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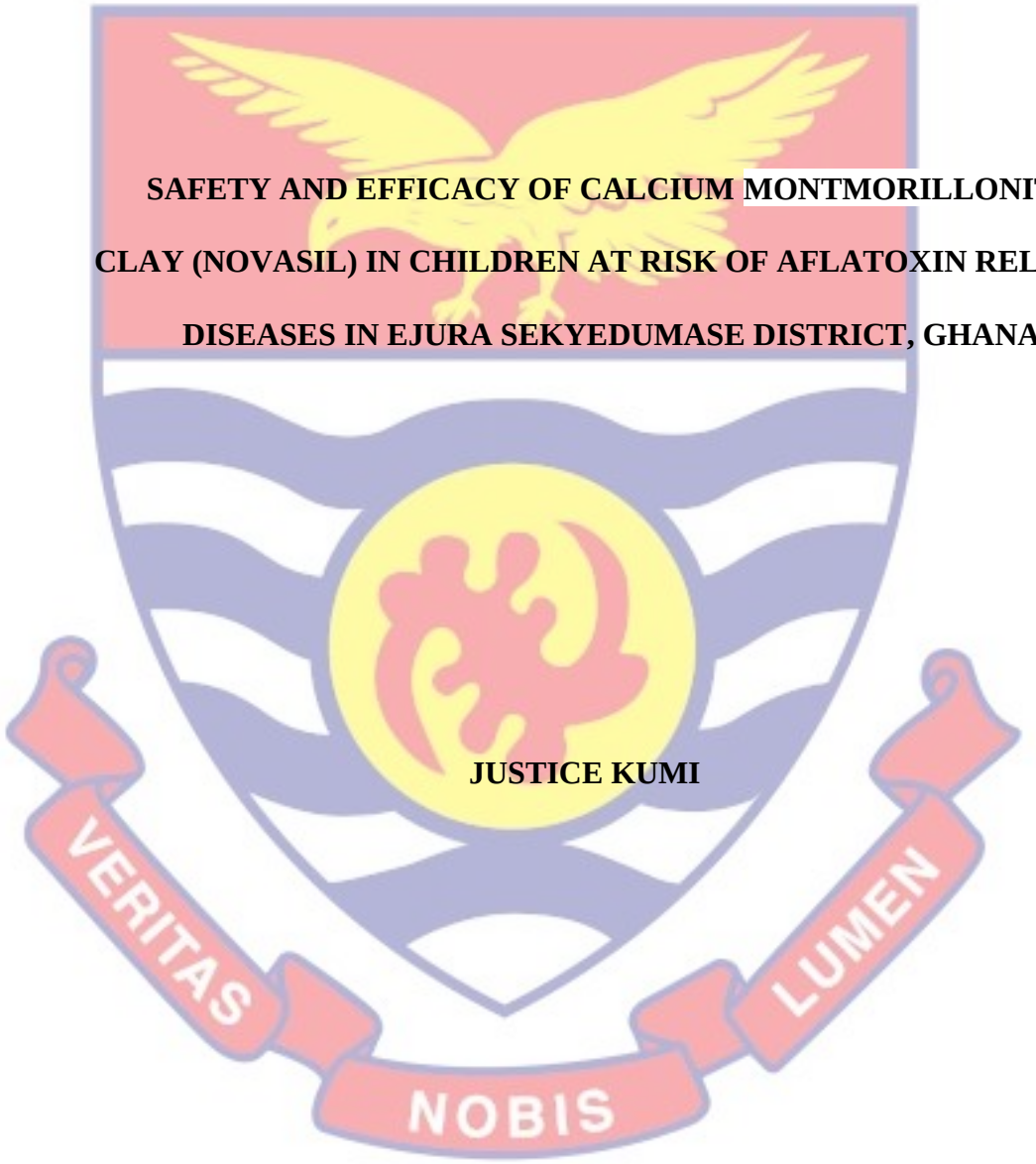

SAFETY AND EFFICACY OF CALCIUM MONTMORILLONITE CLAY (NOVASIL) IN CHILDREN AT RISK OF AFLATOXIN RELATED DISEASES IN EJURA SEKYEDUMASE DISTRICT, GHANA

JUSTICE KUMI

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BY

JUSTICE KUMI

Thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Doctor of Philosophy degree in Drug Discovery and Development

December, 2021

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature: …… Date: …….....

Name: Justice Kumi

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: …… Date:……

Name: Prof. Desmond Omane Acheampong

Co- Supervisor's Signature: …… Date: ……

Name: Prof. Regina Appiah-Opong

ABSTRACT

The use of novasil clay as an intervention technique to prevent aflatoxin poisoning in animals and humans has proven to be effective. In this study, evaluation of the safety and efficacy of novasil clay was carried out in 60 days in children in the Ejura Sekyedumase District, Ashanti Region, Ghana. Also, the relationship between growth indicators and aflatoxin in children was investigated. Stratified cluster sampling was used to recruit participants into the study. Healthy (200 children) within the age bracket of 2-9 years were put into two arms of study, which include 100 test group and 100 placebo group. One arm received 1.5g of novasil (test material) whiles the other arm received calcium sulphate as placebo. Full blood count, reduced glutathione, liver, kidney biomarkers, aflatoxin M1 and growth indicators were measured. Out of the total population of 200 children, 26 (13%) were Stunting 6 (3 %) were underweight. Change in serum biochemistry parameters and calcium levels between the placebo and novasil groups were not statistically significant ($P > 0.05$). At the end of the treatment cycle, reduced blood glutathione levels increased significantly in the novasil treated group. Aflatoxin M1 excretion in urine showed a significant reduction ($P = 0.033$) from a mean of 811.2 AFM_1pg/mg creatinine to 329.1AFM_1pg/mg creatinine at the end of the novasil treatment, representing a 60.7 % reduction. The placebo group demonstrated a significant increase ($P = 0.02$) of 44 % AFM_1 excretion in urine from a mean of 801.3 to 1801.2 AFM_1pg/mg creatinine. At the end of the 60 day intervention study, novasil was safe with no adverse effect and caused a reduction of aflatoxin bioavailability in children.

KEYWORDS

Urine Aflatoxin M1

Children

Efficacy

Novasil clay

Safety

Stunting

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DEDICATION

This work is dedicated to all researchers working to find solutions to aflatoxin poisoning in Ghana.

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LIST OF ABBREVIATIONS

ALB : Albumin

AFB_1: Aflatoxin B1

AFM_1: Aflatoxin metabolite M1

AFTs-G series: Aflatoxin G series

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

ATP: Adenosine triphosphate

AFQ1: Aflatoxin Q1

AOAC: Association of official analytical chemistry

CrCl: creatinine clearance

CYP450: Cytochrome P450

Deoxyribonucleic acid: DNA

EDA: Electron donor acceptor

EDTA: Ethylene di-amine triacetic acid

ELISA: Enzyme linked Immunosorbent assay

GAP: Good Agricultural Practices GGT: Gamma-glutamyl transferase

GFR: Glomerular filtration rate

GST: Glutathione-S-Transferase

GSH: Glutathione

HACCP Hazard Analysis Critical Control Point

Hb: Hemoglobin

HCC: Hepartocarcinoma

HSCAS: Hydrated sodium calcium aluminosilicate

HPLC: High performance liquid chromatography

IARC: International Agency for Research on Cancer

LD_{50}: Lethal dose 50

NAFLD: Non-alcoholic fatty liver disease

NIH: National Institute of Health

NMIMR: Noguchi Memorial Institute for Medical Research

P53: Tumor suppressor gene

WHO: World Health Organization

WBC: White blood cells

CHAPTER ONE

INTRODUCTION

Background to the Study

Aflatoxins exist as metabolic products formed by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* that contaminate common foods like groundnuts and maize. The growth of the fungi and the development of the toxin are aided by a combination of bad food storage conditions and hot humid climates (International Agency Research on Cancer, 2002). Aflatoxin (B1) is carcinogenic and can adversely affect the health and functioning of the liver (Turner et al., 2003; Obuseh et al., 2010). In the liver, the cytochrome p450 enzyme converts aflatoxin b1 into the main cancer-causing metabolite, AFB_1-8,9-epoxide. (Ross et al., 1992). Oxidation of AFB_1 to AFB_1-8,9-epoxide by cytochrome p450 produces two stereoisomers, exo- and endo-AFB_1-8,9-epoxide (Raney, Coles, Guengerich, & Harris, 1992). However, only exo-AFB_1-8,9-epoxide binds to DNA at the guanine atom's N7 position (lyer et al., 1994; Johnson, & Guengerich, 1997). Alternatively AFB1-8, 9-epoxide binds to glutathione, which is essential in protecting tissues from the harmful effects of bioactivated aflatoxin (Iyer et al., 1994).

While nutritional and protein deficiencies are the most common causes of stunting and wasting, aflatoxin, a common maize and peanut contaminant, has also been linked to stunting and wasting, especially in Africa and Asia (Gong et al., 2004; Okoth, & Ohingo, 2004). In African countries like Kenya, Guinea, Benin, Senegal, Togo, and the Gambia with 85-100 percent of children have detectable serum or urinary aflatoxin biomarker levels due to possible dietary exposures

(Gong et al., 2004). A strong negative association between aflatoxin exposure and growth was observed in children aged 16-37 months in Benin (Gong et al., 2004).

Aflatoxins in animal models often induce nutrient modifications such as vitamin A or D, and renders them inaccessible for normal body physiology and thus contribute to nutritional insufficiencies. Aflatoxin in the diet has an effect on zinc and selenium levels (Kalorey, Daginawala, Ganorkar, & Mangal, 1996). Appropriate concentrations of these minerals are important for effective immune systems. According to the World Health Organization (WHO), zinc deficiency affects approximately 31 % of the global population (Caulfield, & Black, 2004).

Although the role of aflatoxin in human zinc deficiency is unknown, animal model evidence indicates that aflatoxin can worsen zinc deficiency, especially in early childhood, when mothers even don't show zinc deficiency (Kalorey et al., 1996). In China, selenium concentrations were found to be inversely associated with concentrations of aflatoxin-albumin adducts in men (Chen et al., 2000). Home-made nutritious food (weanimix) with groundnut, beans, and maize prepared for children in the Ejura Sekyedumase District of Ghana, Ashanti Region had high aflatoxin levels, according to studies by Kumi et al. (2014). Hence, children fed with home-made weanimix are possibly exposed to unsafe levels of aflatoxin B1, and this could harm their health.

Clay has the potential to be a strong absorbent because of its high cation exchange, large surface area, mechanical stability and layered structure, (Sheng, Dong, & Li, 2012). Clay is easily accessible, non-toxic to the environment, and can be manipulated to meet the needs of the user. The discovery of a healthy

enterosorbent based clay (novasil), a natural clay-based of calcium montmorillonite is an indication that intervention strategy to minimize bioavailability of aflatoxin and eradicate aflatoxicosis is feasible in animals and humans (Afriyie-Gyawu et al., 2005; Phillips et al., 2008). Montmorillonite clay can act as ion exchanger for heavy metals among natural clay (Nurdan, & Okan, 2013). Novasil clay in animal models has shown selective enterosorbent action for aflatoxin due to its high binding affinity for aflatoxin B1 and thus significantly decreases aflatoxin bioavailability (Afriyie-Gyawu et al., 2005; Mayura et al., 1998; Harvey et al., 1991). Novasil (up to 2 % w/w) had no overt toxicity in animals when included in feed (Afriyie-Gyawu et al., 2005; Mayura et al., 1998; Harvey et al., 1991). Testing of 3.0g novasil/day is a healthy and safe human intervention studies (phase 1, Texas, USA and Phase IIa, Ejura, Ghana). Haematology review, serum vitamins E and A minerals (iron and zinc), electrolytes, biomarkers of liver and kidney function all show that novasil has no effect on the bioavailability and use of essential vitamins and nutrients in human adults (Phillips et al., 2008). Palatability studies conducted in adults' in Ghana by Mitchell et al. (2013), demonstrated that, taste, aroma, and texture of food are not affected when novasil is added to traditional Ghanaian meals. Another 14-days randomized trial study in children also reported that, the inclusion of novasil in food can effectively reduce the bioavailability of aflatoxin. (Nicole et al., 2014). Increased exposure to aflatoxin-contaminated foods is one potential variable leading to poor child health (Gong et al., 2002). Aflatoxin toxicity and its effect on children's health have a detrimental impact on the outcome of primary health care in Ghana and elsewhere. Maize, beans and groundnuts, all of which have a high

risk of being contaminated with aflatoxin, are important food crops in Ghana for the preparation of children's meals.

Problem Statement

Food consumed in Ghana are mostly cereal base which has the tendency to be contaminated with aflatoxin which interns affect humans. However one cannot stop eating these cereals.Weanimix, an important nutritious cereal-based food made from beans, groundnuts and maize, was found to contain significant aflatoxin contamination levels over 400 ppb (Kumi et al., 2014). Weanimix is particularly prepared for infants during weaning in Ghana.

In a report by Delle (2016), sixty five (37.79%) out of 172 children studied between the ages of 6-59 months were found to be stunted in the Atwima Kwanwoma district in the Ashanti region of Ghana. Ejura-Sekyedumase district is one of the districts located in the Ashanti region. These findings may, among other factors, be due to exposure to aflatoxin in children.

Previous research has found aflatoxin toxicity among residents of the Ejura-Sekyedumase district in Ghana's Ashanti region (Jolly et al., 2006; Robinson et al., 2012). High aflatoxin exposure concentrations were found in 100% of 180 people who tested positive for aflatoxin B1-albumin biomarker and 75% of those who tested positive for aflatoxin metabolite in urine (Wang et al., 2008).

In a 14 days study in the Ejura- Sekyedumase District, Nicole and her colleagues found high concentrations of aflatoxin M1 in urine of children (Nicole et al., 2014). Children are more susceptible to these toxins than adults. Therefore intervention

studies that present therapeutic strategy to prevent aflatoxin bioavailability through the use of novasil clay in children is worth undertaking

Aim

The aim of the study was to determine the safety and efficacy of novasil over a two-month duration, as well as the relationship between growth indicators and aflatoxin levels in children at risk of aflatoxin-related diseases in Ghana.

Specific Objectives

1. To evaluate the impact of novasil intervention on the haematological and biochemical parameters of the participants.
2. To compare the levels of urinary aflatoxin M1 in participants before and after novasil treatment and monitor adverse effect of novasil.
3. To determine the extent of aflatoxin exposure and its relationship to growth indicators.
4. To evaluate the correlation between aflatoxin exposure and critical micronutrient (calcium) before and after novasil action.
5. To investigate levels of blood glutathione in study participants and its relationship to aflatoxin exposure.

Research Questions

This study was guided by the following research questions.

1. What is the prevalence of stunting among children between the ages of 2-9 years who are exposed to aflatoxin in the Ejura-Sekyeduamse District
2. Will novasil be safe if consumed by children between the ages of 2 -9 years.

3. What is the efficacy of novasil in binding aflatoxin when consumed by children between the ages of 2-9 years over a 60 days intervention period.

Hypothesis

1. Novasil inclusion in food would be safe when administered to children from the ages of 2-9 years over a 60-day duration and will result in reduced bioavailability of aflatoxin.
2. Aflatoxin exposure will have an impact on growth indicators of children

Significance of the Study

Increased exposure to aflatoxin-contaminated foods is one potential factor leading to poor child health. The toxicity of aflatoxin and its effect on the health of children has the potential of negatively affecting Primary Health Care outcomes in Ghana. Data from a previous study in the Ejura-Sekyedumase district indicates that, children in this community are highly exposed to aflatoxin from maize, groundnuts and beans, constituents of weanimix, a formula food to prevent malnutrition (Kumi *et al*, 2014). Besides their adverse effect on the liver and the immune system, aflatoxin is also an anti-nutrient and has been associated with growth disorders in infants and children (Gong et al., 2004). Intervention studies that present therapeutic strategy to prevent aflatoxin bioavailability through the use of novasil among children at risk of high exposure to the toxin are worth undertaking.

Ninety (90) days of human novasil intervention trials in Ghanaian adults previously, recorded a reduction of aflatoxin bioavailability as much as 58 % in biomarker levels matched to the placebo group (Wang et al., 2008). The study also

reported that, novasil was safe in the 90 days period. Urinary aflatoxinmetabolite had a 45 % reduction with 3 g of novasil inclusion in food during two weeks palatability studies in Ghanaian adults (Mitchell et al., 2013). Again, in a 14 day safety and efficacy studies in children by Nicole et al. (2014), it was reported that, novasil inclusion in food at a dose of 1.5 g/day was safe and showed a 52 % reduction in aflatoxin bioavailability compared to the placebo group.

In view of the phase I study in children by Nicole and her group in 2014, it is appropriate to broaden the scope of the study (phase II) to confirm whether novasil will be safe and efficacious when it is used for a longer period in children. This is also to confirm the safety and establish proof of principle that novasil will be efficacious for a period of two months in children. The study also will establish whether aflatoxin is implicated in stunting among children in Ghana.

Delimitations

This research is limited to children of 2-9 years within the Ejura-Sekyedumase District in the Ashanti Region of Ghana. The study was mainly focused on evaluating the safety and efficacy of novasil clay in a clinical intervention trial in children and also to determine the prevalence of stunting in children who are are exposed to aflatoxin in the Ejura Sekyedumase District, Ashanti Region of Ghana

Limitation of Study

Time and budget constraints were the limitations to this study

Definition of Terms

Mycotoxins: Mycotoxins are harmful compounds that are naturally produced by certain types of moulds (fungi).

Bioavailability: Refers to the extent a substance or drug becomes completely available to its intended biological destination(s).

Biosynthesis: Is a multi-step, enzyme-catalyzed process where substrates are converted into more complex products in living organisms.

Aflatoxicosis: Is a disease caused by aflatoxin consumption

The Organisation of the Study

The thesis is presented in five chapters. The first chapter covers the introduction of the study. It comprises the background to the study, statement of the problem, the aim of the study, research objectives, research questions, hypothesis and significance of the study. The chapter also looks at the delimitations, limitations, definition of terms used and organization of the study.

Chapter two comprises the literature review which describe the definition of moulds and their importance and also toxic substances they release. The chapter also provides information about physical and chemical structure of aflatoxin as well as how it is produced, synthesis, metabolised and level of toxicity. The chapter also describes how the various biochemical test are performed and its significance to clinical manifestations in humans in disease progressions. Various detection techniques are also described in this chapter. Prevention of aflatoxin contamination by the use of physical and chemical methods are also mention in this chapter.

Chapter three comprises of the study design, sample size estimation, novasil dose selection, selection criteria for participants, field staff who help in participants recruitment and collection of data and the statistical tool used to analyse the results.

Chapter four is made up of the results and discussion of the study which includes results from novasil adverse effect, anthropometry analysis, biochemical analysis and concentrations of aflatoxin metabolite in urine The results and discussions described the findings and its importance in respect to aflatoxin effect and its management

. Chapter five covers summary, conclusion and reecommendations.

CHAPTER TWO

LITERATURE REVIEW

Moulds

Moulds are a diverse group of fungal species that causes fuzzy appearance and discolourration. Metabolic by-products of moulds can be useful and dangerous to humans and animals. Moulds have been used in cheese production as well as the fermentation of beer and wine in early times (Peraica, Radic, Lucic, & Pavlovic, 1999). Antibiotics and other drugs have been developed using secondary metabolites from the same molds. For example, nearly 5000 years ago, moulds were used by the Chinese for obstetrical purposes (Hesseltine, 1979).

Mycotoxins are made up of variety of organic structures containing a number of heteroatom-containing functional groups. These potent organic chemicals that cannot be seen by the naked eye can be found in mould contaminated foods which can be toxic when large quantities are consumed over a long period. Fortunately, the toxicity of these compounds are dependent on the dose (Phillips, Lemke, & Grant, 2002). The production of mycotoxins may be unavoidable during extended periods of drought, resulting in polluted food products that pose substantial health risks to humans and animals (Huebner, Herrera, & Phillips, 2004; Phillips, Lemke & Grant, 2002; Phillips, Afriyie-Gyawu, Wang, William, & Huebner, 2006; Williams *et al.*, 2004).

Food Contamination by Moulds and Human Disease

Though the term "mycotoxin" was not extensively used until the mid-twentieth century, history shows that mycotoxin poisoning of food and major disease outbreaks related to the consumption of mouldy food occurred on regular basis. Harmful effects by moulds can be traced back nearly 5000 years to ancient Chinese civilizations (Ramsbottom, 1953; Van, & Altenkirk, 1974). Over 300 mycotoxins have been discovered and chemically characterized and connected to the etiology of human and animal disease. Aflatoxins an extensively researched mycotoxins have the ability to cause mutations and cancer (Wild, & Turner, 2002; Wogan, 1992; Wild, & Hall, 2000).

Aflatoxins

Aflatoxins are metabolites of moulds formed by *Aspergillus parasiticus* and *Aspergillus flavus* that contaminate common foods like groundnuts and maize. Fungal growth and toxin production are aided by a combination of bad food storage conditions and hot humid climates (IARC, 2002). Aflatoxin was named after a parasite that produced a poison that triggered a disease in England known as "Turkey X malady" in the year 1960, killing about 100,000 young turkeys (Asao, 1963). The parasite was classified as a fungus and given the name *Aspergillus flavus*, while the toxin was designated as aflatoxin. The fungi *Aspergillus nominus*, *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxin. However, the common aflatoxin producer among them is *Aspergilus flavus* with aflatoxins B1, B2, G1, and G2 are the four main forms (Bradburn, Blunden, Coker, & Jewers, 1993). Aflatoxin M1 and M2 are hydroxylated metabolites of aflatoxin B1 and B2,

and the most common source of aflatoxin B1 and B2 is *Aspergillus flavus*. These fungi can be found mainly in soil and decaying plants.

Aflatoxins in Stored Grains

Scientists have shown changing groupings of various aflatoxins in an assortment of nourishments put away in various conditions including rice, wheat, corn and maize (Jayaraman, & Kalyanasundaram, 1990). Jayaraman and Kalyanasundaram (1990) found that, the recurrence of aflatoxin B1 (AFB_1) expanded with time in wheat from untreated or crude rice and parboiled rice. It was found that, the pace of increment of AFB_1 was a lot higher in crude wheat. The development of aflatoxin under the states of test stockpiling of rice grain showed that AFB_1 and AFG_1 gathered in the rice grain in the presence of moisture was more than or equivalent to 16 % (L'vova , Bystriakova, Merkulov, shatilova, & Kizienko, 1984 ; Park, 2002).

Figure 1: Maize infested with *Aspergilus flavus*
Source: Kumar, Ghewande, & Basu (2000)

In 1994 and 1995, pre-harvest maize contained aflatoxin contamination at 42.5 %

and 30 % respectively, according to Setamou, Cardwell, Schulthess, & Hell (1997). Furthermore, Hell, Cardwell, Setamou, & Poehling (2000) discovered that aflatoxin levels greater than 5 ppb were found in 9.9 % to 32.2 % of maize tested in various ecozones in Benin. Bugs were also accounted for assuming a role in aflatoxin propagation and increase of aflatoxin contamination (Setamou, Cardwell, Schulthess, & Hell, 1998). According to Udoh Cardwell & Ikotun (2000), aflatoxin contamination was found in 33 % of maize received from different regions in Nigeria. Moreover in Ghana, levels of aflatoxin in maize from storehouses and distribution centers ranged from 20 to 355 ppb, while aflatoxin levels in matured maize mixture gathered from major localities ranged from 0.7 to 313 ppb (Kpodo, 1996).

Chemistry and Structure of Aflatoxins

Aflatoxin B1, B2, G1 and G2 and metabolites M1 and M2, are the most significant aflatoxins among 13 compounds documented so far (Borzsonyi, Lapis, Day, & Yamsaki, 1984). On account on their UV fluorescence (blue or green) and chromatographic movement in thin-layer chromatography, the four major aflatoxins were labeled as B1, B2, G1 and G2 (Shapira et al., 1996). Dihydrofuran and tetrahydrofuran moieties are linked to a substituted coumarin in their chemical composition.

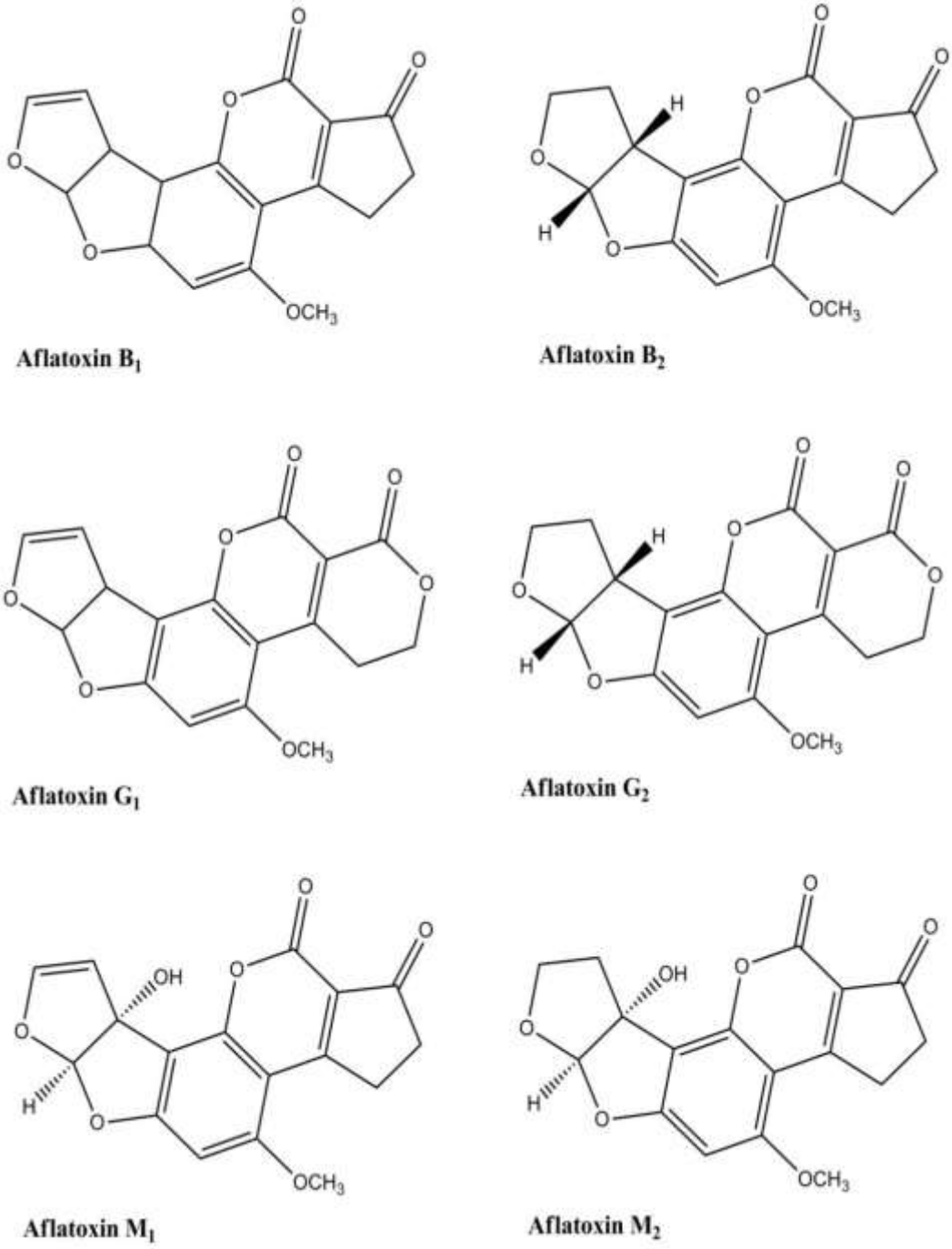

Figure 2: Aflatoxins Structure
Source: Vankayalapati (2018)

Species of *Aspergillus* and Aflatoxin Metabolites

The most common contaminant particularly in agriculture is *Aspergillus flavus*. Aflatoxin is also produced by *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari*, but these are not common (Klich, Mullaney, Daly, & Carry, 2000). The most poisonous is AFB_1, formed by both *Aspergillus flavus* and *Aspergillus parasiticus*, along with aflatoxin B2 (Obuseh et al., 2010; Turner, Moore, Hall, Prentice, & Wild, 2003). In humans and animals, aflatoxin M1 is a metabolite of aflatoxin B1 (Ardic, Karakaya, Ataseve,r & Adiguzel, 2009). Aflatoxin M2, a metabolite of aflatoxin B2 has been found in milk produced by cattle that consume food poisoned with aflatoxin B2 (Prandini et al., 2009). Aflatoxins M1 and M2 are the product of mechanism in animal's liver that aims to make the molecules of the aflatoxin metabolites more hydrophilic so that they can be excreted more easily by the kidney. Other metabolites, such as aflatoxicol, can be produced by biologically reducing aflatoxin B1 as stated by Pawlowski et al. (1977). Other aflatoxins discovered include aflatoxin P1, Q1, B2a, and G2a, which act as biotransforming mammalian products of major metabolites (Heathcote, & Hibbert, 1978).

Properties of Aflatoxins

Physical properties of aflatoxin

Aflatoxin is a crystalline odourless solid that ranges in color from pale white to yellow when isolated. Aflatoxin B1 and G2 have melting points of 268 °C and 190 °C respectively (Waliyar, & Reddy, 2003). In general, the synthesis of aflatoxins appears to be assisted by high pressure of water. *Aspergillus flavus* has been found to grow at temperatures ranging from 10 to 43 °C .Temperatures above 30 °C promote optimal growth. It is not possible to expect an ideal temperature for the development of aflatoxins, in spite of the fact that temperature between 20-30 °C is reported to be fundamentally more appropriate (Pitt, & Miscamble, 1995).

Chemical properties of aflatoxins

Aflatoxins are difuranocoumarins, which have a bifuran group on one side of the coumarin nucleus and a pentanone ring on the other, or a six-membered lactone ring in the case of the aflatoxin B series (AFTs-B series) and a six-membered lactone ring in the case of the aflatoxin G series (AFTs-G series) (Nakai *et al.*, 2008; Bennett, & Klich, 2003). Methanol, chloroform, acetone, and acetonitrile are common polar solvents for these compounds (IARC, 2002). Aflatoxins react with alkaline solutions allowing the lactone moiety to be hydrolyzed. Since recyclization occurs after acidification of solutions containing aflatoxin, this hydrolysis is reversible as noted by (Scott, King, Richard, & Greer, 1993). Ring opening followed by decarboxylation happens at temperatures above 100 °C, and the response may proceed, resulting in the loss of the methoxy group

from the aromatic ring. The acid catalyzes the expansion of hydroxyl through the bond in the furan ring in the presence of mineral acids, aflatoxins B1 and G1 are converted into aflatoxin B2A and G2A. At the sight of acidic anhydride and hydrochloric acid, the reaction continues to acetoxy derivative. With formic corrosive thionyl chloride and trifluroacetic corrosive thionyl chloride, comparative adducts of aflatoxin B1 and G1 are formed (Heathcote, & Hibert, 1978).

Table 1

Properties of Aflatoxins

Aflatoxin	Molecular Weight (g/ mol)	Formula	Melting point (°C)	Name
AFB_1	312.3	$C_{17}H_{12}O_6$	268–269	2,3,6a,9a-tetrahydro-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzo-pyran-1,11-dione
AFB_2	314.3	$C_{17}H_{14}O_6$	286–289	2,3,6aa,8,9,9aa-Hexahydro-4-methoxycyclopenta(c)furo(2',3':4,5)furo(2,3-h)chromene-1,11-dione
AFG_1	328.3	$C_{17}H_{12}O_7$	244-246	7AR,cis)3,4,7a,10a-tetrahydro-5-methoxy-1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)chromene-1,12-dione
AFG_2	330.3	$C_{17}H_{14}O_7$	237–240	1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)(1)benzopyran-1,12-dione
AFM_1	328.3	$C_{17}H_{12}O_7$	299	(6AR-cis)-2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzopyran-1,11-dione
AFM_2	330.3	$C_{17}H_{14}O_7$	293	2,3,6a,8,9,9a-Hexahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1) benzopyran-1,11-dione

Source: Properties of aflatoxin, Vankayalapati (2018).

Conditions for Aflatoxin Synthesis and Production

Aflatoxins are produced during parasitic activity in the course of harvest, stockpiling and food handling. The Food and Drug Administration (FDA) of the United States considers it as inevitable food contaminant (Williams et al., 2004). Aflatoxins can be identified with moistness which impacts the dampness substance of grains. Normal moistness can be used to anticipate aflatoxin generation (Dabbert, & Oberheu, 2001; Williams et al., 2004). When the temperature is between 24 °C and 35 °C, humidity is 18 %, and moisture is >7 %, *Aspergilus parasiticus* and *Aspergilus flavus* species produce these toxins as auxiliary metabolites (L'vova et al., 1984; Williams et al., 2004).

Aflatoxin activity is influenced by physical and natural factors. The activity of aflatoxin are known to be delivered at a temperature of 25 °C to 35 °C, with an acidic pH, relative stickiness of 83 % to 88 %, and a suitable degree of carbon dioxide and oxygen. For example, 20 % CO_2 and 10 % O_2 in the air have a negative impact on aflatoxin production. (Adebanjo, & Bankole, 2003). As far as natural factors are concerned, glucose, sucrose, and fructose are the preferred environment for aflatoxin production. Zinc and manganese are also important for aflatoxin growth. A combination of cadmium and iron however, will stop the growth of the mould and thus affects aflatoxin formation (Gilbert, & Anklam, 2002).

Biosynthesis of Aflatoxins

Polyketide synthase produces norsolorinic acid, an anthraquinone precursor using a biosynthetic pathway. It follows a post-polyketide

synthase series of about 15 steps, producing progressively toxic metabolites (Cleveland, & Bhatnagar, 1992; Hicks, Shimizu, & Keller, 2002). Sterigmatocystina is an associated dihydrofuran toxin (mutagenic and tumorigenic) that is formed as a final biosynthetic product by a variety of organisms, including *Aspergillus versicolor* and *Aspergillus nidulans* (Berry, 1988). Analysis of molecular genetics of sterigmatocystin biosynthesis in the genetically tractable species of *Aspergillus nidulans* offered a valuable model system. The cognate genes for aflatoxin pathway enzymes in *Aspergillus flavus* and *Aspergillus parasiticus* are very similar to the genes of the sterigmatocystin pathway (Payne, & Brown, 1998; Yu, Chang, Bhatnagar, & Cleveland, 2000). According to Cary and colleagues (2001) and Hicks, Shimizu, & Keller (2002), the organization of genes for the sterigmatocystin-aflatoxin pathway in *Aspergillus flavus*, *Aspergillus nidulans*, and *Aspergillus parasiticus* have been studied. The aflatoxin pathway has been silenced in both *Aspergillus sojae* and *Aspergillus oryzae* due to deletions and other genetic defects (Bennett, & Klich, 2003; Takahashi et. al., 2002). A type-1 polyketide synthase (PKS) and a pair of specialized fatty acid synthase subunits (FAS-1 and FAS-2) from a complex to synthesize norsolorinic acid. Two substrates, 1-Acetyl-CoA and 9- Malonyl-coA contribute to aflatoxin biosynthesis. A reaction sequence, norsolorinic acid (NA)-->averantin (AVN)-->5'-hydroxyaverantin (HAVN)-->averufin (AVR), is the early part of a biosynthetic pathway for aflatoxins. Synthesis of aflatoxin requires about 15 steps (Figure 3).

This is followed by a sequence of about 15 redox reaction that is often mediated by cytochrome p450 enzymes. The biosynthesis pathway proceed under the following steps:

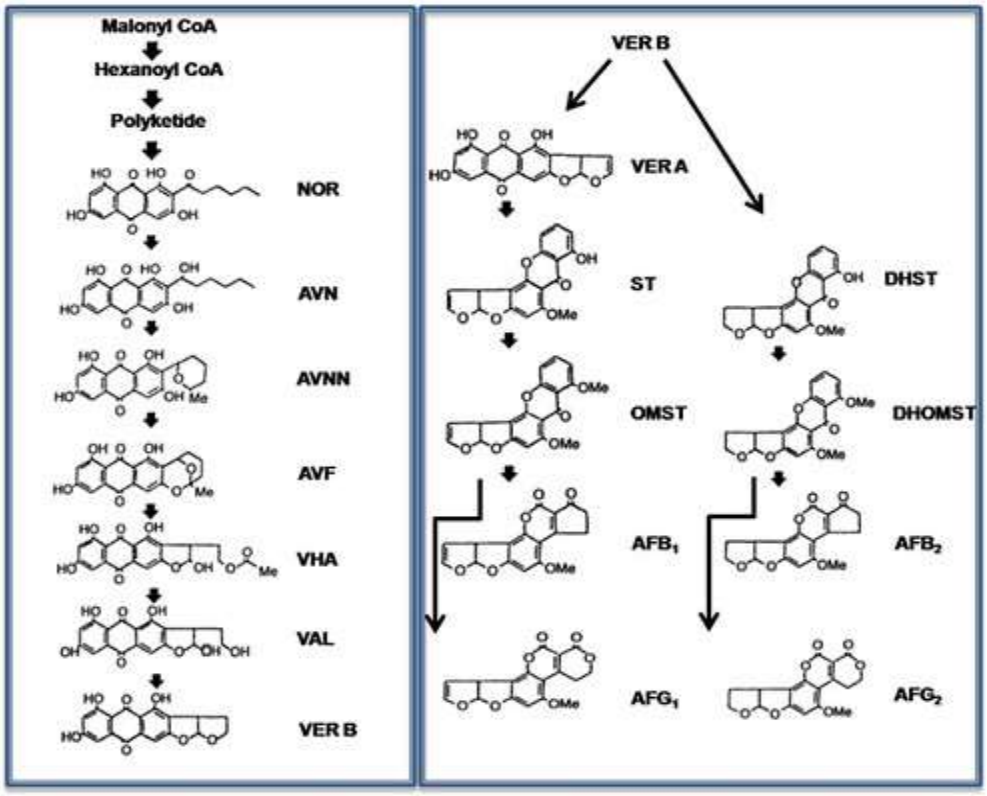

Figure 3. Schematic diagram of aflatoxin biosynthesis pathway.
Source: Caceres et al. (2020)

Synthesis of norsolorinic acid (NOR)

The production of norsolorinic acid is controlled by three proteins: fatty acid synthase (aflA), fatty acid synthase (aflB), and polyketide synthase (aflC). Norsolorinic acid by integrating with malonyl-CoA molecules, plays an important role in the synthesis of the hexanoyl primer. The hexanoyl primer is then transferred to the -ketoacyl synthase region (Herbst, Townsend, & Maier, 2018) and combined with malonyl-CoA to form norsolorinic acid anthrone (NAA). This metabolite is

rapidly converted into NOR by NAA oxidase due to its high reactivity (Frisvad et al., 2019). Norsolorinic, an essential metabolite synthesized in the AF biosynthetic pathway, has a red–orange color in mutant strains of aflatoxin D of *Aspergillus parasiticus* (Moon, Kim, Chun, & Lee, 2018).

Conversion of norsolorinic acid to averantin (AVN)

Aflatoxin D (AflD) protein, which functions as a ketoreductase, converts the NOR 1'-keto group to the AVN 10 -hydroxyl group (Wu et al., 2015). Notwithstanding the fact that its function is known, the mutant strain of aflD does not always result in AVN formation. The other processes that contribute to this reduction are unknown at the moment.

Conversion averantin to 50 -hydroxyaverantin (HAVN)

The breakdown of the 50 -keto group of AVN to the 50 -hydroxyl group of HAVN is catalysed by aflatoxin G (AflG) protein, monooxygenase of cytochrome P450. (Yabe, Hatabayashi, Ikehata, Zheng, & Kushiro, 2015)

Conversion of 50 –hydroxyaverantinv to averufin (AVF)

The dehydrogenation of the HAVN's 50-hydroxyl group to the 50-oxide group of oxoaverantin (OAVN) is facilitated by the HAVN dehydrogenase enzyme (Jahanshiri, Shams-Ghahfarokhi, Allameh, & Razzaghi-Abyaneh, 2015). The ability of the deleted aflatoxin H protein mutant to synthesize OAVN is consistent, implying the involvement of other potential mechanisms (Sakuno et al., 2005).

Conversion of averufin to versiconal hemiacetal acetate (VHA)

Aflatoxin V protein can reduce the hydride group of AVF as a cytochrome P450 oxidoreductase (Wang et al., 2017). The expected compound becomes hydrated, aflatoxin l protein then functions presumably as an oxidoreductase (Li, Muhammad, Yu, Sun, & Zhang 2019). However, aflatoxin W protein monooxygenase is required for the incorporation of O_2 atoms within the 4'-50 ketone groups of HAVN, resulting in VHA.

Conversion of versiconal hemiacetal acetate (VHA) to Versiconal (VAL)

Aflatoxin J protein, an esterase enzyme stimulates VHA acetate suppression, that results in the conversion of VHA to VAL (Kolawole, Meneely, Petchkongkaew, & Elliott, 2021)

Conversion versiconal to versicolorin-B (VERB)

The cyclase that catalyzes the conversion of cyclodehydration of VAL into VERB is aflatoxin K protein (Conradt, Schatzle, Haas, Twonsend, & Muller, 2015). Because the bisfuran ring closes at this point, this conversion phase is critical in the aflatoxin biosynthetic pathway. This phase also serves as the final precursor for the aflatoxin B1 to aflatoxin G1 and aflatoxin B2 to aflatoxin G2 in the biosynthetic pathways. Aflatoxin L protein, a monooxygenase of cytochrome P450, A (VERA)-AFB1-AFG1 pathway is responsible for converting the tetrahydrofuran ring to a dihydrobisfuran ring (Hosseini et al., 2020)

Versicolorin-A conversion to sterigmatocystin and versicolorin-B Conversion to Dihydro Demethylsterigmatocystin (DHST)

Versicolorin B (VERB) is used as a substrate rather than Versicolorin-A (VERA), resulting in Dihydrosterigmatocystin (DHST) formation in the synthesis of AFB_2-AFG_2. Aflatoxin O protein, an O-methyltransferase, is responsible for transmitting the S-adenosylmethionine methyl group in the Conversion of DMST to Sterigmatocystin (ST) and (DHST) (Keller, 2015).

Conversion of O-Methylsterigmatocystin (OMST) to Aflatoxin B1 (AFB_1) and Dihydro-O-methylsterigmatocystin (DHOMST) to Aflatoxin B2 (AFB_2)

Aflatoxin O (AflO) protein, another cytochrome P450 monooxygenase, converts OMST into AFB_1. Yu (2012) proposed a comprehensive metabolic pathway for aflQ replication in C-11 hydroxylation. Aflatoxin L protein may provide oxygen to the keto-tautomer 11-hydroxy of OMST. These reactions however may end in the formation of 370 da metabolites. It is assumed that the aflatoxin Ma protein together with a cytochrome P450 is responsible for demethylating the A-ring as the final stage of the aflatoxin biosynthesis pathway. The 370-da metabolites may act as substrates in the oxidation of aflatoxin U protein, which results in the synthesis of AFG1 and AFG2 (Callicott, & Cotty, 2015). Thus, the NadA gene and aflF protein could be good candidates for increasing aflU activity in the production of aflatoxin G1 and aflatoxin G2.

Absorption, Distribution, Metabolism and Aflatoxin Excretion

Aflatoxins are highly soluble and easily absorbed into the bloodstream from exposure site, typically via the gastrointestinal and respiratory tract Aflatoxin exposure occurs in animals and humans in two ways. First, they are ingested from feed and other animal tissues (Agag, 2004). The second way of aflatoxin exposure is by inward breath of residue particles of aflatoxins particularly aflatoxin B1 in polluted food sources in businesses and industrial facilities (Coulombe, 1994). After entering the body, they are transported to different tissues through blood circulation and then to the liver, which is the primary organ for xenobiotic digestion. Aflatoxins are metabolized by the liver mostly, into a reactive epoxide intermediate or hydroxylated into less toxic aflatoxin M1 (Wild, & Montesano, 2009; Wu, & Khlangwiset, 2010). Aflatoxin-8, 9-epoxide is metabolized by cytochrome P450 (CYP450) microsomal catalysts in humans and animal species to aflatoxin-8, 9-epoxide that binds albumin in the blood forming adducts that causes damage to the DNA. The epoxide formed which can also react with proteins to effect acute toxicity or with DNA to cause liver cancer. (Wild, & Montesano, 2009; Wu, & Khlangwiset, 2010). The human CYP450 isoforms CYP3A4 and CYP1A2 are involved in the digestion of AFB_1. The biotransformation of AFB_1 to reactive exo-8, 9-epoxide is catalyzed by these two enzymes (CYP3A4 and CYP1A2) Cytochrome P 1A2 is responsible for epoxidation of AFB_1 to produce high proportion of endo-epoxide and hydroxylation of AFB_1 to form aflatoxin M1, although is a frail epoxidation substrate (Guengerich et al., 1998) and is less effective than AFB_1 (Wild, &Turner, 2002). In human fetal liver, CYP3A4 is a

major CYP450 protein isoform that metabolizes AFB_1 to the 8, 9-epoxide, which can be fetal (Kitada et al., 1998) and then to aflatoxin Q1 (AFQ_1), a less toxic metabolite. The CYP3A4 protein is the main CYP450 enzyme that converts AFB_1 to an epoxide structure. Aflatoxin B1 is primarily converted to exo-epoxide by CYP3A5, although some AFQ_1 is also converted (Wang et al., 1998). In spite of this, polymorphism studies with CYP3A5 have revealed that, many people especially, Africans express this protein isoform (Wild, & Turner, 2002). Aflatoxin crosses the placental barrier and moves to the fetus, where it disrupts critical steps of development according to studies in Gambian children (Wild, & Turner, 2002).

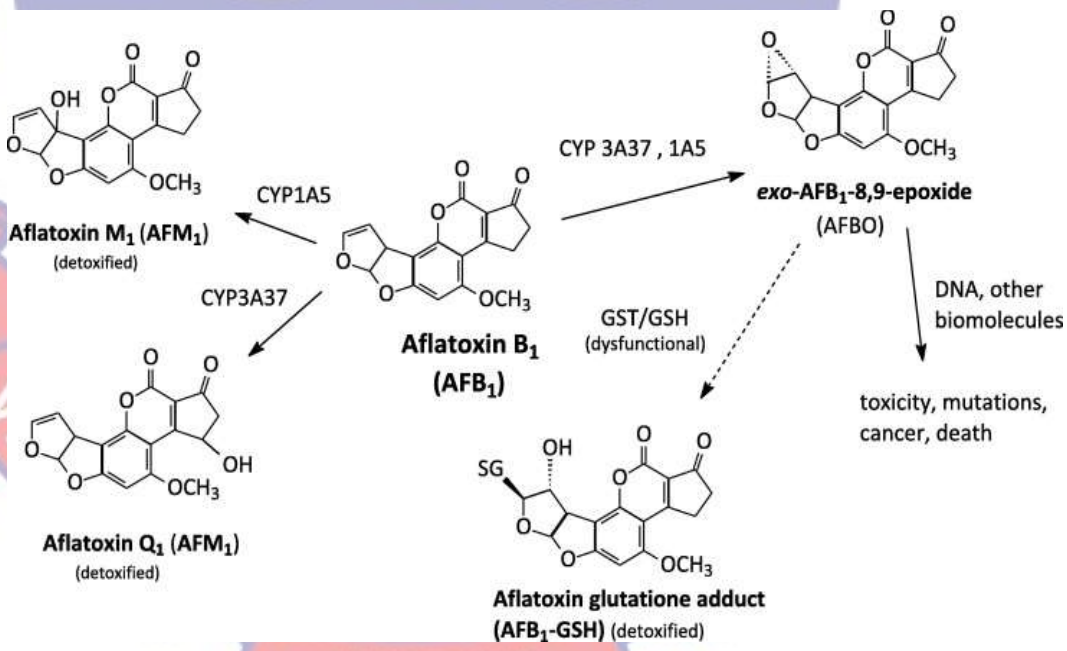

Figure 4: Metabolism of aflatoxin B1 with biomarkers and metabolites
Source: Wild, &Turner (2002)

The epoxidation of AFB_1 to exo-8, 9-epoxide is a fundamental stage in this cancer-causing agent's (AFB1) genotoxic pathway (Raney, Harris, & stone, 1993).

Aflatoxin B1 binding to DNA and AFB_1 exo-8, 9 epoxide DNA adduction have also been linked to practical variations in DNA. The epoxide, extremely unstable forms aflatoxin-N7-guanine when it binds to guanine bases in DNA (Guengerich, 2001). The aflatoxin-N7-guanine has been shown to be capable of influencing the p53 silencer quality in the cell cycle by shaping guanine (purine) to thymine (pyrimidine) transversion transformations in DNA (Bailey, Iyer, Stone, Harris, & cihla, 1996). When there are DNA transformations or signal apoptosis, the p53 gene is essential in stopping cell cycle development. The mutations are thought to have a greater impact on other base pair locations than those in the third base of codon 249 of p53 quality, in the region that corresponds to related proteins and the DNA binding space (Sudakin, 2003). Aflatoxin B1 causes a base G to T transversion in codon 249's third location, and relative mutations is seen in hepatocellular carcinoma (HCC) in people who consumed food with high aflatoxin concentration in East Asia and Africa (Mace et al., 1997).

Glutathione-S-Transferase (GST) and epoxide hydrolase are part in detoxification of AFB_1 in the liver. The GST-catalyzed conjugation of glutathione to AFB_1-8, 9-epoxides, on the other hand, plays a key role in preventing epoxide binding to target macromolecules including DNA and cell proteins (Sherratt, & Hayes, 2019). In the detoxification of AFB_1, the glutathione pathway plays a critical role (Farombi, & Nwaokeafor, 2005). Glutathione forms the exo and endo epoxides of AFB_1 8, 9 to form AFB-mercapturate, this reaction is catalyzes by GST (Farombi, & Nwaokeafor, 2005). Transfer of glutathione-aflatoxin formation from cells is done very fast with an ATP-subordinate multidrug-resistance protein.

Notwithstanding, proclivity for forming a more mutagenic AFB_1 exo-epoxide isomers, moderate limit for GST-catalyzed bio-initiated AFB_1 detoxification in the lungs may be a significant factor in the lung's susceptibility to AFB_1 toxicity (Whitlow, Hagler, & Diaz, 2002).

Epoxide hydrolases (EHs) metabolize highly reactive epoxides with mutagenic and carcinogenic potential to the less reactive corresponding diols and are therefore traditionally viewed as detoxification enzymes during the metabolism of aflatoxin b1(Sherratt, & Hayes, 2019).

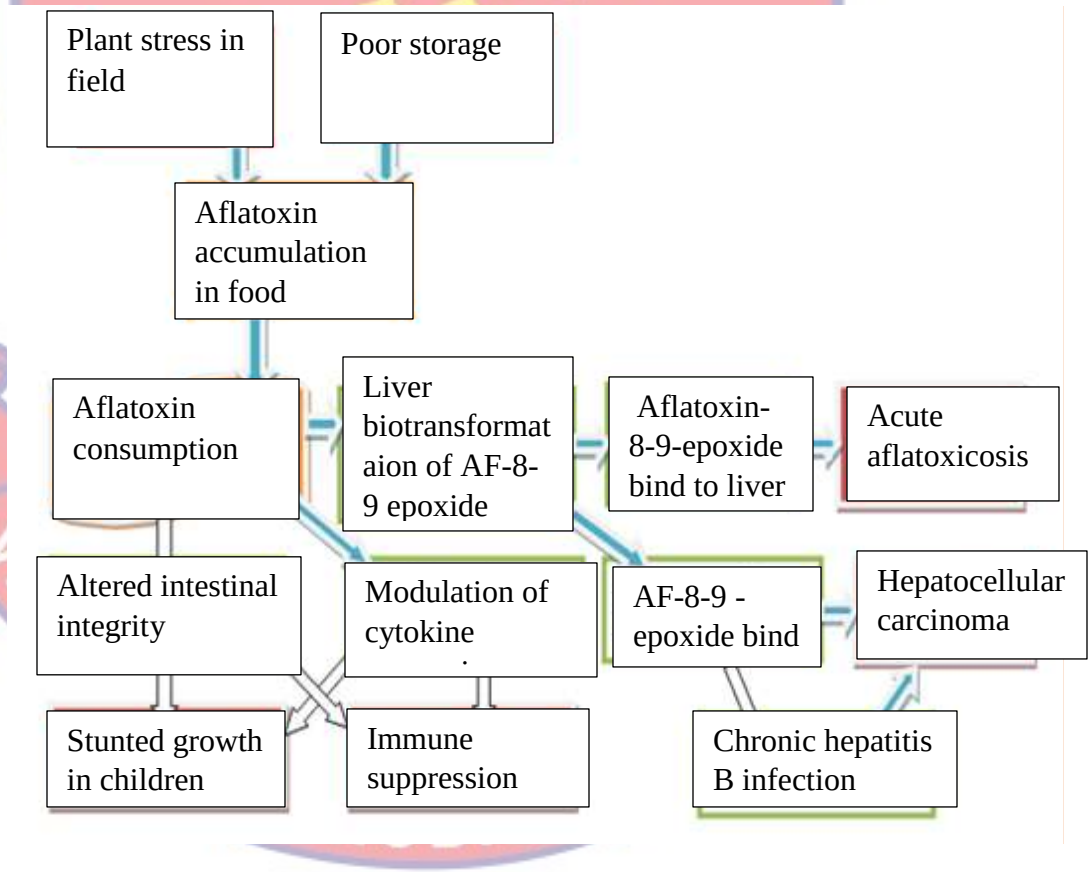

Figure 5: Human Aflatoxin Disease Pathways
Source: Wu, & Khlangwiset (2010).

Toxicity of Aflatoxins

The International Agency for Research on Cancer evaluated the cancer-causing potential of aflatoxin as human group 1 cancer-causing agents (IARC, 1993). *In vitro* studies show that all naturally occurring aflatoxins (B1, B2, G1, and G2), including AFM_1, cause DNA damage in some way (Cullen, & Newberne, 1994). In rat and human cells used for *in vitro* studies, AFB_1 is said to cause chromosomal aberrations, unscheduled DNA union, breaks of DNA strand and the formation of DNA-adducts (IARC, 1987). In addition, *in vivo* studies described the liver as the most common organ to be damaged by AFB_1 in the case of hepatocarcinoma (Miller, & Wilson, 1994).

Toxicity in animals

After the occurrence of "Turkey X disease" (characterized by severe liver toxicity in turkeys) in the year 1960 in England, numerous intensive studies have been directed to determine lethal dose 50 (LD_{50}) values for animals (Council for Agricultural Science and Technology, 1989). Aflatoxins effect on dosimetry, age and nutritional quality are species-specific because of the dependence on metabolic rates (Miller & Wilson, 1994; Newberne, 1986). Ducklings, hares, rainbow trout, and felines, for example, are very sensitive to AFB_1 (Muller, Carlson, Semeniuk, & Harshfield, 1970), while some mouse strains are moderately resistant to AFB_1 in developing cancer (Newberne et al., 1982). Nonetheless, Cullen, & Newberne (1994) found that, weaning rodents are more vulnerable to fatal effects of AFB_1 than other animals (1 year or more). Male rodents are also more sensitive to AFB_1's toxic effects than females (Cullen, & Newberne, 1994). Aflatoxins contributes to

intense toxic effect, especially hepatitis and hepatic disease, in various animal species (Smith & Hamilton, 1970). Animals fed with aflatoxins frequently brings about poor development, weakness and death (Jones, Hagler, & hamilton, 1982; Huff, Kubena, Harvey, & Phillips, 1986; Smith, & Hamilton, 1970). For example, dietary aflatoxin exposure may cause weight loss, uncomfortable hair coat, anorexia, ataxia, tremors, unconsciousness and death in feeder pigs (Coppeck et al., 1989). In dairy cows, ingestion of feed contaminated with aflatoxin is linked to low milk production, tachycardia, and death (Cockcroft, 1995) due to a metabolite called AFM_1 (Hsieh, 1985). Ingestion of dietary aflatoxins has been linked to intense putrefaction and bile conduit expansion in turkeys. Jaundice, stomach pain, edema, anorexia and death are clinical signs of severe aflatoxicosis in hounds (Ngindu et al., 1982). Additional reports demonstrated that, rodents fed with AFB_1 contaminated feed with AFB_1 showed signs of hepatic and renal wounds, gastrointestinal cancers, urogenital danger and liver cancer (Butler, & Hempsall, 1981; Clifford, & Rees, 1967; Goerttler, Lohrke, Schweizer, & Hesse, 1980). Intense and interminable presentation to enormous dosages of AFB_1 can lead to liver problems and death in animals (Roebuck, & Maxuitenko, 1994). Sub-lethal levels of aflatoxins can result in continuous poisoning and results in malignant growth. (Sinnhuber, Hendricks, Wales, & Putman, 1977; Wogan & Newberne, 1967) and liver disease in other animals (Busby, & Wogan, 1984). Aflatoxin (mostly AFB_1) has a variety of effects in animals, including severe hypoproteinemia

immunosuppression, haemorrhagic sickness and sores, impairment blood coagulation, greasy liver and teratogenic effects (CAST, 2003; Cysewski, Wood, Pier, & Baez, 1978; Doerr, Huff, Tung, Wyatt, & Hamilton, 1974; Hayes, 1981; Robens, & Richard, 1992).

Toxicity in humans

Intake of 2-6 mg aflatoxin per day for a month has been shown to cause severe hepatitis and death in humans (Patten, 1981). Aflatoxin poisoning manifests itself in humans primarily as severe hepatitis and is frequently linked to the consumption of contaminated cereals, especially maize and peanuts (Ngindu et al., 1982; Krishnamachari, Bhat, Nagarajan, & Tilac, 1975; Shank, 1977). People who demonstrate intense aflatoxin poisoning normally will have symptoms, which include fever, jaundice, gastrointestinal problems, edema, loose bowels, fatty liver, stomach upset, liver damage and sometimes death (CAST, 2003; Ngindu et al., 1982). An extreme episode of intense hepatitis occurred, because of dietary aflatoxin exposure that happened in India in 1974 (; Krishnamachari, Bhat, Nagarajan, & Tilac, 1975). The death rate of patients who consumed aflatoxin-depleted corn at a concentration of 0.25-15 mg/kg was 25 % during the epidemic. Aflatoxin B1 levels were detectable in liver tissues taken from patients who died, indicating that they had been exposed to aflatoxin contaminated food (CAST, 2003).

A deadly hepatitis flare-up in Kenya, which was attributed to the ingestion of aflatoxin-degraded maize, was another episode that solidified the proof of extreme lethality (Ngindu et al., 1982). Aflatoxin B1 as high as 8,000 ppb were

discovered in 31 samples including maize and maize products from family units in influenced networks, (CDC, 2004). Aflatoxin exposure in children from contaminated food has been associated to Kwashiorkor and Reye's disorder (Hendrickse et al, 1983). Hendrickse et al., (1983), examined tissues of liver from 36 children with Kwashiorkor and identified aflatoxins as the cause of the disease. A hepatitis outbreak in 400 people in India in 1974 resulted in approximately 100 deaths. Aflatoxins were found in contaminated maize with *Aspergillus flavus* up to 15,000 ppb which were responsible for the deaths (Montville, & Matthews, 2008). In the year 2004, one of the most dangerous occurrence of aflatoxin poisoning happened in Kenya, resulting in 125 deaths and 317 cases. The cause of the outbreak was aflatoxin contaminated maize, with aflatoxin B1 concentration as high as 4,400 ppb (Lewis et al., 2005).

Hepartocarcinoma (HCC) is said to be the sixth most commonly reported disease and the third leading cause of cancer death worldwide with 905,677 new cases annually (IARC, 2020). Approximately 80 % of HCC cases happens in developing countries (Wild, & Hall, 2000). Globally, there is a low frequency HCC cases occurring in the Western world, particularly the United States and Western Europe (4 cases per 100,000 people); and a high occurrence of up to 150 cases per 100,000 people, particularly in parts of Sub-Saharan Africa, China, and South East Asia (Manns, & Kubicka, 1997). Epidemiological research in Asia and Africa has revealed a link between HCC and aflatoxin levels in the diet (Jaimez et al., 2000).

The occurrence of aflatoxin and hepatitis B and C, are seen mostly in Asia and sub-Saharan Africa (Qian et al., 1994). Aflatoxin can have synergistic, effect with hepatitis B viral infection, expanding the general danger of liver disease (Jackson, & Groopman, 1999; Qian et al., 1994; Turner et al., 2000). Regardless of the relative risk, the intensity of aflatoxin exposure in people with hepatitis B virus is approximately 30 times higher than in people without hepatitis the B virus (Henry, Bosch, & Bower, 2002).

Other unfriendly impacts that have been connected to aflatoxin presentation is the hereditary change from GC to TA transversions at codon 249 of tumor suppressor p53 protein (Eaton, Ramsdell & Neal, 1994; Smela, Currier, Bailey,& Essigmann, 2001; Wang et al., 1999). It is additionally reported that, even at low temperatures aflatoxin can cause disturbances in the cell and humoral immune response and renders the body's ability fight viral bacterial and other infectious diseases (Miller, & Wilson, 1994 & Turner, Moore, Hall, Prentice, & Wild, 2003). In developing nations aflatoxins levels in humans do not ordinarily happen at levels that can cause intense or clear aflatoxicosis.

Aflatoxin Influence on Selected Nutrients

Prolonged aflatoxin exposure has been shown to affect the nutritional status of livestocks. Aflatoxin has been indicated to reduce nutrients such as vitamin A in the liver (Pimpukdee et al., 2004) and vitamin D in the plasma significantly in poultry (Glahn et al., 1991). Vitamin A deficiency is associated with compromised immune responses and increased disease susceptibility in poultry (Aye et al., 2000; Dalloul, Lillehoj, Shellem, & Doerr, 2002). Vitamin A is necessary for good eye

sight, hematopoiesis epithelial cells, regulation of digestive and respiratory cells, nervous system and immune system, (Gursu, Sari, Sahin, & Sahin, 2002). Vitamin A stimulates lymph proliferation which intends stimulate the immune system. Deficiency of vitamin A reduces the production of specific antibodies and number of circulating lymphocytes, (West, Rombout, Van der Ziypp, & Siytsma, 1991). Vitamin A deficiency contributes to the primary cause of childhood blindness, morbidity and mortality from common infections in developing countries (Fieldler et al., 2000).

Iron (Fe) is important to the body as a cofactor in many metabolic and enzymatic processes (Lehninger, Nelson, & Cox, 1997a). Iron is needed for heme protein synthesis, which includes haemoglobin and myoglobin, metalloenzymes and cytochromes (George-Gay, & Parker, 2003). Iron deficiency (FeD) is one of the world's most common nutritional deficiency issues. Impaired cognitive development, health, anaemia and aerobic work ability in humans have all been linked to iron deficiency (Rogers et al., 2002). Aflatoxin has been shown in animals to have an effect on the physiological levels of iron in serum (Dimri, Rao, & Joshi, 1994; Harvey et al., 1988), but similar studies in humans are not readily available.

Zinc (Zn) is necessary in the body as a cofactor in many enzymes and metabolic functions (Lehninger, Nelson, & Cox, 1997a). Zinc boosts human immunity and cognitive development. Although the role of aflatoxins in human zinc deficiency is unclear, animal data suggests that aflatoxins, particularly during early childhood, may increase the degree of zinc deficiency. (Mocchegiani et al., 1998; Kalorey, Daginawala, Ganorkar, & Mangle, 1996). Further research is

warranted to determine the extent of aflatoxin's effect on zinc in humans, as zinc has a significant impact, particularly in developing countries.

Biochemical Test

Liver chemistries

Liver function test, is a group of tests in the blood that offer information regarding the health of the liver (Lee, 2009). Albumin, bilirubin and other measurements are all part of the liver function test. In patients with any degree of liver injury, the liver transaminases (aspartate transaminase and alanine transaminase) are useful biomarkers of liver damage (Johnston, 1999; Mengel, & Schwiebert, 2005;McClatchey, 2002). Majority of liver disorders have mild symptoms at first, but should be diagnosed in a timely manner. Many diseases may be exacerbated by hepatic (liver) involvement. Some measurements are associated with functionality. Example, albumin for cellular integrity. Some of these methods do not assess activity, they are more suitably referred to as liver chemistries instead of liver function tests (Kwo, Cohen, & Lim, 2017). People with hepatic dysfunction can be assessed and treated using a variety of biochemical tests. These tests can be used to diagnose liver disease, differentiate between different types of liver disease and evaluate the severity of liver disorder and track treatment. These tests are also performed on people who are taking medications to ensure that the medications aren't having a negative impact on the individual.

Total bilirubin

Bilirubin (unconjugated and conjugated) are both measured in total bilirubin. Heme (a component of hemoglobin found in red blood cells) is broken down into unconjugated bilirubin. The organ in charge of removing unconjugated bilirubin from the bloodstream is the liver by modifying (making it water-soluble) with an enzyme called Uridine 5'-diphospho glucuronyl-transferase. A total bilirubin level > 17 µmol/L indicates disease of the liver. When total bilirubin levels exceed 40 µmol/L, bilirubin deposition on the skin and mucous membranes causes jaundice by turning these areas yellow (Shivaraj et al., 2009). Overproduction, decreased hepatic absorption of unconjugated bilirubin, and reduced bilirubin conjugation are all contributors to the rise of in mainly unconjugated bilirubin. Overproduction may occur as a result of ineffective erythropoiesis and haematoma's reabsorption which results in greater destruction of red blood cells (Shivaraj et. al., 2009). Whiles an increase in serum bilirubin levels has been identified in animal species after exposure to aflatoxin. No data on associations between aflatoxin exposure and bilirubin levels in humans is readily available (Clark, Hatch, Miller, & Jain 1984; Cliford, & Rees, 1967).

Alanine transaminase

High levels of alanine transaminase (ALT) is found in the liver. It is also present in muscles, kidneys and heart. Different types of liver disorders can cause an increase in ALT levels. Hepatitis, injury and toxins can all lead to liver damage. Hepatitis C causes a greater increase in ALT levels than Hepatitis A and B. Chronic hepatitis is described as an ALT elevation that lasts longer than six months.

Increased ALT levels have been linked to alcoholic liver disorder, non-alcoholic fatty liver disease (NAFLD), fat accumulation in the liver during childhood obesity and steatohepatitis (Shivaraj et al., 2009). A rise in ALT is also linked to a decrease in insulin sensitivity, decreased glucose tolerance and higher levels of triglycerides and free fatty acids (Shivaraj et al., 2009). Aflatoxin B1 (AFB1) and ALT was measured in 50 blood samples collected from humans in Saudi Arabia, ALT levels were found to be increased in patients with increased aflatoxin levels (Randa, Dujana, Hye-Joo, & Afaf El-Ansary, 2018).

Aspartate transaminase

The isoenzyme aspartate transaminase (AST) is found in two forms: mitochondrial and cytoplasmic. The highest concentration of AST is found in the liver, followed by the heart, muscle, kidney, brain, pancreas, and lungs. (Kasper et al., 2018). Aspartate transaminase is a less precise measure of liver disorder than ALT because of the large variety of organs that produces it. An increase in mitochondrial AST in the blood indicates tissue necrosis in myocardial infarction and chronic liver disease (Shivaraj et al., 2009). The mitochondrial form of the isoenzymes contributes more than 80 % of AST activity in the liver, while the cytoplasmic form of AST contributes 80 % of the circulating AST in the blood. The AST level is particularly high in people who have liver cirrhosis. Mean AST was found to be higher (above normal range) in patients attending hospital in Kumasi with significant levels of levels aflatoxin metabolite (M1) (Afum et al., 2016).

Gamma glutamyl transpeptidase

Gamma glutamyl transferase (GGT) is found in hepatocytes, biliary epithelial cells, renal tubules, pancreas and intestines. Gamma-glutamyltransferase is a microsomal enzyme that aids in glutathione metabolism by conveying peptides across the cell membrane. When cholestasis is present, GGT levels are normally elevated. (Kasper et al., 2018).

Gamma-glutamyltransferase levels in acute viral hepatitis will peak in the second and third weeks of illness and remain elevated for up to six weeks. Gamma-glutamyltransferase levels are also increased in 30 % of hepatitis C patients. Gamma-glutamyltransferase increases tenfold in people with alcoholic liver disease and two to three times in 50 % of people with non-alcoholic liver disease. In a study to estimate the hepatotoxicity of AFB_1 in workers occupationally exposed to wheat flour dust in Egypt. Statistical analysis of gamma-glutamyl transpeptidase (GGT) levels in workers exposed to aflatoxin were significantly higher compared to their controls (Mohgah et al., 2014).

Albumin

Albumin is a protein that is exclusively produced by the liver. It is the body's most abundant protein (the remaining elements are primarily globulins). Liver disease, such as cirrhosis, causes a decrease in albumin levels. It's also lower in people with nephrotic syndrome because it's excreted in the urine (Smith, & Susan, 2017). Decreased albumin can cause oedema, because of the intravascular oncotic pressure being less than the extravascular space. For detecting acute changes, prealbumin measurement is superior to albumin measurement (half-lives

of albumin and prealbumin are around 2 weeks and 2 days, respectively). Aflatoxin binds to albumin in the blood to from aflatoxin albumin adduct. Serum albumin decreased significantly at the smallest aflatoxin dose in chicken (Tung, Wyatt, Thaxton, & Hamilton, 1975). Aflatoxin an active metabolite binds randomly to template deoxyribonucleic acid and inhibits the larger transcribing units such as those for serum albumin and lipids before the smaller transcribing units are inhibited (Tung, Wyatt, Thaxton, & Hamilton, 1975)).

Alkaline phosphatase

Alkaline phosphatase (ALP) is an enzyme found in the cells of biliary ducts of the liver. It's also found on the mucosal epithelium of the small intestine, the proximal convoluted tubule of the kidneys, bone, liver, and the placenta. Alkaline phosphatase required for lipid transposition and bone calcification in the small intestine. Bone accounts for half of all serum ALP function in the blood. Alkaline phosphatase levels normally will be elevated in acute viral hepatitis. Hepatitis A, for example, has elevated ALP due to cholestasis characterized by excessive itching. A mild increase in ALP is caused by cirrhosis, hepatitis, and congestive heart failure. Temporal hyperphosphataemia is a harmless disease that resolves in 4 months in children. Alkaline phosphate levels are low in hypothyroidism, pernicious anemia, zinc deficiency and hypophosphatasia (Shivaraj et al., 2009). Statistical analysis of alkaline phosphatase levels in workers the exposed to aflatoxin were significantly higher compared to their controls in a study to evaluate the hepatotoxicity of AFB_1 in workers occupationally exposed to wheat flour dust in Egypt. (Mohgah et al., 2014).

Kidney Function Test

Creatinine

Creatinine is primarily synthesized in the liver through S-Adenosyl methionine methylation of glycocyamine .Creatinine is extracted from the blood by the kidneys primarily through glomerular filtration and also through proximal tubular secretion. Serum creatinine is a valuable indicator of kidney health, because, it is an easily measurable byproduct of muscle metabolism that is excreted unchanged by the kidneys. Creatinine concentration in blood increase when kidney filtration is inadequate (Shemesh, Golbetz, Kriss, & Myers, 1985). Creatinine clearance (CrCl), which roughly relates with glomerular filtration rate (GFR) can be calculated using creatinine estimation in urine and blood. The GFR can also be determined purely by looking at blood creatinine levels. Since it is a test of kidney function, the GFR is clinically important. However, in situations of severe kidney dysfunction, the creatinine clearance (CrCl) rate would overestimate the GFR because proximal tubule hypersecretion of creatinine accounts greater fraction of total creatinine clearance. (Shemesh, Golbetz, Kriss, & Myers, 1985). Men have more skeletal muscle mass than women which makes their creatinine concentrations high. Daily creatinine excretion can be increased by raising creatine intake or consuming a lot of protein (meat) (Taylor, 1989). Males produce 150-200 µmol of creatinine per kilogram of body weight per 24 hours, while females produce 100-150 µmol/kg/24 hours. Normally, this daily creatinine activity is excreted in the urine. Renal function is assessed using diagnostic serum creatinine tests with 53–115 µmol/L serum creatinine as the reference range (Lewis et al.,

2013). The most popular predictor of renal function is serum creatinine measurement (Taylor, 1989). A rise in blood creatinine is a late indicator, appearing after the nephrons have been severely damaged. As a result, the test is not good for early detection of kidney disease. Calculating the estimated glomerular filtration rate yields a more precise estimate of kidney function (Gross et al., 2005). Feed Consumed by rabbits with aflatoxin contamination 7.5 mg/kg for 90 days caused significant rise in serum creatinine. This increase propose that, aflatoxin has an effect on kidney (Verma, & Ajaykumar,1998).

Urea

The organic compound urea, also known as carbamide, has the chemical formula $CO(NH_2)_2$. Two –NH2 groups are joined by a carbonyl (C=O) functional group in this amide. Urea is the main nitrogen-containing substance in urine of mammal and plays an important role in the metabolism of nitrogen-containing compounds. It is a colorless, solid with no smell, water soluble and when dissolved in water is neither alkaline nor acidic. Urea is used by the body in a variety of ways, the most notable of which is nitrogen excretion. The urea cycle is created by the liver by combining two ammonia molecules with a molecule of carbon dioxide. The blood urea nitrogen test estimates the quantity of nitrogen derived from urea in the blood. Blood urea is inferior to other markers of renal function such as creatinine levels because blood urea is affected by other factors such as diet and dehydration (Wöhrle, 2012). The kidneys' cycling and excretion of urea is an essential part of mammalian metabolism. Aside transporting waste nitrogen, urea is involved in the

nephrons' countercurrent exchange mechanism, which enables water and vital ions to be reabsorbed from urine being excreted. The inner medullary collecting ducts of the nephrons reabsorb urea (Walter, 2005). Thus, the osmolarity in the medullary interstitium covering the loops of Henle's thin descending limb rises, causing the re absorption of water. The operation of the urea transporter 2 causes some of the reabsorbed urea to gradually flow back into the tubule's thin descending limb via the collecting ducts and into the excreted urine (Klein, Blount, & Sands, 2011). The antidiuretic hormone regulates this process, which results in hyperosmotic urine (high concentration urine with dissolved substances than plasma from blood. This function is critical for preventing water loss, maintaining blood pressure, and ensuring sodium ion concentration in blood plasma. Serum urea, was found to be high and increased significantly in aflatoxin fed mice (Li, Xing, Zhang, Wang, & Zheng, 2018).

Sodium

Sodium is an important mineral in humans because it controls flow of blood, pH, blood pressure and osmotic balance. The minimum physiological sodium needed for newborns is projected to be around 120 milligrams per day, increasing to 500 milligrams per day after ten years of age (Northwestern University, 2011). Increased sodium consumption is directly related to higher blood pressure (CDC, 2018). It is projected that lowering sodium intake by 10 % to 17 % will reduced systolic blood pressure by 2 to 4 mm Hg that will result in fewer cases of hypertension (Geleijnse, Kok, & Grobbee, 2004). Serum Sodium levels was found to increase significantly in hens that consumed 1.5mg of aflatoxin in a study to find

the effect of aflatoxin chronic intoxication in renal functions of hens (Martínez-de-Anda et al., 2010).

Potassium

Potassium ranks eighth or ninth in terms of abundant element in the human body by mass (0.2 %), with a total of around 120 g in a 60 kg adult (Abdel-Wahab, Youssef, Aly, & el-Fiki, 1992). Potassium ions can be found in several different proteins and enzymes (Vašák, & Schnabl, 2016). The levels affect a variety of physiological processes in neural, muscular and cardiac tissue, including transmission of action potentials and the resting cellular-membrane potential (Weiner, Linus, & Wingo, 2014; Malnic et al., 2013). Potassium ions are larger than sodium ions because of electrostatic and chemical properties. Cell membrane ion channels and pumps can distinguish between the two ions, actively pumping or passively moving one while blocking the other electrolyte and fluid balance (Lockless, Zhou, & Mackinnon, 2007). Potassium is excreted twice and reabsorbed three times before the urine enters the collecting tubules. Potassium chloride is used to treat and prevent decreased levels of potassium in the blood (WHO, 2009). A study by Zamir-Nasta, Ahmadi, Yazdkasti, Pazhouhi, & Jalili (2021) reported decreased levels of serum potassium in mice exposed to aflatoxin.

Chloride

Chlorides are water soluble salts. Example of chloride salt is sodium chloride. It is an electrolyte found in all body fluids that is responsible for maintaining acid/base balance, transmitting nerve impulses and controlling fluid flow in and out of cells (Green, John, & Sadru Damji, 2001). Chloride plays an

important physiological role in the regulation of osmotic pressure, electrolyte balance, and acid-base homeostasis. It is the most abundant extracellular anion, accounting for approximately 1/3 of extracellular fluid (Rein et al., 2019). Chloride is an important electrolyte for action potential and cell homeostasis transmission that occurs in the neurons (Jentsch et al., 2002). Some proteins, such as the amylase enzyme, contain a chloride ion as a structural feature. Chloride is an important dietary mineral for these functions. The kidneys are the primary regulators of serum chloride levels via a number of transporters found along the nephron (Nagami, 2016). The glomerulus filters the majority of the chloride, which is then reabsorbed by both the proximal and distal tubules (mostly the proximal tubule) via active and passive transport (Shrimanke, & Bhattarai, 2021). Information on the relationship between aflatoxin and chloride is readily not available.

Full Blood Count

Haemoglobin

Haemoglobin (Hb) is an iron-containing metalloprotein that is found in most vertebrate red blood cells (erythrocytes) and some invertebrate tissues (Maton et al., 1993) with the fish family Channichthyidae being an exception (Sidell, & Kristin, 2006). The blood's haemoglobin transports oxygen from the lungs to the rest of the body. It then releases the oxygen, allowing aerobic respiration to supply energy for metabolic process. Haemoglobinemia is a medical condition which is caused by intravascular haemolysis, a form of anemia in which haemoglobin separates from red blood cells which leads to excess of haemoglobin in the blood plasma. Aflatoxin has been implicated in decreasing the level of haemoglobin in

animals' studies (Keçeci, Oguz, Kurtoglu, & Demet, 1998). The study reported that aflatoxin is capable of inducing anemia in animals. Faisal & colleagues (2010) also reported strong association between aflatoxin-albumin adduct and anemia in a study conducted among pregnant women in Kumasi.

White blood cells

White blood cells (WBCs), also called leukocytes, are immune cells that aid in the body's defense mechanism against infectious diseases and foreign invaders.

They are multipotent bone marrow cells that are form and generated from hematopoietic stem cells. Leukocytes are found in all parts of the body, including the bloodstream and lymphatic system (Maton et al., 1997). White blood cells, unlike red blood cells and platelets, have nuclei that distinguish them from other blood cells.

Monocytes

They are type white blood cell that have the ability to differentiate into macrophages and myeloid lineages. In the immune system, monocytes have three primary roles such as antigen presentation, phagocytosis and cytokine synthesis.

Phagocytosis is a process by which bacteria and particles interact being taken in, digested and killed. . Monocytes can phagocytoze pathogens by binding to them directly through pattern-recognition receptors or by using intermediate proteins such as complement or antibodies that coat the pathogen. Monocytes may also destroy infected host cells through antibody-dependent cell-mediated cytotoxicity. White blood cells (lymphocytes) were found to decrease in rams

infected with aflatoxin (Dönmez, Donmez, Keskin, & Kisadere, 2012).

Lymphocytes

Lymphocytes are white blood cells found in vertebrate immune systems (Janeway, Travers, Walport, & Shlomchik, 2001). Lymphocytes account for 18 to 42 % of all circulating leukocytes (Omman, & Kini, 2020). Thymus (T) cells and B cells are the primary cellular components of the adaptive immune. Thymus cells play a responsible role in cell-mediated immunity, whereas B cells primarily play a role in humoral immunity. During the antigen presentation process, T and B cells can recognize distinct "non-self" antigens. Dönmez, Donmez, Keskin, & Kisadere, (2012), reported that white blood cells (lymphocytes) were found to decrease in rams infected with aflatoxin.

Neutrophils

Neutrophils (also called neutrocytes or heterophils) are the most common granulocytes, accounting for 40 to 70 percent of all white blood cells in humans (Actor, 2012). They are a significant component of the immune system, (innate) with various roles in different species (Ermert et al., 2013). Neutrophils are phagocytes that can consume microbes or particles. Antibody opsonization, a function of neutrophil, is the process of coating targets in opsonins so that they can be recognized (Edwards, 1994). Each phagocytic event results in the formation of a phagosome, which secretes reactive oxygen species and hydrolytic enzymes, allowing them to kill a large number of microbes. Neutrophils move to the site of injury in some few minutes of damage and are the trademark of acute inflammation

neutrophils may not be able to resolve such infections without the aid of other immune cells because of the indigestibility of certain pathogens. White blood cells (neutrophils) have been found to decrease in rams infected with aflatoxin (Dönmez, Donmez, Keskin, & Kisadere, 2012).

Platelets

Platelets, also called thrombocytes, are blood components found only in mammals. In response to bleeding from a blood vessel injury, platelets clump together, resulting in a blood clot (Laki, 1972). Platelets are fragments of the cytoplasm derived from megakaryocytes that do not have a nucleus (Machlus, Thon, & Italiano, 2014). They play an important part in innate immunity, inducing and participating in a range of inflammatory developments as well as directly binding and killing pathogens. Research has shown that, many people with severe viral or bacterial infections have thrombocytopenia, which reduces their contribution to inflammation (Jenne, Urrutia, & Kubes, 2013). Platelets can influence inflammatory processes by interacting with leukocytes and secreting cytokines, chemokines and other inflammatory mediators that can be quickly delivered to sites of injury (Wagner, & Burger, 2003; Weyrich, & Zimmerman, 2004). Aflatoxin has been associated to the reduction of blood platelets in rats causing aggregation Gad, Abd Allah, Fararh, & Ayman Samir Farid, 2017)

Economic Impact of Aflatoxin

The main fields of concern are the economic effects of aflatoxin poisoning. Aflatoxins have detrimental effects on human health, production and trade in livestock. Generally, when aflatoxin contaminated feed is fed to animals, there is

reduction on growth, causes disease and sometimes even death. Furthermore, the meat and milk may contain toxic biotransformation products in humans (Pier, Richard, & Cysewski, 1990). The direct economic impact of aflatoxin contamination in crops is primarily due to a decrease in marketable yield due to the rejection of foreign export products. Others are losses incurred through livestock disease, as a consequence of morbidity and mortality, resulting in substantial economic losses in volume and loss of value in the domestic markets (Wagacha, & Muthomi, 2008).

The suggested sanitary and phytosanitary requirements for aflatoxins have a negative impact on the trade of grains in developing countries (Gebrehiwet, Kirsten, & Qangweni, 2007). Products that does not meet aflatoxin requirements are rejected or assigned a reduction in price by distribution networks especially on the international market. Crops contaminated with high concentrations of aflatoxins are diverted to animal feed, resulting in low growth rates and disease in animals (Pier, Richard, & Cysewski, 1990). Toxigenic fungal pathogens are major constraints on crop production, affecting seed quality by spoilage. Groundnut, for example, play a key role in Ethiopia as a cash crop and food as well (Bhat, & Vasanthi, 2003). The produce is currently becoming one of the value crops cultivated in northern Ethiopia. However, aflatoxin contamination has a significant impact on groundnut production.

Aflatoxin Regulation

In order to minimize the exposure to aflatoxins and thus protect human and animal health, more than 100 countries have set regulatory limits on acceptable

aflatoxin levels in human food or animal feed (Wu, & Guclu, 2012). As of 2003, only 15 countries in Sub-Saharan Africa had regulations governing aflatoxin, according to the Food and Agriculture Organization (Strosnider et al., 2006). Nevertheless, almost all foods sold in local markets are not effectively controlled even in regulated countries (Awad, Ghareeb, & Böhm, 2012). The maximum limit for aflatoxin B1 for infant food in the European Union (EU) is fixed at 2.0 µg/kg for products such nuts, groundnuts, tree dried fruits, refined products, cereals and cereal products The highest daily limit for aflatoxin B1 in foods such as almonds, pistachios, and apricot kernels is 12.0 µg /kg (Alhussaini, 2012). More stringent aflatoxin regulations with respect to baby formula and children's food are observed in other countries, including Germany (0.01 µg/kg), Bosnia and Herzegovina (0.025 µg/kg), Turkey (0.025 µg/kg), The United States of America (0.5 µg/kg) and Argentina (0.5 µg/kg) (Bueno, Muñoz, & Marty, 2013). In terms of total aflatoxins, the European limit for infants' food is 4.0 µg /kg for products such as peanuts, tree nuts, dried fruits and their processed products, cereals and cereal products, including processed cereal (Alhussaini, 2012). Japan has one of the world's strictest regulations, where the total aflatoxin level must be less than 10 µg/kg in all foodstuffs (Bueno, Muñoz, & Marty, 2013). Relevant limits for total aflatoxins in primarily nuts are set in countries such as the United States of America, Canada, Australia, and New Zealand, and for foods where no specific maximum limits are set. Countries such as India and the USA set a general maximum limit for total aflatoxin as 20 µg /kg in food (Wu, & Khlangwiset, 2012). The Ghana Standards

Authority has set 15 µg/kg as a limit for maize and 20 µg/kg for groundnuts (Omari et al., 2020).

Methods of Aflatoxins Detection

In cows producing milk, aflatoxin contamination is easily detectable from milk samples (Ellen, 2012). Nevertheless, due to the difference in clinical symptoms, gross anatomy and the involvement of other diseases due to immune system suppression, aflatoxin diagnosis in non-lactating cattle becomes more difficult. (Ellen, 2012). Aflatoxin detection has traditionally relied on absorption and emission spectra as a photophysical properties. At 360 nm, for example, aflatoxins show characteristic of maximum absorption of its ring (Korde, et al., 2001).

Aflatoxin detection and quantification methods include enzyme linked immunosorbent assay (ELISA), electrochemical immunosensors, chromatography, and fluorescence. The competitive enzyme-linked immunosorbent assay is one of common tests performed in biochemistry. It allows the researcher to obtain correct concentrations. However, its disadvantage is necessitating the use of laboratories experts who are well-equipped and well-trained (Dallasta, Ingletto, Corradini, Galaverna, & Marchelli, 2003).

Electrochemical immune sensor aflatoxin detection and quantification have been shown to be effective, simple to use and capable of detecting very low aflatoxin levels. Detection using fluorescence (high-performance liquid chromatography) is effective, with high sensitivity and alternative to traditional techniques. (Cavaliere, Foglia, Pastorini, Samperi, & Lagana, 2006).

Rapid detection assays includes, optical fiber, electrochemical transduction, low injection tracking and biosensors (Carlson et al., 2000). Except for biosensors, most of these still have a scarcity of implementations due to functional inconveniences. The biosensors were created to address the shortcomings of commonly used instruments for detecting and quantify aflatoxins. Aflatoxin testing appears to be a combination of visual, immunochemical, and fluorescence techniques (Carlson et al., 2000).

Prevention and Control of Aflatoxicosis

Public health measures that encourage efficient cultivation, storage and processing of homegrown and commercial grains need to be explored to avoid aflatoxicosis. Furthermore, screening, specific treatment and decontamination procedures for food concentrations of aflatoxin can prevent widespread outbreaks of acute aflatoxicosis (Park, 2002). Other methods that can be employed to prevent aflatoxicosis include chemoprotection and enterosorption (Galvano, Piva, Ritieni, & Galvano, 2001).

To ensure detoxification, chemoprotection focuses on tracking the biochemical processing of aflatoxin. Enterosorption is the process of applying an agent that binds to food and prevent the toxin from being ingested while the food is in the digestive system, and then excreting the combined toxin-sorbent in urine. The technique has been used widely and successfully in the human and animal industry. (Rosa et al., 2001; Mitchell et al., 2013).

It has been demonstrated that the primary enterosorption agent is hydrated sodium calcium aluminosilicate (HSCAS) that has shown to successfully prevent aflatoxin

poisoning in a number of laboratory and farm animals resulting in the decrease of aflatoxin in feaces and urine (L'vova, Bystriakova, Merkulov, Shatilova, & Kizlenko, 1984). Other solutions to prevent aflatoxin production in Africa have been suggested by Bankole, & Adebanjo (2003) which includes smoking, drying, storage of farm produce and educating farmers.

Physical methods of preventing aflatoxin contamination

Drying

To decrease attack and damage fungi, the drying stage is very necessary. To ensure that crops have moisture content at levels that do not promote mould growth, drying is necessary. Twelve percent moisture content for maize, groundnut (in-shell) 9 % and shelled groundnut 7 % is the appropriate moisture content to prevent fungal growth (Hell et al., 2008). According to Awuah, & Ellis (2002), drying groundnut kernels to 6.6 percent moisture content prevented growth of mould regardless of storage. To prevent contamination of grains, it is important to observe good hygiene and sanitary conditions during drying. Crops that are well dried from the field and kept off the ground are less vulnerable to insect damage and the growth of moulds (Hell et al., 2008). Sun-drying of maize and groundnuts is normal practice in Ghana along with the use of raised platforms, the growth of toxigenic fungi such as *Aspergillus, Fusarium*, and *Penicillium* was shown to be reduced (Hell et al., 2008). Lavkor, & Bicici (2015) recorded four separate periods of peanut kernel aflatoxin analysis from harvesting, post-harvesting, drying and pre-storage. The findings of the study showed that, aflatoxin contamination was not found in 96 out of 96 samples that were sun-dried. Post-harvest screening seems to

be a chance of minimizing or removing aflatoxin in defiled seeds. Sun-dried shelled grain reduces aflatoxin contamination compared to undried shelled maize Siriacha, Kawashima, Kawasugi, Saito, & Tomboon, 1989). Majority of farmers in Africa sun-dry their crops, which frequently necessitates longer drying times for the commodity to reach a humidity level of less than 7 %, especially during cloudy weather conditions.

The system of winding groundnut pods after harvest in Ghana ensures that they are exposed to direct sunlight and circulating air. This low-cost method easily and thoroughly dries the pods, ensuring that aflatoxin levels are reduced (Amoako-Attah, Awuah, Kpodo, Fialor, & Jolly, 2007).). The use of solar dryers is a good substitute for open sun-drying, since they dry crops faster and more effectively and provide a regulated environment that provides enhanced sanitation. However, due to high costs, complex operating procedures and reluctance to move from conventional methods, the use of solar dryers among smallholder farmers has been the norm (Ekechukwu, 1999).

Sorting and cleaning

In order to minimize aflatoxin contamination, cleaning and sorting techniques for grains are required. Sorting may typically be performed on the basis of distinction of physical characteristics such as shape, color, density and height, as well as identifying fungal growth in crops that are affected. Sorting operations minimize the levels of aflatoxin and other toxins in food and feed by rejecting degraded and discolored samples. (Fandohan et. al., 2005). High capacity electronic optical sorters are used in groundnuts to extract nuts contaminated by aflatoxin after

simple clean-up of the crop by commercial farmers (Whitaker et al., 2003). In grain sorting, specialized specific gravity seed cleaning equipment has also been used for a long time. For the African value chain, industrial optical sorting equipment for groundnuts needs to be modified for both large and small operations. A research in the Philippines found, that manual sorting decreased aflatoxin concentrations in raw groundnuts from 300 ppb to less than 15 ppb (Galvez, Francisco, Villarino, Lustre, & Resurrection, 2003). Research conducted in Kenya showed that manual sorting of groundnuts purchased from local markets could reduce aflatoxin levels close to 98 %. (Filbert, & Brown, 2012). However, manual sorting needs to be done under hygienic conditions and away from the sand. Based on results from a study in Benin, the removal of clearly mouldy, insect-damaged and broken grains by hand decreased aflatoxin concentrations by 40 % in maize (Fandohan et al., 2005).

The use of computer-based image processing techniques is one of the most promising approaches for large-scale fungal screening of aflatoxin contamination in food and feed. For example, 98 % of aflatoxin infected figs have been documented to be successfully identified and separated by image-based sorting technology involving the use of UV light and color detection systems (Ozlüoymak, 2014).

Smoking

Aflatoxin-producing fungi are effectively prevented from infesting maize by smoking. In Nigeria, between 4 and 12 % of farmers in different ecological zones used smoke to protect their crops, this practice was found to reduce the amount of aflatoxin in farmers' produce (Udoh, Cardwell, & Ikotun, 2000).

Early harvesting

Early harvesting has been promoted as a method of lowering the risk of aflatoxin contamination. McDonald, & Harkness (1967), discovered that, the traditional practice of drying groundnuts in the field predisposes kernels to *Aspergillus flavus* contamination.. Rachaputi, Krosch, & Wright (2002) demonstrated the importance of evaluating aflatoxin risk on a site-by-site basis in order to decide effective harvest timing that minimize aflatoxin levels while maximizing returns. Early harvesting and threshing resulted in low concentrations of aflatoxin than delayed groundnut harvesting under high aflatoxin risk conditions.

Education

The threat that aflatoxin poses to human health and the economy is not widely known among the people of most African countries. The task of raising public awareness on the need to consume food free from aflatoxin should therefore be taken up by the national agency responsible for food safety in these countries. In both towns and villages, private NGOs can also help disseminate information on the hazards of aflatoxin. There is a need for the media to educate people on the dangers of aflatoxin, debate on the subject should be widely covered in regular newspapers and magazines. Extension personnel from Ministry of Food and Agriculture, Ghana (MOFA), for example, should educate farmers or producers on the importance of following Good Agricultural Practices (GAP) in order to produce aflatoxin-free food. Hazard Analysis Critical Control Point (HACCP), a food safety control system centered on thorough detection and valuation of food hazards, as well identifying their control in food processing situations, has proven useful

(Marriott, 1999). Aflatoxins will be reduced in any step of food processing in an ideal HACCP-based method.

Storage structures

Storage facilities made of plant materials (wood, bamboo, thatch) or mud placed on raised platforms, covered with metal roofing sheets are typical farm storage structures used by farmers (Public Private Partnership Program, 2006). The storage structures are primarily built to deter insect and rodent attacks and to prevent moisture intrusion into the grains. Farmers' adoption of high-yielding varieties frequently fails to withstand long storage periods, and conventional storage facilities have found them to be ineffective (Public Partnership Program, 2006). However, because of their high cost, it has been extremely challenging to promote new storage approach, such as the use of metal bins for small-scale farmers. Building and optimizing successful low-capital-input storage systems necessitates research.

Use of plants products

Fungal toxic properties have been demonstrated in a number of historically useful plants. According to Awuah, & Kpodo (1996), the plants *Xylopia aethiopica*, *Occimum gratissimum*, *Syzigium aromaticum*, *Cinnamum verum*, *Piper Monodera*, *Myristica nigrum* and *Cymbopogon citratus* inhibited the development of nonsorbic acid, a precursor to the aflatoxin synthesis pathway. Powder leaves of *Occimum* has been used for 9 months successfully to prevent the development of mould on stored soybeans (Awuah, 1996). The important oil and powder extracts of *Cymbopogon citratus* inhibited the growth of fungi, including dangerous species such as

Aspergillus flavus and *Aspergillus fumigatus* (Adegoke, & Odelusola, 1996). Adegoke, Iwahasi, Komatsu, Obuchi, & Iwahasi (2000), found that *Aframomum danielli* spice monoterpene essential oil had a minimum inhibitory concentration of 78ug/mL on aflatoxigenic mould (*Aspergillus parasiticus*). The terpenoid was found to have detrimental effect on biological membranes of vulnerable species. Awuah, & Ellis (2002) demonstrated the successful use of *Ocimum grattisimum* and cloves of *Syzygium aromaticum* powdered leaf in addition to some packaging products to protect groundnut kernels that had been artificially inoculated with *Aspergillus parasiticus.* The essential oils of *Azadirachta indica* and *Morinda lucida* were established to prevent growth of *Aspergillus flavus* and considerably decreased aflatoxin build up in inoculated maize grains (Bankole, 1997). Bouda, Tapondjou, Fontem, & Gumedzoe (2001) stated that *Sitophilus zeamais* was effectively regulated by the essential oils of species of certain weeds such as *Ageratum conyzoides, Chromolaena odorata* and *Lantana camara* and indicated that they could be used to control insect in stored products. It is worth noting that, regardless of a large body of literature on the efficacy of plant materials in the control of toxigenic moulds, there has been no intensive effort to conduct a large-scale experiment of these plants in the fields of the farmer. According to Udoh, Cardwell, & Ikotun (2000), when regulating mycotoxins using plant materials, caution must be exercised as some of these materials are natural media for *Aspergillus flavus* growth. For instance, Hell, Cardwell, Setamou, & Poehling (2000) discovered that using *Khaya senegalensis* bark to protect maize from insects rather increased aflatoxin development.

Biological control

Biological method to control aflatoxin production is by the introduction of atoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. In the United States, introducing different combinations of atoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* into the soil resulted in a 74.3 to 99.9 % reduction in aflatoxin contamination of peanut (Dorner, Cole, & Blankenship, 1998). The use of a non-aflatoxigenic strain of *Aspergillus flavus* around cotton plant production, resulted in a 68-87 % reduction in aflatoxin contamination (Cotty, 1994). Dorner, & Cole (2002) also demonstrated that field application of non-toxic strains of *Aspergillus flavus* and *Aspergillus parasiticus* had a carry-over effect and reduced aflatoxin contamination 95.9 % after harvest.

Chemical methods of controlling aflatoxin contamination

Several chemical aflatoxin detoxification processes, including ammoniation, ozonation, and nixtamalization have been used. Ammoniation requires the application of ammonium hydroxide or gaseous ammonia to contaminated food (e.g. peanuts, maize or cottonseed) and heat the lactone ring to hydrolyze it (Price, & Park, 2001). Methods using ammonia-based have been advanced and tried. High pressure temperature system with ammonia (0.5-2.0 %) has proven to be the most effective, and presents a comparatively safer product for 20-60 minutes under controlled conditions of moisture (12-16 %), pressure (45-55 psi), and temperature (80-100 ^{o}C) (Park, 2001). If treatment using ammonia is permitted for appropriate period, this will result in decarboxylation and less toxic compounds will occur in the process (Park, Lee, Price, & Pohland).

Nevertheless, it is stated that aflatoxin molecules are subject to chemical modification by the ammoniation process into compounds with decreased or undetectable toxic potential. According to Park (2000), the presence of known aflatoxin/ammonia reaction products in animal feed has no effect on animal health.

Nixtamalization is an alkaline management of maize that has shown to considerably reduce aflatoxin levels Ulloa, & Herrea, 1970). However, aflatoxin b1 may be reformed during acidification (Park, Lee, Price, & Pohland, 1985)
1985), rendering this procedure not effective.

Ozonation (a chemical inclusion of fusing ozone into water) can also help to reduce aflatoxin production (McKenzie et al., 1998). Ozonation of contaminated maize in Turkey successfully decreased toxicity of aflatoxin in feed without upsetting the nutritional assessment (McKenzie et. al., 1998).

Aflatoxin toxicity can be reduced by including specific chemicals or nutrients in one's diet. For example, vitamin A has been shown to inhibit the formation of AFB_1-adducts, and phenobarbital may speed up natural detoxification processes (Leeson, Diaz, & Summers, 1995). Vitamin E has a high affinity for aflatoxin and works by forming a stable relationship with it by reducing its bioavailability (Odin, 1997). Clay minerals, in particular, have been shown to be effective in binding aflatoxin in the animal gastrointestinal tract, reducing the bioavailability and distribution of toxins in the blood and target organs (Phillips, Kubena, Harvey, Taylor, D. R., & Heidelbaugh ; Phillips, 1999).

Clay research

Soil solid particles are grouped into three types, based on their proportions: sand, silt, and clay. Clay minerals typically have particle sizes less than 2 m, whereas silt and sand particles have particle sizes ranging from 0.002-0.05 mm and 0.05-2 mm, respectively (Sylvia, Fuhrmann, Hartel, & Zuberer, 1997). Minerals are classified into groups based on the chemical identity of the dominant anion or anionic group. The main mineral groups include sulphides, sulphosalts, oxides and hydroxides, halides, carbonates, nitrates, borates, phosphates, sulphates, tungstate, and silicate (Hurlbut, & Klein, 1977). Among these groups are more minerals that are subdivided with respect to their structural similarities (Schulze, 1989).

Silicates are the largest known class of minerals, with regards to the number of developed mineral forms and their vast association to the entire mass crust of the earth (Schulze, 1989). The simple structural unit for silicates is a silicon oxygen tetrahedron with Si4+ in the center and four O2- mounted at the apices. By sharing four O_2-ions, the tetrahedral structures can be linked together. Rings (cyclosilicates), sheets (phyllosilicates), chains (inosiliates), and three-dimensional structures (tectosilicates) are among the more complex structures that can be formed (Schulze, 1989). In observation, tetrahedra (for example, SiO_4) and octahedra (for example, Al_2O_3) are common structural components in most mineral structures.

Uses of clay

Although some critics regard it as a maladaptive behaviour motivated by cravings, some clays are believed to have therapeutic properties and that can be of

benefit to humans and animals (Johns, & Duquette, 1991; Krishnamani, & Mahaney, 2000). Geophagy's potential health benefits include enterosorption and reduced bioavailability of harmful chemicals and pathogenic microbes from polluted water and food. Geophagy (eating of clay) could be explained by the belief that, the practice provides vital minerals and/or that the body can detoxify toxic substances (Johns & Duquette, 1991). Peruvian macaws, in particular, eat clay after consuming alkaloid-rich seeds and unripe fruits (Diamond, 1999). Clays have been used medicinally as laxatives and anti-diarrhea agents in humans (Carretero, 2002). Though many different clay types and zeolitic minerals are regularly used in animal feed for several reasons, it is important to note that, not all clays are useful. Clinoptilolite inclusion in pregnant rats infected with AFB_1 diets, resulted in severe liver disease that was not observed when aflatoxin B1 alone was present (Mayura et al., 1998). Enzymes and essential nutrients can bind by non-selective or flexible sorbents and can release toxins, rendering prolonged use of sorbents inadvisable (Patterson, & Staszak, 1977). This is a substantial challenge, particularly during prenatal development, when mothers and fetuses are especially vulnerable to nutritional deficiencies. A previous study, discovered that the levels of haemoglobin, hematocrite, and red blood cells in rats fed with 20 % of kaolin during the gestation period significantly decreased compared to controls, indicating maternal anemia caused by kaolin. Babies born to these rats had lower birth weights (Patterson, & Staszak, 1977). As a result, the safety of any potential additive must be thoroughly evaluated prior to routine use in animal feed or human diets.

Phyllosilicate clay minerals

Tetrahedral and octahedral sheets are found in phyllosilicate clay minerals. The tetrahedral sheets are composed of tetrahedral SiO_4, organized in a way that the three neighboring tetrahedral components are shared with three O^{2-} ions of each tetrahedron. This arrangement of O^{2-} ions applies to the same plane in all directions, and the oxygen involved is referred to as basal oxygen (Bailey, 1980)

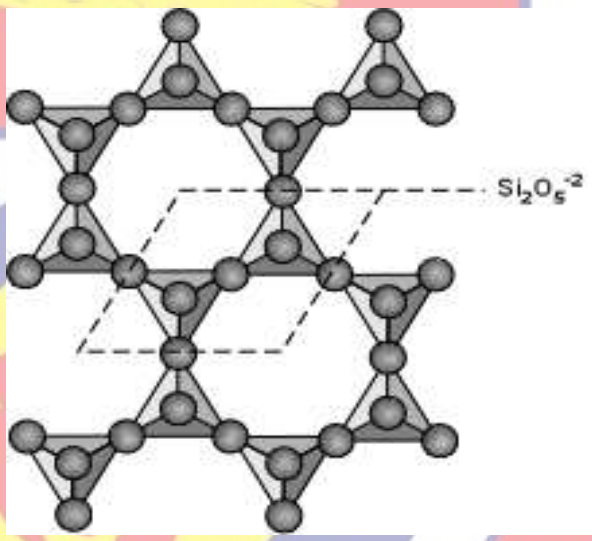

Figure 6: Phyllosilicate Clay in a Tetrahedral Form
Source: Bailey (1980)

First, any available octahedral space must be filled by a divalent cation (e.g., $Mg^{2+)}$ to create a trioctahedral arrangement. In this case, three of every three octahedral sites are filled, resulting in a formula of $Mg_3(OH)_6$ or $Mg(OH)_2$, which keeps the sheet electrically neutral because the balanced charge. A second possibility is that a trivalent cation (e.g., Al^{3+}) fills only two of every three possible octahedral sites, resulting in a dioctahedral arrangement. To keep the charge sheet electrically neutral, each Al^{3+} ion is surrounded by three OH- ions, that results in the formula

$Al(OH)_3$. The apical oxygen from the SiO_4 tetrahedral layer will replace the OH-groups from the octahedral layer in phyllosilicate minerals and matches with the octahedral layer's metal cation, thus linking the octahedral and tetrahedral layers. The hydroxyl ion, OH, is found in the middle of the six-membered rings in the majority of phyllosilicates. As a result, the group is $Si_2O_5(OH)^{-3}$. The apical oxygens and (OH) ions are shared by other cations bound to SiO4 sheets, forming octahedral coordination with the other cations. This results in the formation of an octahedral layer of cations, usually Fe^{+2}, $Mg^{+2,}$ or $Al^{+3,}$ which are in octahedral coordination with the O and OH ions of the tetrahedral layer. The triangle (figure 5) are the faces of octahedral groups that can give rise to tetrahedral layers (Bailey, 1980).

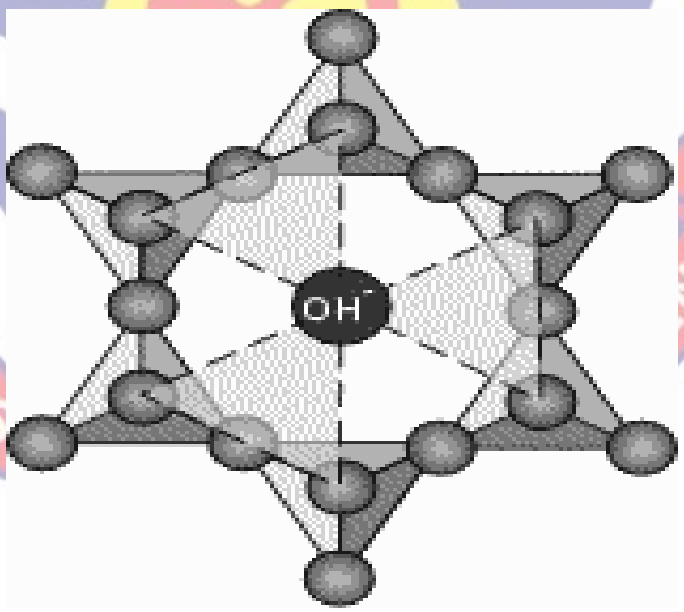

Figure 7: Phyllosilicate Clay with a Hydroxyl (OH) Group
Source: Bailey (1980)

The tetrahedral-octahedral bonding arrangement usually occurs in one of the two ways, depending on the number of tetrahedral and octahedral sheets involved in the layer structure. The 1:1 layer structure is formed when one tetrahedral layer is bonded to one octahedral layer, and the 2:1 layer structure is formed when two tetrahedral layers are bonded to one octahedral layer, one on either side of the octahedral layer. Phyllosilicates with a 1:1 layer structure include kaolinites and halloysites, whereas those with a 2:1 layer structure include talc, pyrophyllites, micas, vermiculites, smectites, and chlorites (Schulze, 1989). The phyllosilicate mineral series is classified according to the amount of substitution and, as a result, the charge per layer.

Kaolinite is a common mineral in the soil, and is probably the most common component of phyllosilicates of the 1:1 type. They are electrically neutral. Electrostatic bonding between the basal oxygen of the tetrahedral sheet and the OH- ions of the octahedral sheet holds the neighboring layers together. The tetrahedral and octahedral sheets of these minerals have very little isomorphic substitution, resulting in very small cationic exchange potentials and surface areas. Kaolinites are widely used as a paper filler and coating in the ceramic industry. Similarly, vermiculite, which becomes very light and translucent when heated, is used in construction as a concrete filler as well as a thermal and sound insulator.

One of the abundant smetitic minerals in the soil are the dioctahedral because of their large surface area and adsorption capacity. These smectites are common minerals found in soils throughout the world's temperate zones (Schulze, 1989). Smectites swell when wet and shrink when dry, which is an important

property to consider. The interlayer charge after substitution is primarily responsible for smectites' optimal swelling properties. Chlorites, palygorskite, and sepiolite are other phyllosilicate minerals with 2:1 layer structures (Schulze, 1989).

Montmorillonites clays are part of smectite with octahedral sheet Isomorphic substitution, resulting in the general formulation Nax[(Al2-xMgx)Si4O10(OH)$_2$]. These clays are naturally abundant, with surface areas of 800 m^2/g, making them ideal sorbent materials (Borchardt, 1989). The clay minerals on their platelets have a negatively charged surface.

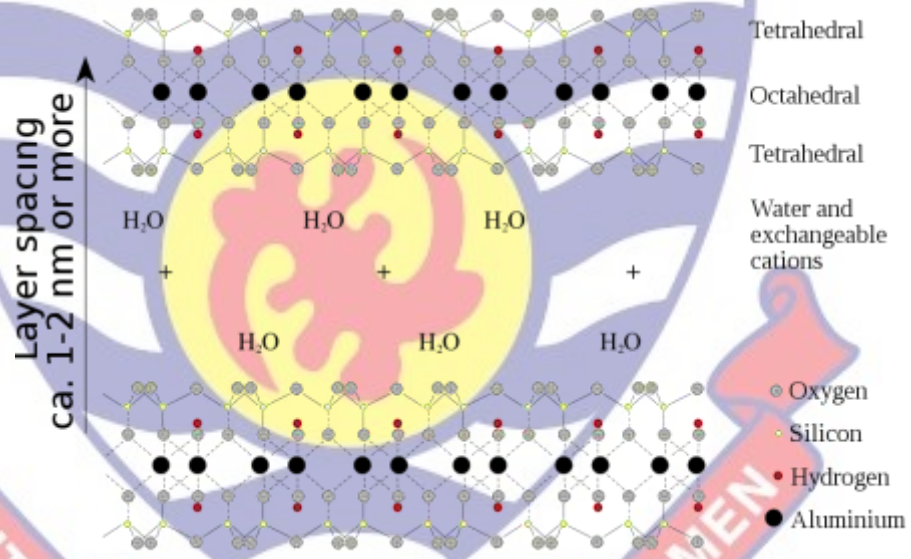

Figure 8: Structure of Montmorillonite Clay
Source: Wikimedia Commons, the free media repository (2007)

This negative charge surface of the clay is caused by either isomorphic substitution of cations within the platelet surface or dissociation of hydrogen (H+) ions from the hydroxyl groups attached to silicon or aluminium atoms. Montmorillonite clay has been used in industry as a decolorizing agent for filtering water and food

products (Millot, 1979). The functionality of this class of minerals is responsible for the distinct structural and chemical properties of silicate layers. Novasil, a calcium montmorillonite clay, is a phyllosilicate clay with a 2:1 layer lattice. These 2:1 clay forms are made up of layers between two tetrahedral silica sheets and one octahedral alumina sheet. The structural morphology of the 2:1 layer-lattice dioctahedral montmorillonite clays suggests that SiO_4 tetrahedral is held together with oxygen ions (three) shared by each tetrahedron with three adjacent tetrahedral ions. This structure expands to form a basal oxygen plane in all directions. Aluminum ions are octahedrally coordinated with SiO_4 sheets, which limits aluminum bioavailability in the center of clay platelets (Phillips, Lemke, & Grant, 2002). Isomorphic substitution occurs when a Si or Al atom is naturally replaced by a cation with identical geometry but a lower charge (e.g., $Mg^{2}+$ for $Al^{3}+$ or $Al^{3}+$ for Si^{4+}) due to negative charge. This phenomenon causes a negative net charge to be generated and distributed across the platelets. Overall, the negative charge on 2:1 clays is caused primarily by isomorphic substitution and, to a lesser extent, dissociation of H+ ions from hydroxyl groups. The negative charge typically neutralizes cations (e.g. Na^{+} and Ca^{2+}) attracted into interlayer regions (Huebner et al., 1999). The interlayer cations are prone to the formation of hydration layers, water enters and causes the clay's interlayer regions to swell (Bohn, McNeal, & O'Connor, 1979). The interlayer area and other surface properties of novasil (Montmorillonite clay) have been found to be particularly important in its binding efficacy for aflatoxins (Grant, & Phillips, 1998).

Clay-based enterosorption detoxification

Novasil

A method currently of interest for aflatoxin reduction has been the use of materials to adsorb or covalently bind aflatoxin. In particular, in the prevention of aflatoxicosis, calcium montmorillonite clays (e.g. novasil) is reported to tightly adsorb and inactivate aflatoxin b1 in the gastrointestinal tract of different species of animals (Mayura et al., 1998). Studies have shown that, including novasil as an anti-caking agent in animal feed has been shown to protect various animal species from the harmful effects of aflatoxin (Mayura et al., 1998; Kubena et al., 1991; Phillips et al., 1988; Kubena et al., 1990a; Bingham, Phillips, & Bauer, 2003; Harvey et al., 1991a; Harvey et. al., 1991b). Furthermore, novasil clay (at 1.0 % feed level) reduced aflatoxin M1 levels in milk without changing the nutritional components or producing overt toxicity on its own (Ellis et al., 1990; Harvey et al., 1991). Inclusion levels of 0.5 % novasil in aflatoxin-contaminated diets of animal species (i.e. young chickens, turkeys, pigs, lambs, and rats) effectively reduced the health manifestations of aflatoxin poisoning (Phillips, Sarr, & Grant, 1995). Novasil effectively reduced the acute effects of AFB_1, as evidenced by low levels of AFM_1 in aflatoxin-exposed rats' urine (Sarr, Mayura, Kubena, Harvey, & Phillips, 1995). In a chemisorption process, the edge sites, basal surfaces, and especially the interlayer surfaces of novasil (NS) were hypothesized to interact with the -dicarbonyl system of AFB_1 (Phillips., Sarr, & Grant, 1995). In many animal studies, the sensitivity of novasil for aflatoxins has been well characterized. For example novasil has been shown ineffective in sorbing dicetoscirpenol (Kubena et al., 1993a), ochratoxin (Huff, Kubena, Harvey, & Phillips, 1992), or

deoxynivalenol in chicks (Patterson, & Young, 1993). Novasil clay effectively protected against aflatoxicosis when the combination of both of these mycotoxins (ochratoxin and deoxynivalenol) with aflatoxin was checked in these same tests, indicating the specificity of this clay for aflatoxin (Patterson, & Young, 1993).

Novasil human studies have been conducted in adults in Texas in the United States (phase I) and adults in Ghana (phase II), and phase I studies have also been conducted in Ghanaian children. Novasil was confirmed to be safe and reduced the bioavailability of aflatoxin in both studies. (Nicole et al., 2014; Phillips et al., 2008).

Mechanisms of aflatoxin sorption to novasil

For novasil sorption of aflatoxin, an electron donor acceptor (EDA) mechanism has been proposed for aflatoxin B1. Because of isomorphic substitution, the platelets of novasil clay are negatively charged, they attract positively charged ions to balance this charge. Platelets can also be drawn to compounds that have areas of electron deficiency (partial positive areas) (Haderlein, Weissmahr, & Schwarzenbach, 1996). Aflatoxin sorption on novasil may favor an orientation with the furan aligned away from the surface. The partially positive carbons in aflatoxins that comprise the dicarbonyl system have been demonstrated to be critical for the adsorption process (Haderlein, Weissmahr, & Schwarzenbach, 1996). The importance of AFB_1 spatial orientation was demonstrated by the fact that stereochemical differences in some aflatoxin analogues had a significant impact on binding tightness. According to adsorption isotherms on heat-collapsed novasil, the interlamellar region of novasil is the primary site of binding, with external surfaces responsible for only minor sorptions

of aflatoxins. Other mechanisms of AFB_1 sorption to novasil surfaces include possible chelation of interlayer cations (particularly $Ca^{2}+$) and various edge-site metals (Grant, 1998; Phillips; 1999; Phillips, Afriyie-Gyawu, Wang, Williams, & Huebner, 2006).

CHAPTER THREE

MATERIALS AND METHODS

Study Design

Randomized control experiment

The design of the study was a randomized control experiment. Ejura Sekyedumase district was segmented into four communities.

A computer generated numbers were given to four group leaders chosen at random to represent the four communities in Ejura Sekyeduamse district. The leaders were asked to picked from the letters A to D. Group A and B was put together as the test group whiles C and D was put together as the control group using the stratified randomization method. The test group received 1.5g of novasil, the intervention being tested, whiles the control group, received 1.5g of calcium carbonate (placebo). The groups were monitored for 60 days under the conditions (as stated under adherence and adverse events monitoring) of the experiment design to assess the effectiveness of the intervention. The control group was used to assess safety and efficacy.

Study area

The research was carried out in the Ejura-Sekyedumase district in the Asahnti region of Ghana. The district is located in the transition zone between the country's northern and southern regions. The climate is typical of the agro-ecological zone of savannah transition. During the rainy season, the relative humidity in the district is high, with mean monthly values ranging from 80-88 % in the morning to 70-75 % by mid-day. During the dry season, the humidity drops to

about 75% in the morning and 45 % by mid-day (Dedzo, 1998). The average annual precipitation is 1400 mm, with two peak seasons, but it can be unimodal at times (Dedzo, 1998). Temperatures are uniformly high, with an annual mean minimum temperature of about 20ºC and maximum temperature of 32ºC. In terms of employment and revenue production, agriculture is the leading industry. Approximately 69.7% of the population is employed in this industry (Population and housing census report, 2010). In the district, a variety of crops are grown. The Ejura Sekyeduamse district produces a lot of crops mostly cereals which have been found to have aflatoxin contamination and its exposures in people in the area (Kumi, Dotse, E, Asare, G, & Ankrah, 2015; Mitchell et al., 2013).

Materials

Vicam AflaTest kits were used according to the Association of Official Analytical Chemists' method (AOAC) for aflatoxin (in AOAC official method 993.31, V1 series 4). Sodium chloride (NaCl) and methanol (HPLC grade) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AflaTest columns were purchased from VICAM (Watertown, MA, USA). Novasil was manufactured by Engelhard/BASF Chemical Corporation (Jackson, MS, USA). The product was donated by Texas A&M University in the USA. All other chemicals and reagents used were of the best available purity and obtained commercially from standard suppliers.

Scientific and ethical consideration

The Noguchi Memorial Institute for Medical Research Scientific and Technical Committee, University of Ghana, Legon first reviewed the scientific importance of the study and subsequently approved the study protocol, which was assigned the code 1(5) 2018-19 (Appendix A). The Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana and the Institutional Review Board (IRBs) of the University of Cape Coast (UCC), both granted ethical clearance with protocol approval number IRB 0001276 and UCC/RB/CHAS/2019/109 respectively (Appendix B and Appendix C).

Consent

Informed written consent were obtained from all participants in accordance with human experimental guidelines of the NMIMR (IRB) and UCC (IRB) (Appendix D). Confidentiality was observed throughout the research process and the data made accessible only to the Principal Investigator and Study Supervisors.

Participants' selection

The cluster sampling method was used to select qualified participants for the study. Ejura-Sekyedumase District was divided into four clusters (communities). A cluster was chosen using a simple random sampling technique, and all eligible subjects within households who consented were included. Homes had traditional house numbering systems in the communities, and a household was numbered serially within a home. If more than one household exists within a home, the first household interviewed was designated as house number/001, the second as

house number/002, and so on if there are more eligible households (with children 2-9yrs) within the same home. All households within a given cluster were eligible for inclusion. To agree for his or her child to be recruited into the research. One parent adult member of each household within a home was interviewed to provide consent for the child in relation to the study, and this continued until the number of subjects to be screened, 220 (based on NIH sample size for Phase II clinical trials) was obtained on the basis of the initial report by Nicole and her group (2014), who reported 100% aflatoxin exposure. Fifty-five (55) participants from each community were selected to obtain a total of 220 participants. Two hundred healthy children of both sexes (age: 2-9 years old, where children are likely to start weaning) who fulfilled the inclusion criteria were selected for the intervention study after the parents or legal guardians of the participants signed/thumb printed the informed consent**.** A computer generated numbers were given to four group leaders chosen at random to represent the four communities in Ejura Sekyeduamse District. The leaders were asked to picked from the letters A to D. Group A and B was put together as the test group whiles C and D was put together as the control group using the stratified randomization method.

Screening of participants

Potential participants were screened based on the following procedure

- Physical examinations (Appendix E)
- Normal levels of hemoglobin (Appendix E)

If an adverse medical condition was discovered as direct results of the initial screening process, the participant was excluded but noted by the personnel and directed to contact a physician for follow-up.

Inclusion and exclusion criteria

Inclusion criteria

- Healthy (upon physical examination by a physician)
- No chronic illness
- No use of medications for acute illnesses or chronic illness
- Aflatoxin M1 ($AFM_{1)}$ > 0.5ug/kg) previous results indicates 100% exposure (Nicole et al., 2014).
- Normal hemoglobin level (greater than 10.5g/dL)

Exclusion criteria

- Abnormal hemoglobin level (below 10.5g/dl)
- Chronic illness
- Use of medications for chronic or acute illness.

Guidelines for participants recruitment

The study recruited 200 healthy children who met the inclusion criteria on voluntary basis (2-9 years) of both sexes living in Ejura Sekyedumase District who passed the inclusion criteria. The sample size was calculated using the standard (100-300) number of participants per treatment required by NIH (USA) guidelines

phase II clinical trials (https://www.nih.gov/health-information/nih-clinical-research-groups) trials-you/common-terms-glossary) and a sample size estimation procedure for a placebo controlled randomized trial (Shein-Shung, Jun, & Hansheng, 2003).

A community leader translated directly the inform consent from English to a local language for parents to understand. After a signed or thumb printed consent form has been received from the parent or legal guardian, computer generated numbers were placed in an opaque envelope and assigned randomly to participants with respect to novasil treatment group and placebo group.

Sample size estimation

A placebo-controlled randomized experiment is proposed to evaluate the efficacy of a drug. A previous study found that 52 % of subjects treated with novasil (drug A) was efficacious (Nicole et. al., 2014), and a clinically significant difference of 19 % when compared to placebo is acceptable. Level of significance = 5 %, Power = 80 %, Type of test = two-sided

Formula of calculating sample size:

$$n = [(Z_{\alpha/2} + Z_{\beta})^2 \times \{(p1\ (1-p1) + (p2\ (1-p2))\}]/(p1 - p2)^2$$

Where:

n = sample size required in each group,

p1 = proportion of subject cured by Drug A = 0.52,

p2 = proportion of subject perceived to be cured by Placebo = 0.33,

p1-p2 = clinically significant difference = 0.19

$Z_{\alpha/2}$: This depends on level of significance, for 5% this is 1.96

Z_{β}: This depends on power, for 80% this is 0.84

The sample size required per group, according to the above formula, was 100. As a result, the total sample size required is 200. Using a two-tailed z-test of proportions between two groups with 80 % power and a 5 % level of significance, a sample size of 200 children, 100 in each group, was sufficient to detect a clinically significant difference of 19 % between groups in treating aflatoxin with novasil. The 19% difference represents a 52 percent cure rate with novasil (Shein-Shung *et al.*, 2003).

Recruitment and training of field assistants

The study recruited 8 field assistants which included: a medical doctor, 2 medical laboratory scientist, 4 study monitors and a nurse. Field assistants were taken through how sampling and data collection was going to be done by the principal investigator. Training included how to properly complete the physical examination form by the medical doctor, completion of daily diary sheet by the study monitors, adverse report form by study monitors and proper blood and urine sampling techniques by laboratory Technicians.

All field assistants were informed of the strict confidentiality of the study participant not to disclose any information regarding the health status of the study participants to other person(s) except to the medical officer involve in the study and the principal investigator.

Field Assistants

Medical Doctor

The medical doctor was responsible for determining if participants meet the pre-defined inclusion criteria, especially the physical examination. Also responsible for follow ups on study participants to determine an adverse condition if any.

Medical laboratory Scientists

Two medical laboratory scientists were responsible for venous blood collection from the upper arm and also processing of blood and urine sample for analysis.

Nurse

The nurse assisted in collecting data from participants, especially anthropometric measurements of children.

Study monitors

Study monitors (4) were responsible for administration of novasil and placebo to participant's food, daily diary worksheet monitoring and monitoring of any adverse effect and documenting them.

Dose selection

A dose level of 1.5 grams of novasil/day (0.25% w/w) was selected for the study based on data from previous safety and efficacy study (3 months) in adults who received up to 3.0 grams novasil/day (Phillips et al., 2008). Also in a phase I intervention study in children, 1.5 grams of novasil was safe for a period of

14 days (Nicole et al., 2014). More important, no adverse effect was found in animals with doses 10 times higher than that proposed for the study (Phillips et al., 2008; Afriyie-Gyawu et al., 2005; Mayura et al., 1998). Before the study, doses were weighed into identical packages put into one package each in a brown enveloped labeled A and B at the NMIMR to ensure that monitors and participants were unaware of the treatment material. However, the research investigators were not blinded to the study. The brown packages were labeled A and B. Envelope A was the treatment material whiles B was the control material. The packages were sent to the treatment site (Ejura Sekyedumase district). The trained study monitors were employed to mix novasil (ash colour) and placebo (white colour) to approximately 600g daily intake of participant's respective food, prior to consumption. Each participant in the study received 1.5g of either placebo (calcium carbonate) or novasil in their breakfast meal. Breakfast consisted of a corn-based porridge known as ''koko." Koko is their staple breakfast food. However, others who preferred milo was provided. Approximately 600g daily breakfast was consumed by participants. The breakfast was a vehicle for the test and control material. Most importantly participants consumed 1.5g of novasil or placebo on daily bases.

Adherence

Adherence to the research was determined by reviewing a daily worksheet (Appendix F) filled out by monitors, regarding consumption and any potential side effects associated with novasil. The study monitors witnessed the consumptiob of food with novasil/placebo and encouraged the study participants to

eat the entire novasil/placebo added food. They also discussed any possible side effects that may occur. Visits and meetings with study participants were scheduled daily by study monitors during the study period.

Adverse events monitoring

Significant toxicity was not anticipated as a result of treatment with novasil based on established scientific literature documenting the ingestion of dioctahedral smectite clay in adults and children (Phillips et al., 2008; Nicole et al., 2014). However, during the entire study period, research personnel and medical staff were on site to observe for possible adverse effects and to take participants out of the study. Study monitors were equipped with regular diary worksheets and symptoms checklists (Appendix G) as evaluation methods for tracking adverse effects and completed once a day after ingestion dose of novasil and placebo. Adverse effects were calculated as a percentage of the total number of reports of adverse events per treatment and placebo group out of the total number of daily diary worksheets completed. In the event of an adverse effect or an unrelated illness at any point during the research period, the participant was provided with free medical care by the Ejura-Sekyedumase district hospital with funds from the research. To determine whether or not there were any symptoms, the following criteria were used: mild (grade 1), mildly relieved by symptomatic treatment; moderate (grade 2), disturbing and interfering with behavior and only slightly relieved by symptomatic treatment; extreme (grade 3), interfering with daily activity and not relieved by symptomatic treatment; It was recommended that any individual experiencing a serious symptom seek urgent medical attention. For chronic symptoms, laboratory analysis and a

physical examination were done. Any symptoms associated with the research confirmed by the medical doctor would result in immediate withdrawal of treatment.

Anthropometry measurements

Electronic bathroom scale measurements were used to measure weight (Precision Health Scale UC-300). The measurements of weight were in line with the definition by WHO (WHO, 1995). The weight in kilograms of each study participant was measured in triplicates using a weighing scale and reported to the nearest 0.1 Kg and the true weight of the participant was assumed to be the average weight. The height of each participant was taken in a standing position using a stadiometer in compliance with normal procedures (WHO, 1995). Heights were determined to the nearest 0.1 cm in triplicates. It reported the average of the three readings as the true value. Stunting and underweight were determined using WHO anthro survey analyzer 2019 (Appendix H).

Specimen Collection and Processing

Blood

Blood (5 mLs) were taken from the anticubital fossa vein (by venipuncture) in the upper arm of all participants at days 0 and 60 by a trained medical laboratory scientist from Ejura Sekyedumase District Government Hospital treatment site. The blood was divided into two, 2 mL into EDTA and 3 mL into serum clotter tube labeled with participant's name and identification number. The clotted blood was centrifuge at 2000 revolution per minute for 10 minutes to get the serum. The serum was stored in aliquots in freezing vials, labeled with the participant's

identification number. Whole blood and serum samples were stored at 4 ^{o}C and -20^{o}C respectively prior to transporting to NMIMR. Whole blood and serum samples were used to assess safety of novasil treatment based on hematology and chemistry parameters. Whole blood specimen was used to analyze reduced glutathione and complete blood count (white blood cells, hemoglobin level, red blood cells, and platelets). Serum whiles samples were used to analyze for aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein, gamma glutamyl transferase (GGT), bilirubin, sodium, creatinine, urea, chloride and calcium.

Urine

Spot urine (20mls) samples were successfully obtained from 200 participants by parents or guardians into a sterile specimen cup. The urine samples were frozen and transported to NMIMR in a leak free cool box. The urine were kept at -20 ^{o}C prior to analysis of aflatoxin M_1 (AFM_1) and creatinine levels at NMIMR.

Laboratory Investigations

Laboratory investigations were done at the Clinical Pathology Department (NMIMR), Ejura Sekyedumase District hospital and the University of Ghana hospital, Legon.

Sample analysis

Complete blood count

Complete blood count parameters analyzed included haemoglobin level (Hb), total white blood cell count (WBC) and total red blood cell count

(RBC). White blood cell count analysed were lymphocytes, neutrophils, monocytes and platelets. These analyses were done at the Ejura-Sekyedumase district laboratory using the KX Sysmex 21, haemaltology analyzer.

Kidney and liver biomarkers

The levels of kidney and liver biomarkers in serum were analysed using an automated colorimetric method by Flexor E, endpoint automation, Vital Scientific, Netherlands. Liver markers analysed included; aminotransferase (ALT), aspartate aminotransferase (AST), albumin, gamma-glutamyl transferase (GGT), total protein and total bilirubin whiles kidney markers analyzed includes, creatinine, urea, sodium, chloride and potassium. Serum calcium was also measured. These were done at the medical laboratory of University of Ghana hospital, Legon.

Urinary aflatoxin M1 (AFM_1)

The levels of aflatoxin M1 in 200 urine samples were determined using immunoaffinity column purification (AflaTest) and HPLC-flourescence detection at 365 nm excitation and 425 nm emission wavelengths.

Affinity chromatography

Each urine sample (5 mL) was acidified with 0.5 mL of 1.0 M ammonium formate (pH 4.5) and diluted with deionized water to a volume of 10 mL. The samples were then allowed to flow by gravity through the immunoaffinity columns at a rate of 1-2 drops per second. Before elution of AFM_1 with 1 mL of 80 % methanol, immunoaffinity columns were washed with 5 mL of phosphate buffer saline (PBS) and 10 mL of deionized water. The eluants were then dried with

nitrogen gas before being resuspended in 1mL of methanol: 1mL of ammoniumformate (ratio 1:1) solution for analysis on a Shimadzu HPLC system with fluorescence detection (Shimadzu Corporation, Japan).

High performance liquid chromatography

A 250 x 4.6 mm LiCrospher RP-18EC end-capped column with a pore size of 100 A and a particle size of 5 m (Alltech) was used to identify aflatoxin metabolites. The mobile phase contained 22 % ethanol in deionized water, which was buffered with 20 mM ammonium formate (pH 3.0). Samples (100 µL) was injected at a rate of 1 mL/min elution rate. The detection limit was 0.5 pg AFM_1/mL urine. The retention time was resolved using an aflatoxin M1 standard which was analysed with the samples. Aflatoxin M1 levels were quantified using fluorescence detection procedure with 365 nm excitation and 425 nm emission wavelengths. The study of AFM_1 was carried out at Clinical Pathology Department, NMIMR. Chromatogram of aflatoxin M1 standard and urinary aflatoxin M1 sample with their retention time is shown in appendix I and J.

Glutathione (GSH) measurement in whole blood

The blood samples were homogenise and diluted 200X with GSH buffer before use. The reaction mixture was of 50 µL of GSH buffer (0.1M NaH_2PO_4, 5mM EDTA, pH 8.0 with 1N NaOH) with 50 µL of the blood sample

and 10 µL of 0.75 mM O-Phthaldehyde (OPT). The mixture was incubated for 15

minutes at room temperature in the dark, and fluorescence was measured at 340 nm for excitation and 460 nm for emission (Paul and Russell, 1976). The experiment was carried out in triplicate. The reduced glutathione was calculated using calibration curve. Reduced glutathione obtained was expressed in terms of glutathione equivalent (mg/mL).

Statistical Analysis.

Statistical analysis was conducted using Sigma Stat 3 (Jadel Scientific Software, 2003, USA). A descriptive analysis were employed to look at the mean, standard deviation and the range. Data were represented by tables, pie chart and line graphs. The overall objective of the intervention study was to evaluate if novasil clay consumed by children was safe. Therefore, statistical valuation was based on comparing the treatment group at baseline and day 60.

Anthropometry analysis to check for growth indicators and z score calculations were done using WHO Anthro Plus software 2007 which looked at stunting and underweight.

Paired T test were used to analyse AFM_1 data, anthropometry, full blood count biochemical parameters for the placebo and treatment group. Signed Rank test was used to analyze significance between data where it was not normalized among the intervention group and the placebo group. Statistical significance was defined as a two-tailed p value < 0.05. Pearson correlation test was used to calculate correlation coefficients to estimate the relationship between biochemical parameters, anthropometry, and AFM_1 levels.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

Results

Characteristics of Study Participants and Compliance

A total of 200 children between the ages of 2-9 years were enrolled in the study. Only two participants (one from treatment and one from placebo) could not give blood at the post-intervention stage. Also, two participants within the placebo group were diagnosed and treated for malaria. All the participants completed the study.

Table 2 contains anthropometry data of study participants. There were no statistically significant differences between the treatment and placebo groups in terms of height, age and weight (Table 2). Stunting was described as a growth indicator for height-for-age which is given as a z-score value less than minus two standard deviation (< -2SD) whiles underweight was described as a growth indicator of z-score value for weight-for-age of any value greater than minus two standard deviation (<-2SD) according to WHO Anthro Survey Analyzer 2019.

Table 2.

Anthropometry Analysis of Participants

Participants	Treatment Group	Placebo Group	P-Value	Range
Age (years)	*5.6 ± 1.8	*5.9 ± 1.9	0.261	2 - 9
Gender				
Male (number)	50	48		
Female (number)	50	52		
Height (m)	*1.1 ± 0.1	*1.1 ± 0.2	0.204	0.8 – 1.31
Weight-for-age z-score	*-0.4 ± 0.9	*-0.3 ± 1.0	0.245	-2.35 – 1.9
Height-for-age z-score	*-0.4 ± 1.4	*-0.4 ±1.3	0.909	-3.6 – 2.5
Body Weight (kg)	*18.6 ± 5.0	*19.4 ± 4.6	0.250	10.3 – 40

*mean ± SD. $P < 0.05$: Significance
Note: All the data are baseline values for participants without follow –up.

Table 2 shows the average z score analysis of participants. The average z score for weight-for-age for the treatment group was −0.4SD with a range of −2.35 – 1.9 SD, whiles the placebo had an average z score for weight-for-age as -0.3SD with a range of -2.35-1.9 SD. The average height- for- age z score was -0.4SD in a range of -3.6-2.5 SD in the treatment group. The placebo group also showed an average z-score value of -0.4 for height-for-age in a range of -3.6 - 2.5SD. Of the 200 participants, 26 (13%) exhibited stunted growth, whiles 6 (3%) were found to be underweight (Figure 9). However, 84 % of the total population had normal growth indicators (Figure 9).

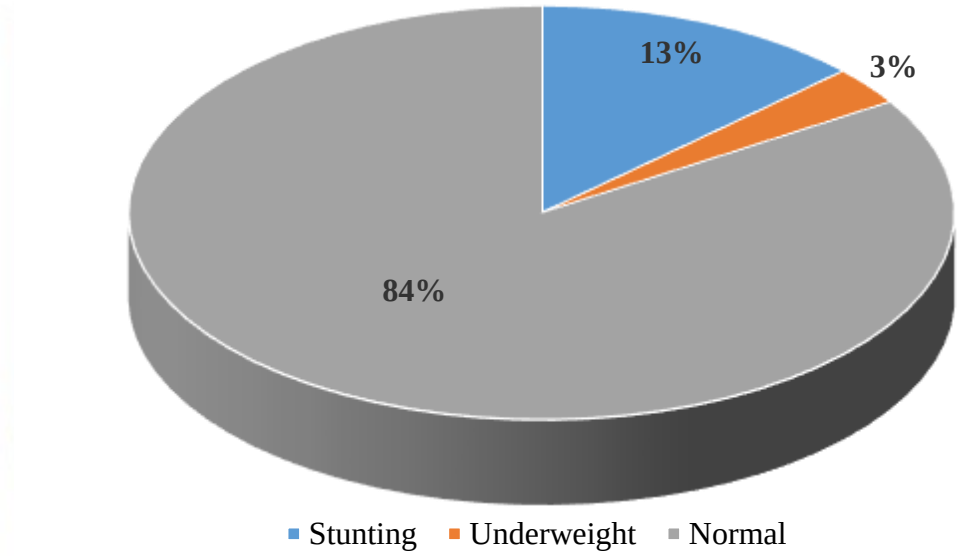

Figure 9: Degree of growth indicators amongst participants

Adverse measures monitoring and side effects

Table 3 shows the list of adverse outcomes reported during the study. Participants were administered with 1.5 g/day novasil during the study. During the 60-day study period, the most common adverse symptom was fever, which was reported by two individuals. After clinical examination and subsequent laboratory testing at the hospital, these two participants who were in the placebo group were diagnosed with malaria and treated. Notwithstanding, they were able to complete the clinical trial due to the physician's advice. More importantly, the temperature normalized after commencement of medication for malaria. The two participants who were diagnosed with malaria corresponds to 4% out of a total population of

200 study participants. This is less than the World Health Organization's estimated prevalence rate (> 75 per 1,000) which is estimated for the area (WHO, 2012).

The severity of side effects (Table 3) was generally described as mild (Apppendix F). Importantly, no major variations in the number of adverse effects were found between the treatment and control groups (P = 0.746) or severity of symptoms reported (P = 0.700).

Table 3

Reported Adverse Measures

Reported Symptoms	Treatment Group	Placebo Group	Total
Indigestion	none	none	0
Heartburn	none	none	0
Vomiting	none	none	0
Constipation	none	none	0
Loss of Appetite	none	none	0
Insomia	none	none	0
Nausea	none	none	0
Dizziness	none	none	0
Diarrhea	none	none	0
Bloating	none	none	0
Abnormal Discomfort	none	none	0
Other	none	2†	0
Incidence Total	none	2*	2*
Severity			
Mild	none	2	2
Moderate	none	0	0
High	none	0	0

*Shows the number of times an adverse event was reported.
† Shows participant has been diagnosed with malaria, according to medical results.

Analysis of haematology parameters

Tables 4 and 5 show analysis of haematology results. After 60 days of novasil administration, blood haemoglobin and platelets in the treatment group

showed a significant statistical difference of haemoglobin and platelets compared to baseline (P=0.005 and P=0.032 respectively) (Table 4). However, the placebo group showed no significant differences in blood hemoglobin and platelets. Haematological analysis (white blood cells, neutrophils, lymphocytes and monocytes) samples of blood showed no significant difference within the placebo group and treatment group (Table 4). All the blood cells with the exception of monocytes and lymphocytes were above the normal reference values across the treatment and placebo groups (Table 5).

Table 4

Analysis of Haematology Parameters with P-Values

	Treatment Group			Placebo Group		
	Before	After	p-Value	Before	After	p-Value
Haemoglobin (g/dL)	11.6	11.9	0.005*	11.4	11.3	0.477
WBC (1000/mm^3)	7.6	7.5	0.482	7.5	7.3	0.470
Platelets (1000/mm^3)	306	332	0.032*	310	312	0.660
Lymphocytes (%)	49.1	49	0.796	47.8	48.9	0.612
Monocytes (%)	9.4	9.6	0.643	8.6	8.2	0.138
Neutrophils (%)	37.1	37.0	0.923	35.9	34.4	0.335

Data are means (N= 200) and p-Values. P < 0.05 shows significant difference.
*Significance

Table 5

Analysis of Hematology Parameters with Reference Ranges

	Treatment Group		Placebo Group		Normal Reference Range
	Before	After	Before	After	
Hemoglobin(g/dL)	11.6 ± 0.8	11.9 ± 0.8	11.4±0.7	11.3 ± 0.6	11.0- 14.5 (A)
WBC(1000/mm^3)	7.6 ± 2.1	7.5 ± 1.8	7.5 ±1.7	7.3 ±1.1	3.4 - 12.0 (A)
Platelets(1000/mm^3)	306 ± 89.3	332.0 ±100	310 ± 85.8	312.1 ± 75.2	150.0 – 450 (A)
Lymphocytes(%)	49.1 ±10.0	49.0 ± 10.6	47.8 ±12.2	48.9 ± 9.0	28.0- 48.0 (B)
Monocytes (%)	9.4 ± 3.3	9.6 ± 3.2	8.6 ± 2.7	8.2 ± 2.3	3.6 - 6.0 (B)
Neutrophils (%)	37.1±10.2	37.0 ±10.9	35.9 ± 10.6	34.4 ± 8.7	32.0 - 67.0 (B)

Data is showed as reference ranges and mean ± SD (N = 200). Pediatric comparison range of values of Mayo Clinic are shown as A. Clinics in Minnesota and Children's Hospitals reference value ranges are indicated by the letter B. Male and female reference ranges are combined. WBC = white blood cell

Serum biochemistry

Serum biochemistry analyses indicative of liver function tests are shown in Tables 6. These biomarkers were found to be in the normal reference ranges before and after novasil administration among the treatment and placebo groups (Table 6). The difference between treatment and placebo groups for biomarkers such as albumin, total protein, total bilirubin, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine indicated no significant difference (Table 6). However, Gamma-glutamyl transferase (GGT) showed a significant difference within the novasil treatment group (P=0.004) on day 60. The placebo group also indicated no significant difference in biomarkers such as AST, urea,

creatinine, total protein and total bilirubin (Table 6). Values for ALT within the control were relatively high and showed a statistical difference (P= 0.02) (Table 6). However, the values were found to be in the normal reference range.

Table 6

Serum Biomarkers with Reference Range

	Treatment Group		Placebo Group		Normal Reference Range
	Before	After	Before	After	
Albumin(g/L)	45.3 ± 5.9	45.5 ± 2.7	45.3 ± 5.2	44.6 ± 4.0	35.0-50.0 (A)
ALT(U/L)	20 .1 ±7.2	21.4 ± 6.0	20.1 ± 7.8	22.5 ± 7.6[a]	7.0-55.0 (A)
AST(U/L)	28.3 ± 10.9	29.0 ± 9.4	28.2 ± 15.3	30.0 ± 13.6	8.0-60.0 (A)
GGT(U/L)	19.8 ± 10.1	19.0 ± 9.4[a]	21.0 ± 8.1	20.8 ± 7.2	7.0-29.0 (A)
T. Bil (µmol/L)	7.4 ± 3.4	7.6 ± 4.3	8.0 ± 4.7	8.3 ± 4.3	1.7-17.1 (A)
T. Protein (g/L)	75.8 ± 9.7	76.1 ± 6.1	76.3 ± 5.4	75.4 ± 4.4	63.0-79.0 (A)
Urea (mmol/L)	3.1 ± 0.9	3.0 ± 0.8	3.2 ± 0.5	3.1 ± 0.5	2.5-7.1 (A)
Creat. (µmol/L)	59.3 ± 7.0	60.5± 8.1	61.7 ± 6.5	60.6 ± 6.9	50-110 (D)

Data is showed as reference ranges and mean ± SD. Pediatric comparison range of values of Mayo Clinic are shown as A. Reference ranges of The Royal College of Physicians and Surgeons of Canada are shown as D. Reference ranges are combined for female and male values. T. Bil = Total Bilirubin and T. Protein = Total Protein. N=200, [a] indicates statistical significance within the treatment and placebo group. P< 0.05 indicates statistical significance. Creat. : Creatinine.

Analysis of minerals in blood

Tables 7 show analysis of minerals and aflatoxin M1 in urine. The levels serum calcium (Ca^{2+}), potassium (K+), sodium (Na+) and chloride (Cl−) were not statistically significant in both treatment and placebo groups at the end of the study (Table 7). The treatment showed a slight increase in Ca^{2+} and the placebo group

showed no change Ca^{2+} levels at the end of the study (Table 7). However, sodium, potassium and chloride were within the normal reference ranges in the treatment group whiles the placebo recorded lower sodium level (Table 7).

Analysis of blood glutathione and urine aflatoxin

The difference in blood reduced glutathione (GSH) levels within the treatment group from baseline and after treatment was statistically significant (P= 0.033) (Figure 10). Aflatoxin metabolite M1 level was statistically significant within the novasil treatment group at baseline and day 60 (P=0.02) (Figure 11). There was no change in the concentrations of GSH within the placebo group from day 0 to day 60 with respect to blood glutathione (P=0.384) (Figure 10). The placebo group showed significantly higher AFM_1 levels on day 60 of the study period (P=0.013) (figure 11). In the course of the study, all urine samples (placebo and treatment) had detectable AFM_1 in the range of 0.035 to 28,019.46 pg/mg creatinine. Urinary aflatoxin M1 showed a reduction from mean 811.2 AFM_1 pg/mg creatinine at baseline to 329.1 AFM_1 pg/mg creatinine on day 60, representing a 60.7 % reduction in the treatment group (Figure 11), whereas urinary aflatoxin M1 in the placebo group showed an increased mean of 801.3 AFM_1 pg/mg creatinine at baseline to 1801.2 AFM_1 pg/mg creatinine at representing 44.0 % of AFM_1 pg/mg creatinine on day 60 (Figure 11).

Table 7

Mineral Analysis in Serum

	Treatment Group		Placebo Group		Normal Reference Range
	Before	After	Before	After	
Sodium(mmol/L)	135.4 ± 6.1	135.9 ± 5.3	132 ± 8.3	133 ± 10.0	135.0-145.0 (A)
Potassium (mmol/L)	3.6 ± 0.4	3.7 ± 0.5	3.4 ± 0.4	3.4 ± 0.4	3.6 -5.2 (A)
Chloride(mmol/L)	102.0 ± 7.2	101.8 ± 4.5	103 ± 7.9	104.6 ±7.4	102.0-112.0 (A)
Calcium(mmol/L)	2.2 ± 0.1	2.3 ± 0.2	2.2 ± 0.1	2.2 ± 0.1	2.4-2.7 (A)

Data is showed as reference ranges and mean ± SD. (N=200). Pediatric comparison range of values of Mayo Clinic are shown as A. Reference ranges are combined for female and male values. (N=200). $P < 0.05$ indicates statistical significance within the treatment and control group. $P > 0.05$, No statistical significance was found within the placebo and test group.

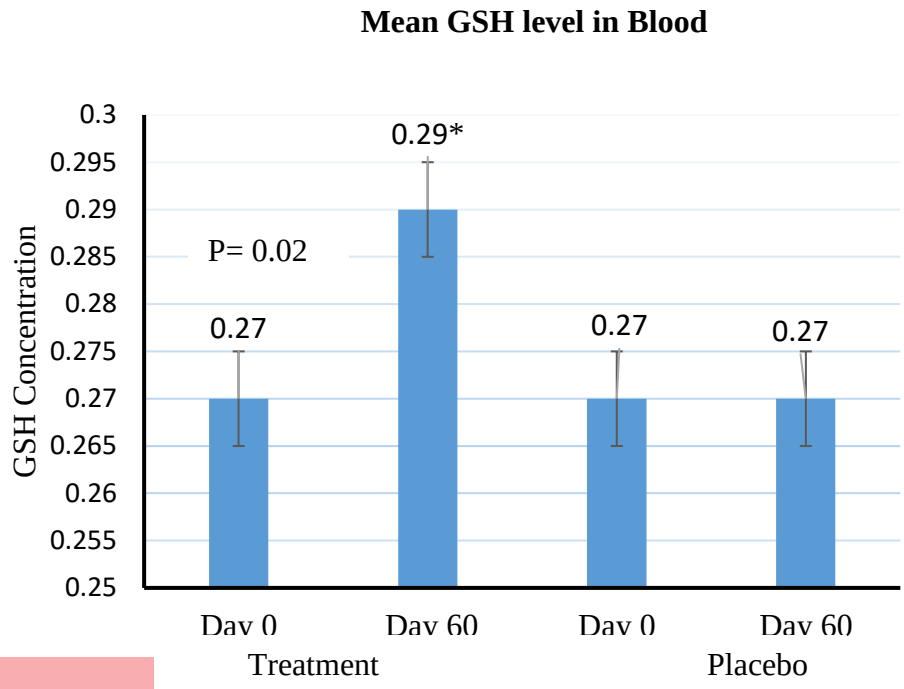

Figure 10: Data is represented as Mean GSH in blood. N=200.
P < 0.05 = * Significance

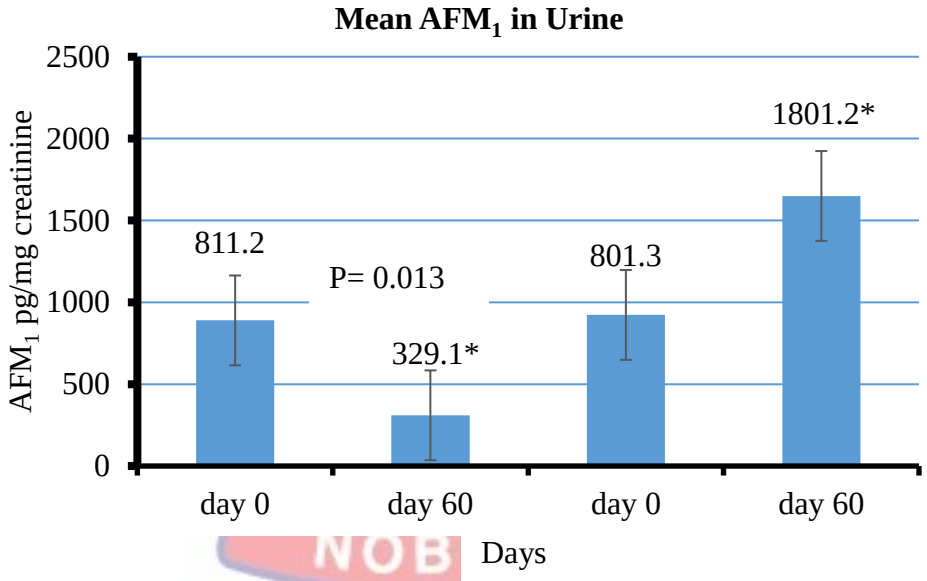

Figure 11: Data is represented as Mean Aflatoxin M1 in Urine. N=200.
P < 0.05 = * Significance

Correlation between Urinary Aflatoxin M1 and Stunting, reduced Glutathione and Serum Calcium

Correlation analysis (Pearson Correlation) to find the relationship between urinary aflatoxin M1 baseline values of participants against their height-for-age z-scores (stunting) at baseline was done. No significant relationship was found between AFM_1 and stunting parameters (Figure 12). The relationship between urinary aflatoxin M1 and serum calcium and blood reduced glutathione was evaluated after novasil treatment using correlation analysis. No significant relationship was found between urinary aflatoxin M1, glutathione and serum calcium ($P > 0.05$) (Figure 12, Figure 13 and Figure 14).

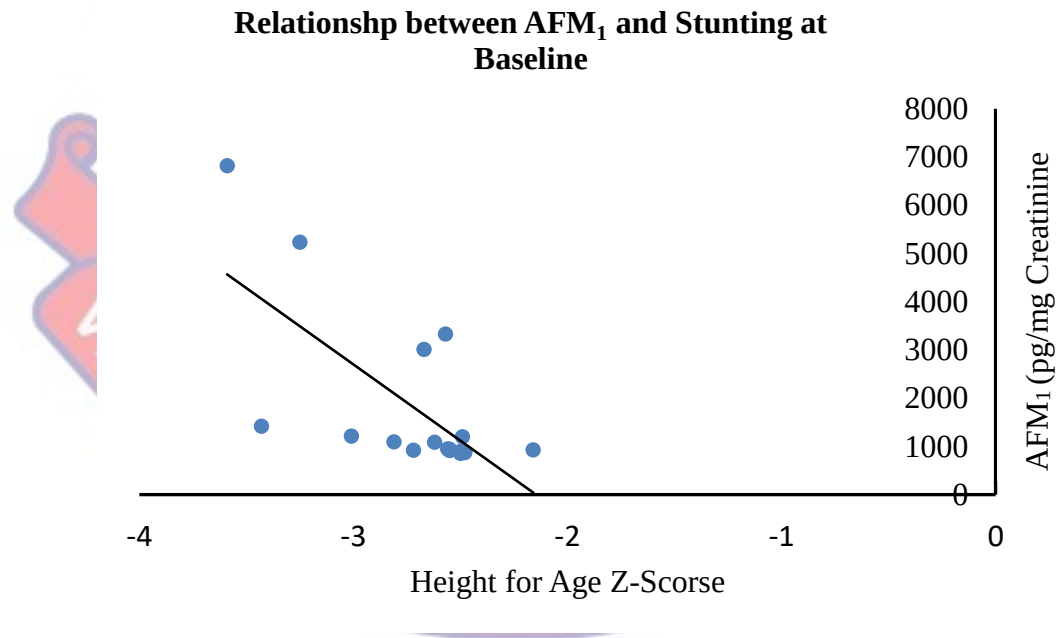

Figure 12: Data is represented as Mean AFM 1 in Urine and Height for Age Z Scores. N=26. $P < 0.05$ = Significance between stunting and AFM1. No significant relationship found ($P > 0.05$).

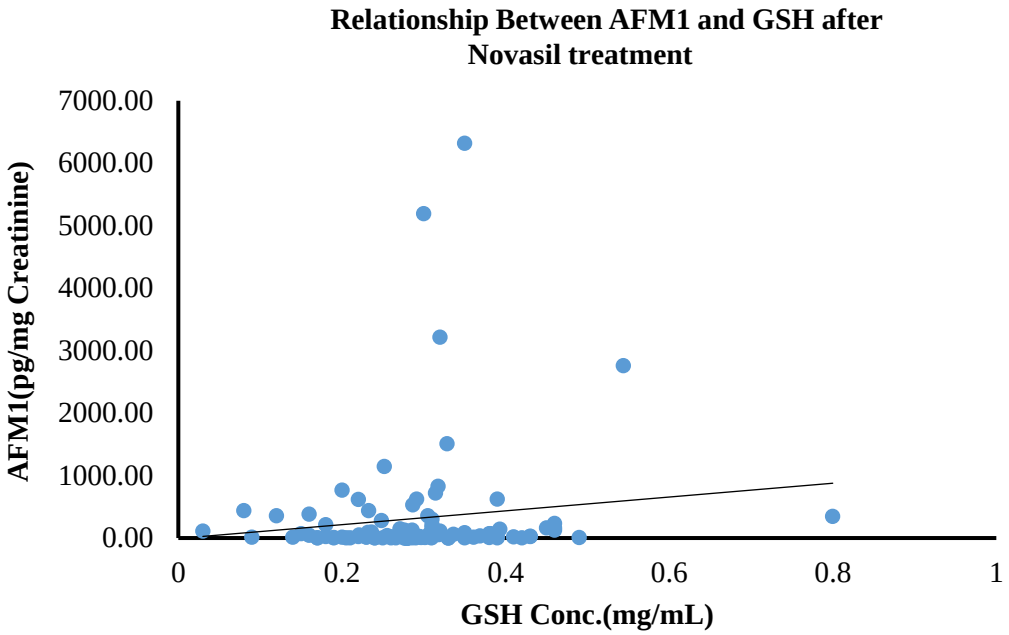

Figure 13: Data is represented as AFM $_1$ in urine and blood GSH for novasil treatment group. N=100. P < 0.05 = Significance relationship between AFM_1 and GSH within the treatment group No significant relationship found (P >0.05)

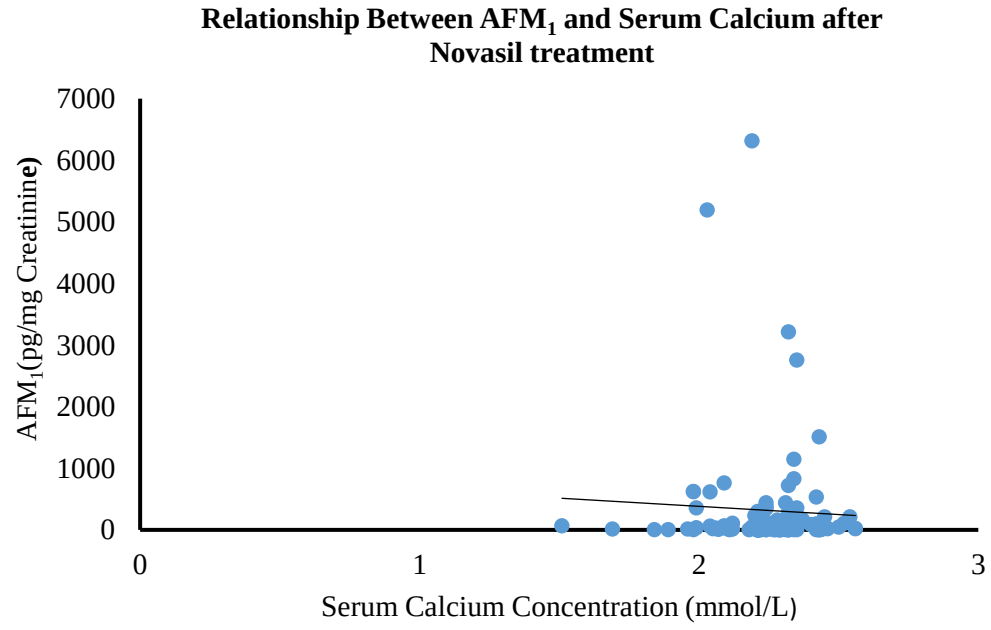

Figure 14: Data is represented as AFM $_1$ in urine and serum calcium for novasil treatment group. N=100. P < 0.05 = Significance relationship between AFM_1 and Ca^{2+}. No significant relationship found (P >0.05)

Discussions

Aflatoxin exposure in children over the years has been due to biological and environmental factors resulting in stunting of child growth, especially in sub-Sahara Africa. While nutritional and protein deficiencies are the most common causes of stunting and wasting, aflatoxin, a common maize and peanut contaminant has also been linked to stunting and wasting, especially in Africa and Asia (Gong et. al., 2004; Okoth, & Ohingo, 2004). Aflatoxin exposure has been reported over the years in the Ejura Sekyeduamse District (Wang et al., 2008; Kumi et al., 2014 ; Mitchell et al., 2013).

A survey conducted in Ghana in 2013, reported that 496 (19 %) children from the Ashanti region of Ghana where Ejura- Sekyeduamse is one of the districts were stunted amongst 2,896 households (Ghana Demographic Health Survey, 2014). In a study conducted in Benin and Togo among 480 children of ages between 1 and 5 years, aflatoxin adduct levels were found in 99 % of the samples (range: 5 to 1,064 pg/mg creatinine) (Gong et al., 2003). Also, stunting was found to be prevalent in 33 % of the children with 29 % of children being underweight (Gong et al., 2002). Gong and his colleagues 2002 reported an inverse relationship between aflatoxin-albumin adducts and growth, with stunted children having mean aflatoxin adduct concentrations 30 to 40 % more than children with normal z-scores. Rasheed et al. (2021), in estimating the burden of aflatoxin-related stunting in the population, found that 3–36 % of stunting was due to aflatoxin exposure. Similarly, the results of the current study found aflatoxin exposure in all the 200 participants with 13 % children being stunted and 3 % underweight at baseline data

(Figure 9). The current study is comparable to the study by Gong et al. (2002) and Rasheed et al. (2021). In the year 2002 a study involving 479 children from Benin and Togo by Gong and his colleagues, discovered an important dose-response correlation between AFB_1-albumin concentrations and weight-for-age z-scores (WAZ) and height-for-age z –scores (HAZ) (Gong et al., 2002). An 8-month longitudinal study accompanied the study in 2002 by Gong and his group, which revealed a significant negative relationship between AFB_1-albumin adduct concentrations and stunted growth (Gong et al., 2004). In contrast to the present study where the same growth indicators were used (WAZ and HAZ), there was no significant relationship between exposure of aflatoxin and growth indicators of the children in the current study (Figure 12). This could be due to only the baseline analysis of growth indicators employed in the present study and also participants were not followed up for a longer period as compared to the 8 months study by Gong and his group in 2002. In a study by Phillips et al., 2020 in Tanzania, change in growth indicators in correcting stunting could be seen in aflatoxin control diet of less than 5ug/kg at 18 months and beyond. The current study lasted for two months hence improvement in stunting among the participants could not have been realized in 60 days of novasil intervention as indicated by Phillips and her group. The percentage of stunting and underweight discovered in the present study was mainly based on baseline anthropometry data. Another district in the Ashanti Region reported a prevalence of stunted children up to 54.9 % (Inungu, 1995). Though the high rate of 54 % stunting recorded by Inungu (1995) was thought to be caused by a lack of nutrition and protein in their diet, however, there is still a challenging issue

as 26 (13 %) of the children in the current study were found to be stunted. One potential variable leading to poor health of children is exposure to aflatoxin-contaminated diets (Gong et al., 2002). Weanimix, a nutritious food made locally with maize, groundnuts and beans in the ratio (0.5:0.5:4) is one example. The product designed to prevent malnutrition in Ghanaian children aged 6 months to 2 years after weaning, was discovered to be contaminated with aflatoxin concentrations as high as 500 parts per billion (range, 14 – 500 ppb) (Kumi et al., 2014). Samples of urine collected from children before and after 21 days of eating weanimix showed higher urinary aflatoxin M1 levels (Kumi et. al., 2015). Weanimix is a common nutritional food in the Ejura Sekyedumase District. Aflatoxin has been implicated in causing stunting in children in Ghana according to the present study. Strategies to reduce aflatoxin poisoning are therefore necessary to alleviate the nutritional and growth challenges of children in this district while preserving the use of this important nutritional food.

Enterosorption therapy in reducing the bioavailability of aflatoxin could be a valuable approach in dealing with aflatoxin exposure in endemic areas where food insecurity leads to a lack of diversity (Phillips et al., 2008; Shephard, 2003). Research using dioctahedral smectite clays at doses up to 6 g/day for the treatment of acute diarrhea in children found few side effects, with mild constipation being the main side effect (Madkour et. al., 1993; Lexomboon, Harikul, & Lortholary, 1994; Szajewska, Dziechciarz, & Mrukowicz, 2006). Nicole et al. (2014) discovered that giving dietary novasil powder to healthy children (ages 3–9 years) at concentrations ranging from 0.75 to 1.5 g/day for 14 days resulted in minimal

side effects. In the study, neither dose-dependent adverse effects nor serious clinical indications were linked to novasil intake. Similarly, the findings of this study show that giving healthy children (ages 2–9) dietary novasil powder at a concentrations 1.5 g/day for 60 days was safe and has limited side effects (Table 3). The findings of the current study are consistent with those of earlier studies (Madkour et al., 1993; Lexomboon, Harikul, & Lortholary, 1994; Szajewska, Dziechciarz, & Mrukowicz, 2006; Nicole et al. 2014). Enterosorption method using novasil intervention has been assessed in a number of research (Afriyie-Gyawu et. al., 2005; Harvey et al., 1991; Mitchell et al., 2013; Mayura et al., 1998; Phillips et al., 2008; Nicole et al., 2014). Novasil, a natural clay based calcium montmorillonite, presents a feasible intervention strategy to minimize bioavailability of aflatoxin and eradicates aflatoxicosis (Afriyie-Gyawu et al., 2008; Phillips et al., 2008). Serum biochemical and haematological changes may be used to diagnose chronic aflatoxicosis before clinical symptoms (Oğuz et al., 2000). Smaller quantities of aflatoxin are hazardous to animal health because of its negative impact on certain biochemical and hematological principles. (Keçeci, Oguz, Kurtoglu, & Demet 1998).

Haematological changes have higher prognostic value for toxicity in humans, so blood parameter analysis is important in toxicological risk assessment in human and animal studies (Adeneye, Ajagbonna, Adeleke., & Bello, 2006; Olson et al., 2000). At a dose of 3.0 g/day in human adult intervention studies (phase 1, Texas, USA and Phase II, Ejura, Ghana), haematological analysis suggested that, novasil does not have adverse effects on blood cells of human and does not impair

the function of the body's immunity (Phillips *et al.*, 2008). A phase 1 study in children carried out by Ncole et al. (2014) similarly reported that, novasil inclusion in food has no adverse effect on blood cells of children, neither does it affect their immunity. The current study reports similar findings. Thus, novasil inclusion in food is safe and does not have adverse effects on blood cells. All blood cell parameters were within the reference ranges across the treatment and placebo group except monocytes and lymphocytes which were slightly above the normal ranges (Table 5). However, no statistically significant difference was found between total blood cell count across the placebo and the treatment groups (Table 4).With reference to the study by Nicole et al. (2014) who observed a slight increase of lymphocytes and monocytes in a phase 1 study, the current study observed the same pattern in the same district both in the placebo and treatment groups. This suggests that the slight increase above the normal ranges of lymphocyte and monocytes were not as a result of novasil inclusion in food after 2 months of children consuming 1.5 g/per day, since the same pattern was seen in the placebo group. Further investigations are warranted to confirm the slight increase of lymphocytes and monocyte in children from the district.

Haemoglobin, the protein molecule is also essential for the red blood cells to retain their form which is important for intake of oxygen. It is found in red blood cells and transports oxygen from the lungs to the body's tissues. Aneamia is a medical disorder defined by a low red blood cell count or haemoglobin level. Studies have linked presence of aflatoxin in mammals to anemia. The findings of a study by Keçeci, Oguz, Kurtoglu, & Demet (1998), indicated that animals who

received 250 µg/day of aflatoxin had a decreased level of haemoglobin than normal when compared to the control group. The study by Keçeci, Oguz, Kurtoglu, & Demet (1998) reported that aflatoxin is capable of inducing anemia in animals. A strong association was found between aflatoxin-albumin adduct and anaemia in a study by Faisal et al. (2010) conducted among pregnant women in Kumasi, close to where the current study was carried out. In the present study a statistically, significant increase in haemoglobin was seen after day 60 of novasil intervention in the treatment group (Table 4). The findings of Keçeci, Oguz, Kurtoglu, & Demet 1998) as well as Faisal et al. (2010) concur with the present study. The increase of haemoglobin in the present study could be due reduction of aflatoxin bioavailability since aflatoxin has been implicated in causing anaemia as reported by Faisal & colleagues, 2010. Reducing aflatoxin bioavailability may help to improve hemoglobin levels. These findings need to be explored further to establish a clear relationship between aflatoxin and anemia in humans.

Assessing haematological and biochemical parameters is an important method of evaluating health in both animals and humans (Milner et al., 2003). Blood biochemical analyses have been used by researchers in determining the safety of novasil (Phillips et al., 2008; Mitchell et al., 2013; Nicole et al., 2014). Serum liver biochemical analysis of the present study indicated that serum liver biomarkers levels were found to be within the normal reference ranges before and after novasil administration among the treatment and placebo groups (Table 6). Gamma-glutamyl transferase (GGT) showed a significant decrease in the novasil treatment group (P=0.004) on day 60 but values were within the normal reference

ranges, therefore the significant decrease is of no clinical importance. Aflatoxin has been seen to cause a rise GGT levels in workers exposed to aflatoxin (Mohgah et al., 2014). The intervention of novasil to reduce bioavailability of aflatoxin could have caused the decrease in GGT levels in the treatment group since the group had a significant reduction to aflatoxin. All other parameters such as AST, urea, creatinine, total protein and total bilirubin both in the treatment and placebo group showed no significant differences. The current study concurs with a study by Phillips et al. (2008) and Mitchell et al. (2013) who stated that novasil inclusion in food for consumption by adults in the same community did not adversely affect liver function. In a 14-day phase 1 study in children in the same community, levels of serum liver biomarkers showed that novasil was safe and did not affect liver function (Nicole et al., 2014). The current study is in agreement with the findings of Nicole & colleagues (2014), who indicated that novasil inclusion in food for children from the ages of 2-9 years was safe against liver function. However, Nicole & colleagues (2014), reported an increase in ALT in the placebo group. The current study also found a similar trend in the values of ALT (Table 6), although they were within the normal reference ranges (Table 6). A study by Randa, Dujana, Hye-Joo, & Afaf El-Ansary (2018) recorded increased significant levels of ALT in humans exposed to aflatoxin in Saudi Arabia. The significant increase in ALT levels in the placebo group could be due to high concentrations of aflatoxin exposure in the community. The significant increase of ALT in the 14-day clinical intervention study in the same community by Nicole et al. (2014) remains unclear since all other liver parameters were within the normal reference ranges as seen in the current

study. Whiles an increase in serum bilirubin levels has been identified in animal species after exposure to aflatoxin, to the best of our knowledge no associations between aflatoxin exposure and bilirubin levels in humans have not been found (Clark, Hatch, Miller, & Jain, 1984; Cliford, & Rees, 1967). Although AFB_1-albumin adduct is a useful aflatoxin assessment biomarker in the blood, it is a long-term biomarker of exposure that does not change as rapidly as the urinary biomarker with recent exposure. As a result, urinary AFM_1 is a more accurate marker for correlating with complex serum components such as bilirubin. Since stunting of growth and underweight are usually observed in most types of chronic liver disease, future research in Ghana to determine the relationship between liver function parameters associated with children with stunted growth and underweight and aflatoxin exposure will be important (Sokol, & Stall, 1990). Furthermore, since direct bilirubin can be detected in the urine of individuals who have liver disorders, it could be a good non-invasive biomarker to check, in clinical aflatoxin research.

Within the treatment period (60 days), sodium, potassium, and chloride ion levels were all within the normal reference ranges in both the treatment and placebo groups, with no significant differences over the study period (Table 8). In comparison to the research of Nicole et al. (2014), the current study found a similar pattern in potassium, sodium and chloride levels that were within the normal reference range, as shown in Table 7.

Total calcium levels in serum in both treatment and placebo groups were found to be within the normal reference range (Table 7). Total serum Ca^{2+} levels in the treatment group were increased in the current research after 60 days of novasil

treatment (Table 7). There was no significant difference in total serum $Ca^{2}+$ in the placebo category (Table 7). The findings of this study support those of Afriyie-Gyawu et al. (2005) and Marroquin-Cardona et al. (2011), who also found increased calcium levels after novasil administration in animals. No relationship was found between AFM_1 and calcium ions in the treatment arms of the present study (Figure 14). Calcium is one of the most important minerals for the human body. It promotes the development and maintenance of healthy teeth and bones, particularly in children. Maintaining a healthy calcium level in the body over the course of a lifetime will aid in the prevention of osteoporosis. Calcium levels in plasma have been found to decrease in animals exposed to aflatoxin (Nassar, Galal, Mohamed, & Hafez, 1985). The administration of novasil to rats increased levels of calcium ions ($Ca^{2}+$). The breakdown of calcite and exchangeable $Ca^{2}+$ ions from montmorillonite clay was the cause of this (Afriyie-Gyawu et al., 2005; Marroquin-Cardona et al., 2011). Nicole et al., 2014, in 14-day (phase I study) novasil intervention study reported that, calcium levels were reduced in both the placebo and treatment group in clinical trials conducted in the same district as the current study. Nicole et al. (2014) reported that the overall decrease in calcium serum levels may be due to dietary changes made during the intervention study. Since novasil clay commonly acts as a calcium supplement, it was likely that the increase in total serum calcium levels seen in the treatment group in this study was due to the addition of novasil to the food. Calcium levels in the placebo group did not see any change in levels though the placebo also received calcium sulphate needs further

research. To conclude whether novasil could affect micronutrient absorption, longer safety trials, controlling for critical dietary nutrient intake are required.

Glutathione is an antioxidant produced by liver cells. The three amino acids glutamine, glycine, and cysteine make up the most of it. A variety of factors, including poor diet, environmental toxins, and stress, can lower glutathione levels in the body. Glutathione (GSH) is essential in protecting tissues from the damaging effects of free radicals of bioactivated aflatoxin (Iyer et. al., 1994). Aflatoxin caused a reduction in the level of blood GSH in an animal study (Ankrah, Archibald, Addo, & Ekuban, 1995). The study reported a reduction of 24 % blood GSH in aflatoxin infected mice. The findings of Ankrah, Archibald, Addo, & Ekuban, (1995) suggest that, reducing aflatoxin levels in animal studies will help improve the level of blood GSH. The current study found an increased level of blood GSH in the treatment group at the end of novasil intervention (Figure 10). A significant difference was found between blood GSH at day 0 (baseline) and after 60 days novasil treatment period in this study (Figure 10). The findings of the present study suggest that reducing aflatoxin bioavailability of blood in human studies could help improve the concentrations of blood GSH. No correlation was seen between AFM_1, and blood GSH as reported in the present studies (Figure 13). This could be due to the duration of the study and sample size. The placebo group in the current study showed no significant difference in levels of GSH in blood (Figure 10). However, future control research involving two arms of study with experimental group who received glutathione and a control group without

glutathione is warranted to determine the relationship between aflatoxin and GSH in humans.

Montmorillonite clay (novasil) has been identified as the active ingredient in bentonites for aflatoxin binding (Marroquin-Cardona et al., 2009; Kannewischer, Arvide, White, & Dixon, 2006). Aflatoxin adsorption in the gastrointestinal tracts of animals and humans (primarily onto the interlayer spaces of montmorillonite) reduces toxin bioavailability to organs and blood, according to the proposed mechanism of action (Phillips, 1999). A decrease in AFM_1 percentage excretion in urine (45%, 55%, and 58.7%) has been reported previously in adult studies using novasil (Mitchell et al. 2013; Wang et. al., 2008). At a dose of 1.5 g novasil/day, a significant reduction of 52 % in urine excretion of AFM_1 was observed in the novasil treatment group as compared to the placebo group (Nicole et al., 2014). The findings of the present study recorded a significant decreased ($P = 0.013$) of 60.7 % of AFM_1 in urine excretion in the treatment group (Figure 11) compared to 44% increase of AFM_1 urine excretion in the placebo group (Figure 11). The findings of the present study showing a decrease in AFM_1 excretion in urine in a 60-day intervention with novasil is comparable to the outcomes in earlier studies (Wang et al., 2008; Nicole et al., 2014). It is worth noting that, there has been an improvement in the level of AFM_1 excretion in urine from the 52 % recorded by Nicole *et al.* (2014) in a 14-day study compared to the 60.7 % AFM_1 excretion in urine (Figure 11) in the current 60-day intervention studies in the same district. This difference could be due to longer intervention studies employed in this research. We can infer from the two studies that, increasing the intervention period using novasil could

correlate with reduced percentage of aflatoxin bioavailability. The outcome from this clinical intervention study demonstrates that, novasil ingestion by children between the ages of 2–9 years is safe at a dose of 1.5 g/day for 60 days. The addition of novasil to cereal/grain based weaning foods is beneficial and could reduce the amount of aflatoxin B1 in contaminated diets and bioavailability in humans. This could reduce the negative effects of aflatoxin exposure and also improve the value and bioavailability of nutrients present in cereal/grain and legume foods.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

The end point of the study indicates that, novasil inclusion in food was beneficial to children. The study concluded that, after 60 days of a phase II novasil clinical trial, no adverse effects was recorded in response to novsil treatment. More importantly, no major variations in the number of adverse effects were found between the treatment and control groups.

Stunting was found among 13 % out of a total population of 200 study participants which could be due to high aflatoxin exposure. Three percent (3%) of the study population recorded underweight.

Haemoglobin levels increased in the treatment arm of the study after novasil inclusion in food for a 60-day period as seen for the first time.

Glutathione levels were found to be higher for the first time in human studies in the treatment group after novasil intervention for 60 days.

Aflatoxin M1 pg/mg creatinine was found in all study participants.

The outcome from this clinical intervention studies demonstrates that novasil consumption by children (ages 2–9 years) for the first time in a phase II studies was safe at a dose up to 1.5 g/day for 60 days of intervention and reduces bioavialabilty of aflatoxin by 60.7 % . Novasil promises to be used as aflatoxin binder as food supplement/fortification to control exposure of aflatoxin in human thereby preventing aflatoxin related health problems.

Recommendations

With reference to the conclusions of the current study the following recommendations are made:

1. A phase II clinical trials without larger sample size, monitoring important dietary nutrients such as zinc, iron and selenium for intake are needed to establish whether novasil could interfere with micronutrient or mineral absorption in children.
2. Longer safety and efficacy study (phase III) will be of paramount interest to assess whether novasil efficacy in aflatoxin binding will improved.
3. Future control research involving two arms of study with experimental group who received glutathione and a control group without glutathione is warranted to determine the relationship between aflatoxin and glutathione in humans.
4. Future studies is needed to establish whether novasil can have an impact on growth and anthropometric indicators in children in a longitudinal study.

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APPENDICES

APPENDIX A

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979
A Constituent of the College of Health Sciences
University of Ghana

Phone: +233-320-501180/513202 (Direct)
+233-320-501178/9 (S/board)
Fax: +233-320-502182/513202
E-mail: Director@noguchi..ug.edu.gh

Post Office Box LG 581
Legon, Accra
GHANA

My Reference: DF5

Your Reference:

9th May, 2019

Mr. Justice Kumi
Department of Clinical Pathology
NMIMR
University of Ghana
Legon

Dear Sir,

APPROVAL OF PROTOCOL

The Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research at its meeting on 9th April, 2019, reviewed the protocol entitled: "Safety and Efficacy of Calcium Montmorillonite Clay (Novasil) in Children at risk of Aflatoxin related Diseases in the Ashanti Region of Ghana", STC Paper 1(5) 2018-19, which was submitted by Mr. Justice Kumi.

The Committee after the review approved the protocol on Thursday, 9th May 2019.

The Scientific and Technical Committee avails to you the assurances of its highest consideration.

Thank you.

Yours faithfully,

Professor Abraham Kwabena Anang, PhD
DIRECTOR

APPENDIX B

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

Phone: +233-302-916438 (Direct)
E-mail: nirb@noguchi.ug.edu.gh
Telex No: 2556 UGL GH

My Ref No: DF22
Your Ref. No:

Post Office Box LG 581
Legon, Accra
Ghana

8th January, 2020

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 001/19-20 **IORG 0000908**

On 8th January 2020, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your revised protocol titled:

TITLE OF PROTOCOL : **Safety and efficacy of calcium montmorionite clay (Novasil) in children at risk of aflatoxin related diseases in the Ashanti Region of Ghana (Phase II)**

PRINCIPAL INVESTIGATOR : **Justice Kumi, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 7th January, 2021. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB CHAIR)

APPENDIX C

UNIVERSITY OF CAPE COAST

INSTITUTIONAL REVIEW BOARD SECRETARIAT

TEL: 0558093143 / 0508878309/ 0244207814
E-MAIL: irb@ucc.edu.gh
C/O Directorate of Research, Innovation and Consultancy

OUR REF: UCC/IRB/A/2016/582
YOUR REF:
OMB NO: 0990-0279
IORG #: IORG0009096

7TH FEBRUARY, 2020

Mr. Justice Kumi
Department of Biomedical Sciences
University of Cape Coast

Dear Mr. Kumi,

ETHICAL CLEARANCE – ID (UCCIRB/CHAS/2019/109)

The University of Cape Coast Institutional Review Board (UCCIRB) has granted **Provisional Approval** for the implementation of your research protocol titled **Safety and Efficacy of Novasil in Children at Risk of Aflatoxin Related Diseases in the Ashanti Region of Ghana.** This approval is valid from 7th February, 2020 to 6th February, 2021. You may apply for a renewal subject to submission of all the required documents that will be prescribed by the UCCIRB.

Please note that any modification to the project must be submitted to the UCCIRB for review and approval before its implementation. You are required to submit periodic review of the protocol to the Board and a final full review to the UCCIRB on completion of the research. The UCCIRB may observe or cause to be observed procedures and records of the research during and after implementation.

You are also required to report all serious adverse events related to this study to the UCCIRB within seven days verbally and fourteen days in writing.

Always quote the protocol identification number in all future correspondence with us in relation to this protocol.

Yours faithfully,

Prof. P.K. Buah-Bassuah
UCCIRB Chairperson

CHAIRPERSON
INSTITUTIONAL REVIEW BOARD
UNIVERSITY OF CAPE COAST

APPENDIX D

NMIMR-IRB PARENTAL CONSENT FORM

Title: Safety and Efficacy of Calcium Montmorillonite Clay (Novasil) in Children at Risk of Aflatoxin Related Diseases in the Ejura- Sekyedumase District, Ghana

Student: Justice Kumi

Address: Department of Biomedical Science, School of Allied Health Sciences, University of Cape-Coast

General Information

This study is entitled, "Safety and Efficacy of Calcium Montmorillonite Clay (Novasil) in Children at risk of a poison from fungal and related diseases in the Ashanti Region of Ghana". Your child is being asked to take part in this research study because we want to learn more about how Novasil clay study in Ghanaian children, will help reduce fungal poisoning.

Previous findings shows that, Novasil study in children (ages 2–9 years) from Ejura-Sekyedumase Community, for two weeks was safe and also showed a decrease in the poison level. If you agree to be on this study, your child's food will be mix with half teaspoon of novasil every day for 90 days. Not every child wil receive novasil in his/her food. Some of the children would be put into a control group. Your child will be asked to provide blood sample (one teaspoon full) and urine sample (about 6 teaspoons full) before adding novasil to his/her food and on day 90 where novasil will not be added to his/her food. A trained biomedical laboratory scientist will draw the blood from one of the arms of your child. Your child's blood sample will be use to test how healthy his liver is and also for some

nutrients you need in your blood to grow well. Urine (6 teaspoons full) samples will also be taken from your child into a clean urine cup. The urine samples will be used to test fungal poison. Your child's height and weight will be taken to calculate how your child is growing. The researchers will share the results with you.

Possible Risks

The risk that comes along with your child's participation is a slight pain from the collection of blood. The process of blood drawing for laboratory test sample can also be associated with rare risks including bruising, bleeding or skin infection. Before blood collection, your child's arm will be cleaned with alcohol and a new hollow needle/plastic tube will be used to take the blood samples. Well trained laboratory staff will draw the blood to decrease the chances of those risks/harm happening and alcohol wipes will be used before drawing to avoid contamination/ infection. However, if an improper event happens, your child will be immediately managed by a medical doctor who already works with the hospital at Ejura and your child would be provided with free medical care in hospital.

Possible Benefits

Including your child in this study will result in no direct benefits. However, as part of the objectives of the study; we hope that the information from the study will form the firm basis to find solutions that will help reduce the amount of parasite in the urine of your child. Your child's participation would also help informed the effectiveness of the treatment.

Confidentiality

We will protect information about your child to the best of our ability. Your child will not be named in any reports. The records of your child will be kept under lock and key for a maximum of two years after which it would be destroyed. However, the leader of the study team and the person who will work on your child's records may sometimes look at your child's records.

Compensation

The study will not give your child any compensation. However, your child would be given a tin of milo, a tin of milk and a packet of sugar as an appreciation for the time spent.

Voluntary Participation and Right to Leave the Research

Your child can stop being part of this study at any time if he/she feels uncomfortable. No one will be angry with you if you do not want to participate.

Contacts for Additional Information

You may ask me any questions about this study on behalf of your child. You can call me at any time on 0244876215 or talk to me the next time you see me. Please talk about this study with your wards before you decide whether or not to participate.

Your Child's Rights as a Participant

This research has been reviewed and approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB). If you have any

questions about your child's rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.ug.edu.gh

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title: Safety and Efficacy of Calcium Montmorillonite Clay (Novasil) in Children at risk of aflatoxin related diseases in the Ashanti Region has been read and explained to me on behalf of my child. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

_____

Date

Name and signature or mark of parent or guardian……..

If volunteers cannot read the form themselves, a witness must sign here:

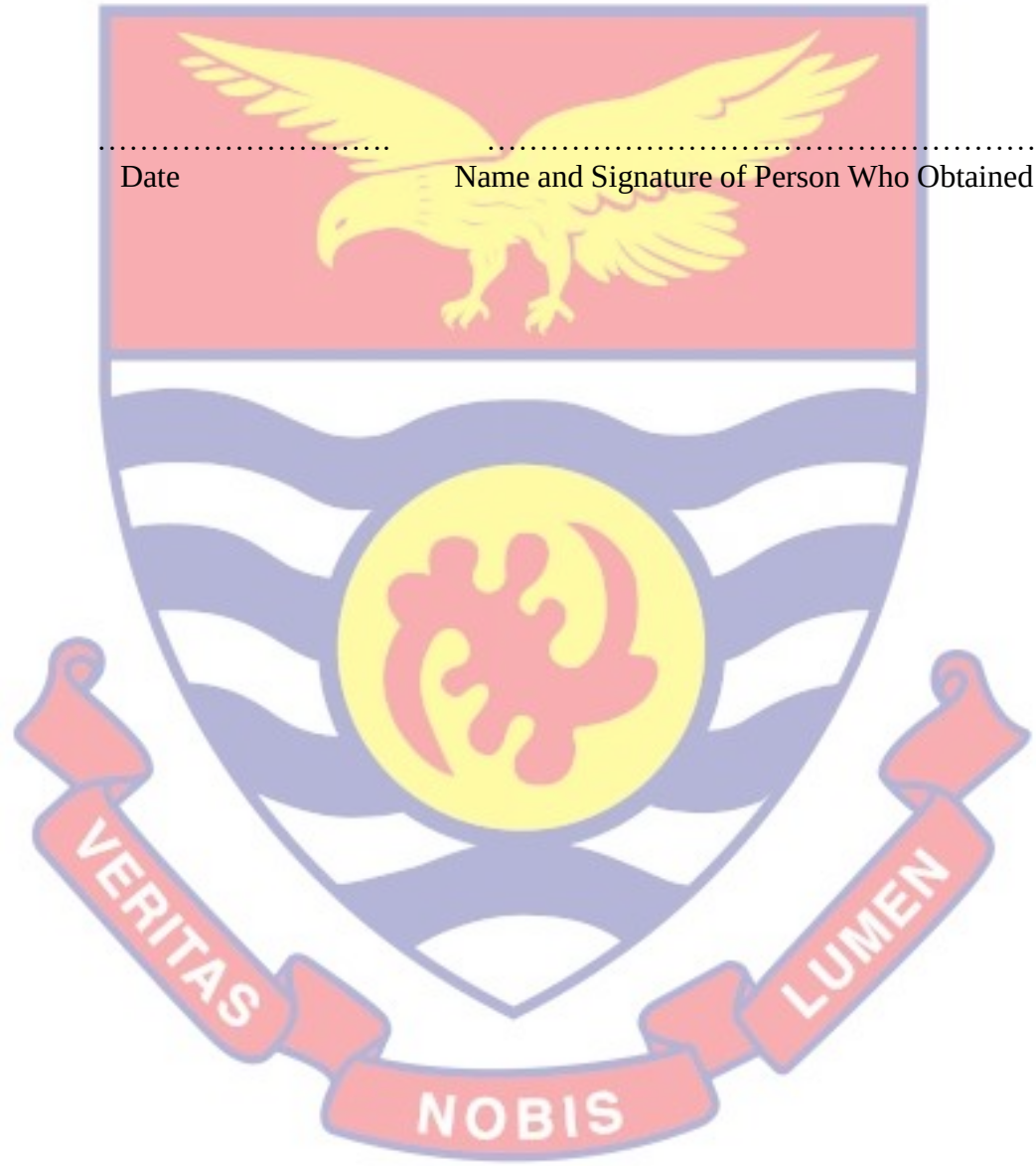
I was present while the benefits, risks and procedures were read to the child's parent or guardian. All questions were answered and the child's parent has agreed that his or her child should take part in the research.

_____

Date

Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.



…… ……

Date Name and Signature of Person Who Obtained Consent

APPENDIX E

PARTICIPANTS SCREENING

PHYSICAL EXAMINATION FORM

A. Screening ID Code…….

1. Name……

2. Gender: male…….female……..

3. Age…….

Address……
……

4. Date of Birth…….yy…….mm……dd

5. Date of Visit……..yy…….mm……dd

6. Informed consent for screening study signed?

Yes….. No……

B. Physical Examination Finding:

7. Height……..cm

8. Weight…….kg

Circle appropriate response	**Normal**	**Abnormal**
9. Skin	0	1
10. Eyes	0	1
11. Lymph nodes	0	1
12. Extremities: edema	0	1

13. BMI…….

C. Blood Hemoglobin……..g/dl

APPENDIX F

DAILY DIARY WORRKSHEET

Study Monitor (initials)	Day	Test Agent Taken	Side Effects	Grade 1, 2, 3…	Treatment 1,2,3,4
	1	Y N			
	2	Y N			
	3	Y N			
	4	Y N			
	5	Y N			
	6	Y N			
	7	Y N			
	8	Y N			
	9	Y N			
	10	Y N			
	15	Y N			
	16	Y N			
	17	Y N			
	18	Y N			
	19	Y N			
	20	Y N			
	21	Y N			
	22	Y N			
	23	Y N			
	24	Y N			
	25	Y N			
	26	Y N			
	27	Y N			

Start Date……

Side Effects

0= Other (Specify)	1= Indigestion	2= Nausea	3= Vomiting	4= Constipation	5= Diarrhea	6= Flatulence	7= Loss of Appetite

8=Abdominal Discomfort	9= Heartburn	10=Dizziness	11=Insomnia	12=Bloating	13=None

Grade: 1=Mild 2=Moderate 3=Severe Treatment**:** 1=No treatment 2=Self Treatment 3=Called research team only 4=Sought immediate medical attention.

Dispensing Records(s)

Initials …… Signature……

APPENDIX G

ADVERSE EVENT REPORT FORM

A. Clinic, participant and visit identification

1. Participant's ID code……..
2. Date of Visit……..
3. Laboratory ID code……
4. Cycle number……
5. Date specimen analysed (yy/mm/dd)……..
6. Sequential number of this Adverse Event form first form completed on any one date is number 01; if more forms are needed, number additional forms sequentially…….

B. Adverse Event of Toxicity

Use this event codes and severity grades from the side effect/toxicity monitoring from to complete this form whenever possible.

7. Adverse event

a. Specify…….

b. Laboratory value (if applicable; include units)……

c. Adverse event code:……

8. Onset date (yy/mm/dd)……

9. Is this the first report or a follow-up report regarding this adverse event ?

First report…..1 Follow-up…..2

10. Did the event occur during or within one-half hour of ingesting the study material?

Yes…..1 No…….2 Unkownn……..3 N/A…….4

11. Is the event associated with the study material toxicity?

Yes…….1 No…..2

If not related specify the underlying condition:……
……
……
……
……..

12. Severity of event of toxicity (check only one):
For follow-up reports, check current severity.

…….. Life-threatening (grade 4)

..…… Severe (grade 3)

..…… Mild (grade 2)

…… Mild (grade 1)

…… Resolved (follow-up reports only)

13. Did the event or toxicity result in any of the following?

	No	Yes
Emergency room visit	……..	……
Hospital admission	……..	…….
Severe of permanent disability	……..	…….
Death	……..	…….

14. Was participant treated with prescription drug?

Yes…..1 No….2

If yes, specify
treatments……

15. Current status (check only one) →

……Resolved Date resolved (mm/dd/yy)……

……...Active

……Unknown

16. Has the study drug been modified because of adverse events? (Check only one)

…….No modifications → go to question 18

…….Dose reduced

…….Drug stopped

…….Other, specify……..

17. Date of study material modification (mm/dd/yy)…….

18. Did event resolve after study material stopped or dosage reduced? (Check only one)

Yes…..1 No….. 2 Uncertain…..3 Not applicable…….4

APPENDIX G (cont'd) – ADVERSE EVENT REPORT FORM

INSTRUCTION:

SUBMIT this form when a participant:
1. Fails to start assign therapy
2. Discontinues therapy

Participant's Name……..

Study Number……..

Date for completed (mm/dd/yy)……

Date the discounted drug were taken for at least part of the course……

Last course from which the drug were taken at least part of the course ……..

Reason the powder was discontinued below:

Toxicity, specify conditions…..1…….

Other Medical condition, specify……2…….

Other non-medical reason, specify….3……

EXIT INTERVIEW RESULTS

Date of interview (mm/dd/yy)…….

Interviewer……...

Reason given for withdrawal from study……

Compliance interventions attempted prior to study withdrawal……

……
……..

If the participant has permanently discontinued therapy and an exit interview was not conducted, please describe

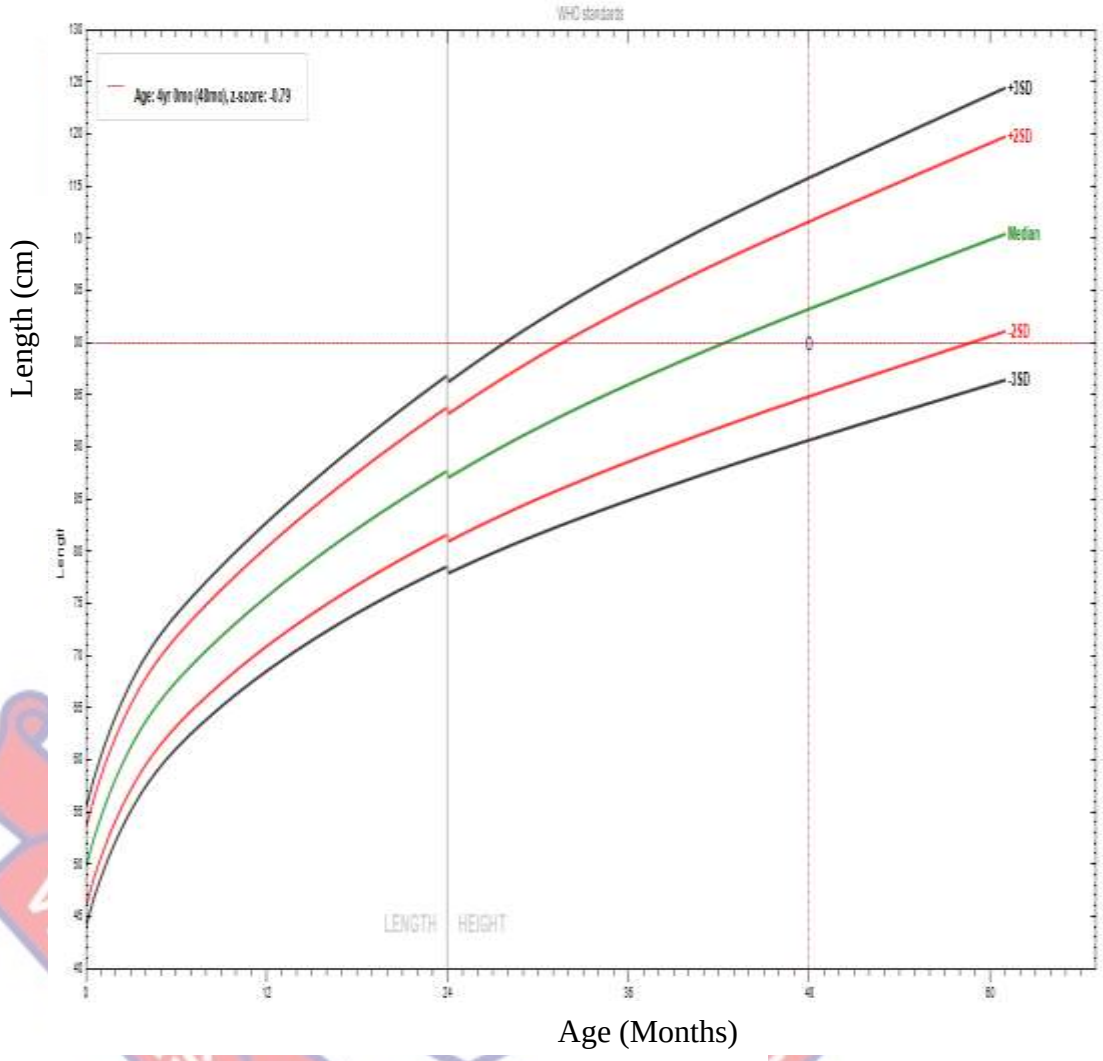
why:……
……
……

COMMENTS (IF ANY)

APPENDIX H

WHO ANTHROPOMETRY STANDARD CURVE

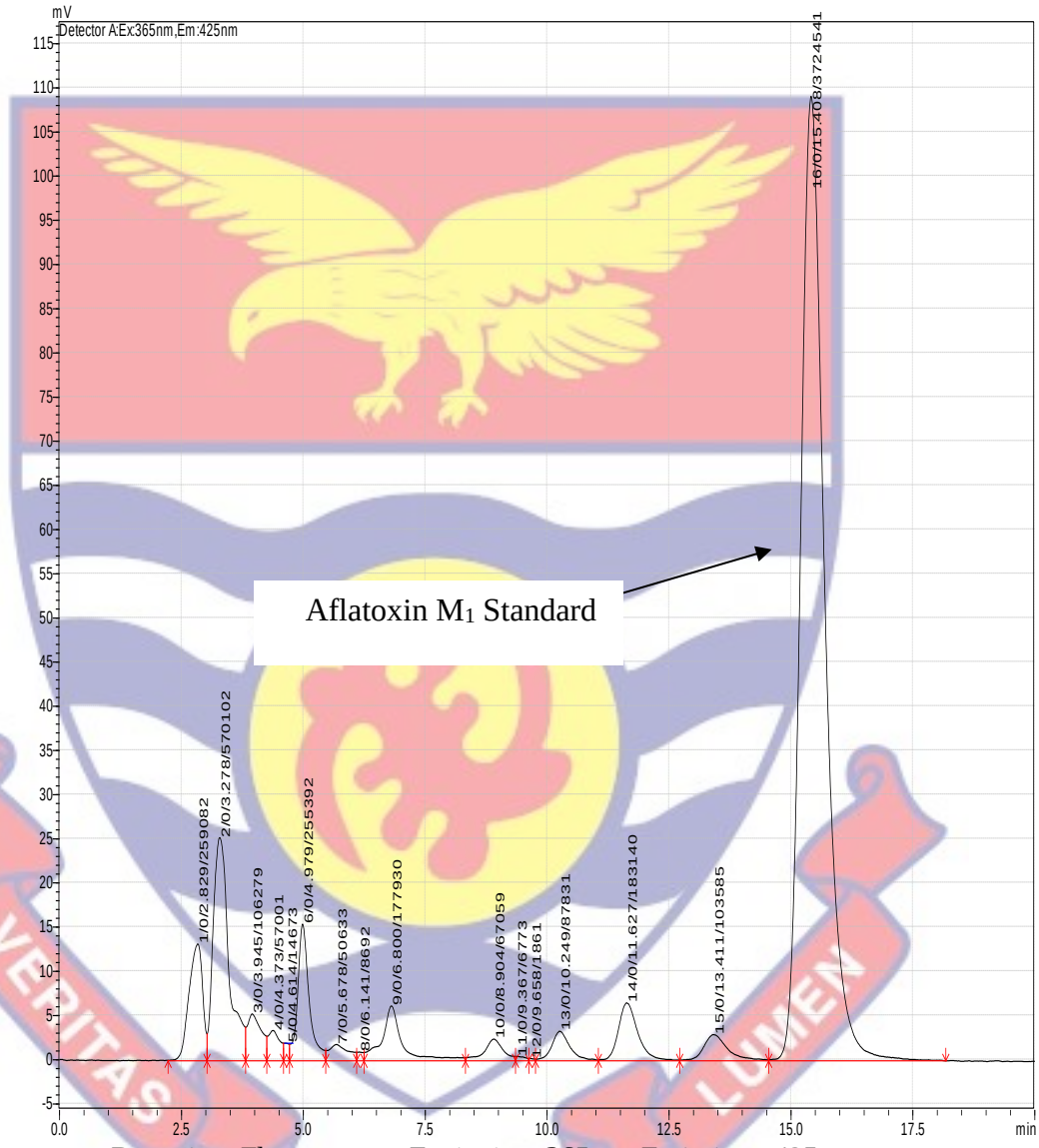
HEIGHT FOR AGE (HFA)



APPENDIX I

HPLC CHROMATROGRAM SHOWING

AFLATOXIN M_1 STANDARD

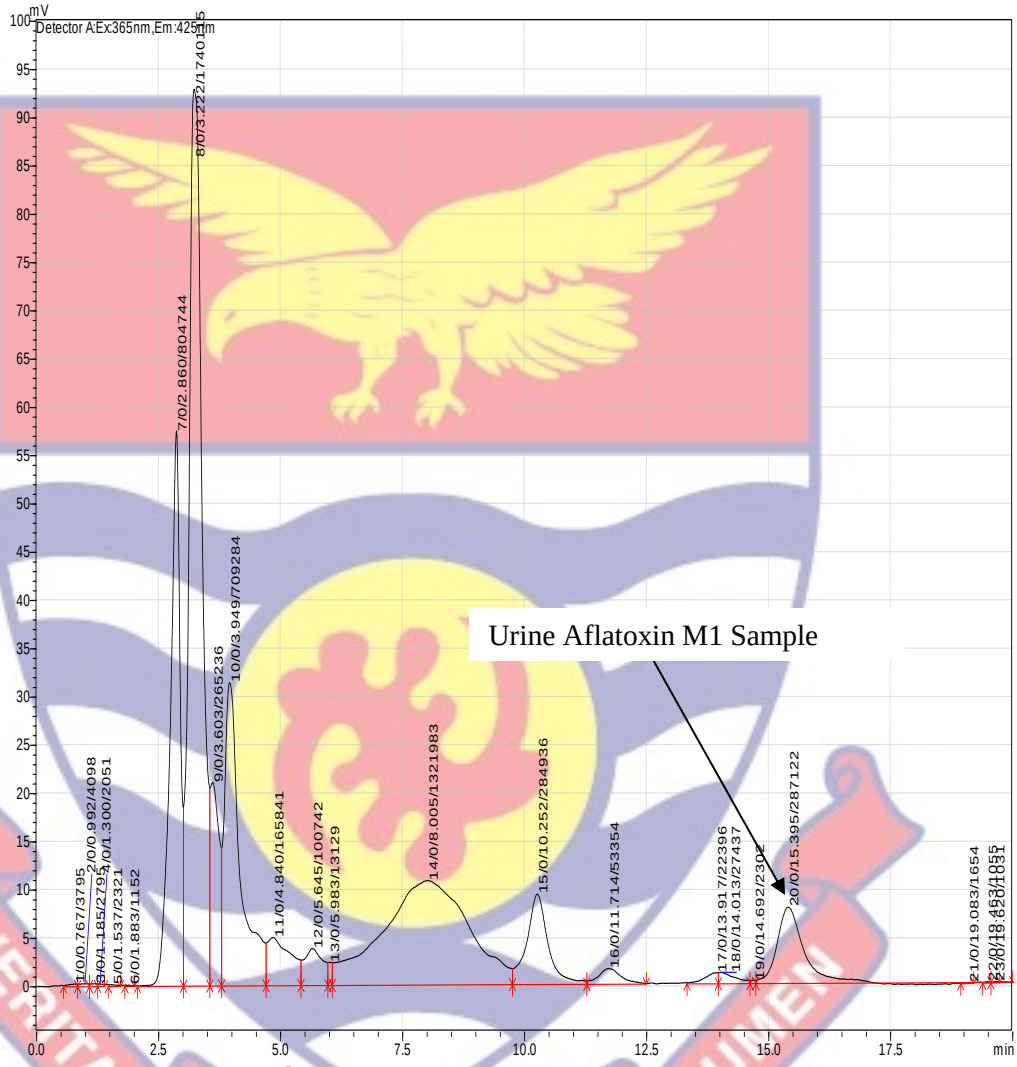


Detection: Fluorescence, Excitation: 365nm, Emission: 425 nm,
Retention Time: 15.4 min

APPENDIX J

HPLC CHROMATROGRAM SHOWING

AFLATOXIN M_1 IN URINE



Detection: Fluorescence, Excitation: 365nm, Emission: 425 nm
Retention Time: 15.4 min