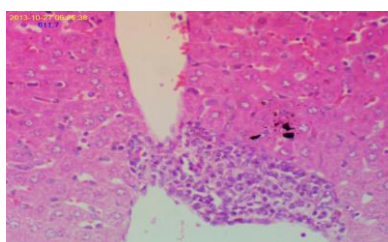


(a) x 814.5

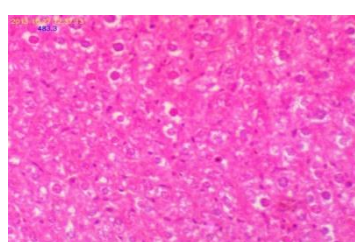


(b) x1433.8

Fig. 4: Showing the kidney tissue sections of control and AoG extract treated mice. Photomicrographs of Kidney sections H&E stain at indicated magnification (a) 0 mg/kg body weight.; observed normal kidney architecture with normal proximal and distal tubules, glomerulus, blood vessels and interstition .the blue arrow shows tubules while the red arrow shows a glomerulus. Podocyte, urinary pole and corpuscular space appear within normal range (b): 5000 mg/kg body weight; observed normal kidney architecture with normal features of proximal and distal tubules, glomerulus, blood vessels and intersitition as indicated for control (0 mg/kg body weight)



(a) x 611.7



(b) x 521.2

Fig.5: Showing the Liver tissue sections of control and AoG extract treated mice. Photomicrographs of Liver sections from control dose group;(a) 0 mg/kg body weight and treated group ( b) 5000 mg/kg body weight: H&E Stain at indicated magnification: Normal architecture of liver showing the bile duct and central vein in the center of hepatic lobule filled with blood. Hepatocytes are arranged in the form of cords which are rounded in a polyhedral shape and radiate peripherally. They show nucleus with clear nuclear membrane and nucleous cords are separated by sinusoids with kupper cells. Portal tract is seen in the section of the control.

haemocrit, mean corpuscular haemoglobin, mean corpuscular volume, mean corpuscular haematocrit count) in treated rats were not significantly different from the controls.

Macropathology and Histopathology. Macroscopic examination of the vital organs (kidney, heart and liver) of treated animals revealed no abnormalities in the colour or texture when compared with the organs of the control group. The light microscopy

examinations of the transverse sections of organs of AoG extract treated and the control group animals are shown in Figures 3, 4 and 5. Histopathological examination of the control group and AoG extract treated animals showed normal structure and absence of any gross pathological lesion in organism the center of hepatic lobule filled with blood. Hepatocytes are arranged in the form of cords which are rounded in a polyhedral shape and radiate peripherally. They show nucleus with clear nuclear membrane and nucleous cords are separated by sinusoids with kupper cells. Portal tract is seen in the section of the control.

#### **DISCUSSION.**

The extraction procedure undertaken gave a high yield of 10.63 gram from 12.50 gram of the crude exudate AoG form. Further purification of this extract yielded (9.00 gram). These values were indicative of the abundance of this biomaterial from the crude form.

Though there is an increasing and preferential shift to the use of natural products specifically polysaccharides such as gums and mucilages over synthetics as excipients for pharmaceutical formulations due to their biodegradability, biocompatibility, inertness and purported non-toxicity, evaluation of their safety (microbial and toxicity) is critical to the elucidation of their excipient usefulness. Extracted AoG being of plant origin and a polysaccharide could be contaminated by microbes as well as support microbial growth which could therefore affect this biomaterial safety for human use. This indicates the rationale and importance of the microbial load assessment undertaken in this study. Microbial load is the qualitative and quantitative estimations of the specific viable microorganisms present in a biomaterial. This assessment has indicated that extracted AoG did not support microbial growth and had microbial load profile that conformed to the Pharmacopeia (USP, 2015) limits for natural plant products. Pathogenic organisms (both enteric and fungal microbes) were absent which implied its good, acceptable and safe microbial profile. The microbial load was also not affected by storage period as the obtained microbial profiles of AoG stored at room temperature for both 6 and 12 months were not statistically significantly different to the value obtained for the freshly prepared AoG. The outcome has indicated compliance of the microbial load profile to the official limit (Pharmacopeia) regardless of storage time. This has implied the stability and safety of AoG as a biomaterial and inferred its potential as an excipient.

Regardless of its good microbial profile and its purported benefit as a potential pharmaceutical excipient in formulation system such as drug delivery, detailed knowledge on the acute and chronic toxicity of this polysaccharide which is essential for excipient development type is lacking. Thus for the evaluation of the safety profile of AoG, acute and repeated oral toxicity (chronic) studies are needful. Acute oral toxicity study of a test substance forms the crucial pace in the description of any chemical or biomaterial in the drug development process (Chitra, et al., 2015). Hence, this study was undertaken to assess the acute toxicity of the bark exudate of AoG extract in an animal model. In the evaluation of the toxic characteristics of natural products that are pharmacologically active or pharmaceutically useful as excipients, the determination of LD50 is usually an initial step. This is because data from the acute toxicity study may be useful in any or all of the followings : (a) it could serve as the basis for classification and labeling; (b) it could provide an initial information on the mode of toxic action of a substance; (c) it could help to arrive at a dose of a new compound; (d) it could help in dose determination in animal studies; and (e) it could help in determination of the LD50 values that would provide many indices of potential types of drug activity, Ukwuani et al. , 2012. Moreover, should a high dose (e.g., 5000 mg/kg) is found as survivable, no further acute testing would be conducted (NRC, 2006). The 5000 mg/kg limit test guideline used for this acute toxicity evaluation was based on the polysaccharide nature, hence assumption of its inertness and non-toxicity of this biomaterial.

In this study, AoG at a dose of 5000 mg/kg had no adverse effect on the tested mice up to 14 days of observation. The physical examination during the experimental period indicated that all animals were healthy. In addition, the treated mice did not show any significant alteration in water or food consumption (data not shown). There were no significant changes ( $p > 0.05$ ) in the relative weights of the organs (liver, heart, kidney and spleen in the AoG treated group) which indicated its non-toxicity on the organs. Therefore, this study outcome has suggested that AoG would not cause acute toxicity effects at the dose tested and that it had an LD50 value greater than 5000 mg/kg. In principle, the limit test method undertaken was not intended for determining a precise LD50 value, but it served as a suggestion for classifying the extracted exudate gum based on the dose level the animals were expected to survive (Roopashree, 2009). According to the chemical labeling and classification of acute

systemic toxicity recommended by OECD, the extract of AoG has been assigned class 5 status (LD50 > 5000 mg/kg), this being the lowest toxicity class/level. This assertion is in accordance with the findings of study undertaken by Kennedy et al., (1986) which indicated that substances with LD50 values higher than 5000mg/kg by oral route should be regarded as safe or practically nontoxic. General behavior and body weights are one of the critical parameters for the evaluation of first signs of toxicity (Sireeratawong et al., 2008, Ezeja et al., 2014). In this acute study, there were no observable signs of significant toxic effects of morbidity or mortality to the experimental animals (mice) on acute oral administration of AoG at dose levels of between 5 mg-5000 mg/kg throughout the 14-day study period, which was an indication that this exudate gum was well tolerated by the mice. Therefore, it is concluded that there was no observed adverse effect level (NOAEL). In this study it was at least 5000 mg/kg/day for the animals used. This was collaborated with the no comparable significance of values in the hematological parameters evaluated with the control and test groups. This was because after 14 days of treatment, there was no significant ( $p > 0.05$ ) treatment related changes in the hematological parameters between control and treated groups, indicating that extracted AoG was not toxic. Analysis of blood parameters could be used to determine the extent of adverse effect of foreign compounds including plant extracts (Agbaje et al., 2009). Changes in the hematopoietic system have a higher predictive value for human toxicity when data are translated from animal studies (Olson et al., 2000). The hematology results obtained from this study suggested non-significant effects on these parameters when used for human.

Gross examination at autopsy and histopathology evaluations of the various organs stained with haematoxylin and eosin revealed no significant difference in the tissue morphologies (macroscopic or microscopic changes in these internal organs or tissues of the control and the treated mice groups (Figs. 3, 4 & 5). These results have indicated the healthy status of the heart, liver and kidney in the treatment mice.

The LD50 of extracted AoG has therefore been estimated to be more than 5000 mg/kg. The limit test is primarily used in situations where an information that the test material is either non-toxic or of low toxicity; this being the guideline laid by the Organization for Economic Co-operation and Development (OECD) for chemicals/ Section 4: Health Effects Test No 423: Acute Oral toxicity-Acute Toxic Class Method; Organization for

Economic Cooperation and Development, Paris, France, 2002.

The finding, therefore has suggested that extracted AoG dispersion at the 5000 mg limit dose tested was essentially non-toxic and safe in oral formulations. This has indicated that extracted AoG is relatively safe as classified by Organization for Economic Cooperation and Development (OECD, 2001 and Tarkang, et al., 2012).

This result is in agreement with the findings of Okoye et al., 2012 who reported that the LD50 of AoG was more than 5000 mg/kg. The fact that the LD50 value of extracted AoG was more than 5000mg/kg demonstrated and implied its relative safety for oral use. This has thus inferred its pharmaceutical usefulness as a potential excipient. Acute toxicity data are however of limited clinical application due to the fact that cumulative toxic effects could occur even at very low doses (Abotsi et al., 2011).

Though no toxic effects were found during the acute toxicity study of this extracted AoG, further evaluation would be conducted to evaluate the sub-chronic and chronic toxicity of AoG up to 90 days in an animal model for the preparation of a comprehensive toxicology data of extracted AoG. Sub-chronic and chronic studies assess the undesirable effects of continuous or repeated exposure of natural product extracts or compounds over a portion of the average life span of experimental animals such as rodents. Specifically, they provide information on target organ toxicity and are designed to identify any observable adverse effect level (NRC, 2006). Sub-chronic and chronic evaluation could also help to determine appropriate dose regimens for longer term studies.

It has been indicated that the data of the acute and sub-chronic toxicity studies on natural products should be obtained in order to increase the confidence in their safety to humans particularly for use in the development of pharmaceuticals (Ukwuani, et al., 2012). It is intended therefore, that a further study on the sub- chronic and chronic toxicity of extracted AoG would be undertaken.

#### **CONCLUSION**

High yield of extracted AoG was obtained from the crude bark exudate, which has indicated the abundance of this biomaterial in the exudate substance. The microbial and acute toxicity profiles of this extracted biomaterial have been assessed on experimental animals. Extracted AoG did not permit microbial growth and had pharmacopeia acceptable microbial load values, thus microbiologically safe. There was also no observance of signs of acute

toxicity such as abnormal behavioural patterns, morbidity and mortality in all the doses administered. The LD50 was above 5000 mg/kg. There was no significant difference in both the haematological parameters and organs histopathology of the control and treated animal groups as evidenced by the absence of treatment related abnormalities in the haematological profiles and organs histopathology. This preliminary study has suggested a safety profile for extracted AoG which thus portends its pharmaceutical excipient usefulness in drug formulation systems such as drug delivery.

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