EFFECTS OF AQUEOUS EXTRACT OF *Spirulina plantesis* ON IMMUNOLOGIC DYSFUNCTION AND INFLAMMATION ASSOCIATED WITH AFLATOXIN B1-INDUCED TOXICITY IN MICE

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JUNE 2024

DECLARATION

This thesis is my original work and has not been presented for award of a degree in any other university or for any other award.

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DEDICATION

With whole-hearted devotion, I dedicate this thesis to my adorable family. Their unwavering backing and kind words during my studies have always given me a boost of motivation. This would not have been possible without the tireless support and love they have shown me.

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ABBREVIATIONS AND ACRONYMS

AFB1	Aflatoxin B1
ANOVA	Analysis of Variance
ANVISA	Agência Nacional de Vigilância Sanitária *(National Health Surveillance Agency Brazil)*
BALB	Bagg Albino c
CDC	Centre for Disease Control
cDNA	Complementary DNA
CTMDR	Center for Traditional Medicine Research
CYP450	cytochrome P450
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	Generally Regarded as Safe
GSH	Reduced glutathione
GSTs	glutathione-S-transferases
HCC	Hepatocellular Carcinoma
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
IFNγ	Interferon Gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
KEMRI	Kenya Medical Research Institute
MMUST	Musinde Muliro University of Science and Technology
mRNA	messenger RNA
ppb	parts per billion
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription polymerase chain reaction
TNFα	Tumor Necrosis Factor Alpha
WHO	World Health Organization

ABSTRACT

Kenya is among African countries that face the burden of food contamination by aflatoxin. High levels of aflatoxins have been reported to kill 157 people in Kenya while also contributing to cancer burden. Aflatoxins are produced by *Aspergillus flavus, Aspergillus parasiticus, Aspergillus* and *Aspergillus nomius.* These toxins majorly affects cereal grains. This study aimed at bioprospecting *Spirulina plantesis* in ameliorating immune dysfunction and inflammation caused by aflatoxin B1 (AFB1). *Spirulina plantesis* have been consumed for decades as food supplement as it has been proven to have strong antioxidant and anti-inflammatory properties. Male BALB/c mice weighing 28 to 34 g were divided into six groups at random and given the following oral treatments: Group 1 was only provided with food and water during the treatment course. AFB1 was given orally to Group 2 at a dosage of 200µ g/kg body weight. AFB1 was administered orally to Group 3 an hour after receiving 1g/kg of activated charcoal. Groups 4, 5, and 6 each received 200µg/kg of AFB1 orally an hour after receiving *Spirulina plantesis* at doses of 50, 100, and 150mg/kg respectively. The mice were treated daily for 14 days. During the last day of the treatment schedule, mice were aseptically dissected and tissues isolated for immunological studies. The results indicate that intervention with spirulina at doses of 100mg/kg and 150mg/kg were enough to increase the body weight of mice significantly ($p<0.05$). It was also demonstrated the blood levels of interleukin 4 and interleukin 2 were not affected significantly when AFB1-induced mice are treated with spirulina extract ($p>0.05$). Interferon gamma (IFN- γ) and interleukin 2 (IL-2) blood levels were significantly lower in the group not treated with Aflatoxin b1 ($p\leq0.05$). The findings also indicate that immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) serum levels were unaffected by treatment with spirulina extract at various dosages ($p>0.05$). In addition, TNF and IFN-γ mRNA expressions were also highly up-regulated, while interleukin 4 (IL 4) was down-regulated. The results further show that the over-production of TNF and IFN due to Aflatoxin B1 is correctable upon spirulina treatment ($p<0.05$). To sum up, the results suggest that spirulina treatment can be an innovative approach to correcting the aflatoxin B1-mediated immune aberration and inflammation.

CHAPTER ONE

INTRODUCTION

1.1 Background

Immunological dysfunction often develops as a common complication of various diseases, including those caused by foodborne pathogens. Even though infections from pathogens in humans can lead to fatal diseases and death, the way toward devising therapeutic products that can provoke the immune system against such infections has been focused upon by most studies in recent years (Elbehiry *et al.,* 2023).

Aflatoxins were first characterized in the late 1950s and early 1960s as the cause of the epidemic known as "turkey X" sickness, which led to the deaths of many birds whose diets were supplemented with high levels of peanut feed from South America (Kensler *et al.,* 2011). They are present in various foods and feeds and are known to cause severe health implications to human beings and other animals. So far, the pathogenic effects, including liver damage and immune suppression and the increase in the risk of liver cancer, have been studied in detail (Long *et al.*, 2018). However, the potential use of naturally occurring substances to modulate their effects has yet to be fully explored. Aflatoxin-related illnesses are distinguished from other diseases by their unique liver toxicity, specifically the potential for harm to liver cells and the potential for carcinogenesis (McCullough and Lloyd, 2019). Aflatoxin-related illnesses are distinguished from other diseases by their unique liver toxicity, specifically the potential for harm to liver cells and the potential for carcinogenesis (McCullough and Lloyd, 2019).

The AFB1 is a toxic compound synthesized by certain types of fungi, most notably *Aspergillus flavus*. Aflatoxin contamination of food has been a major concern for decades because it causes serious consequences to the liver, kidneys, and immune system in both humans and animals (Negash, 2018). Aflatoxin B1 is characterized as a Group one cancer-causing agent by the International Agency for Research on Cancer (Saad-Hussein *et al*., 2014). In an article published by Eskola *et al*. (2020), between 60 and 80 percent of the world's consumable food may contain fungal toxins. This estimate delayed the often known 25% estimate ascribed to the UN's Food and Agricultural Organization (FAO). Guidelines have been set on the allowed concentration of total aflatoxins in food. For example, the U.S has set the limits to be 20 µg per kg. The European Union has stringent guidelines, setting the limits to as low as 4 µg per kg of total aflatoxins. In developing nations, the levels of total aflatoxins have been set to between 10-20 µg/kg, with most countries setting the permissible levels to be 10 µg/kg (Knowledge Platform, 2015). The Kenya Bureau of Standards has established regulations for the maximum level of aflatoxin acceptable in foods. The highest allowed aflatoxin level in Kenya is 10 parts per billion for maize which is considered main meal in the country and 15 parts per billion for peanut butter (Rödl and Partner, 2020). However, Kenya has been struggling with elevated concentration of aflatoxins despite these guidelines. In 2004, The Standard, one of the leading dailies in Kenya, reported that 125 individuals died due to consumption of food contaminated with aflatoxins (Kalua, 2017). This implies that these guidelines are not effective and there is need to explore options to address this public health concern.

Kenya is one of the countries in Africa that is affected by contamination of food by fungal toxins. For the food supply to be safe, it is crucial to test for aflatoxins in food. Aflatoxin tests are available; however, they are typically performed in laboratories and are not available to individuals or at homes. Consumption of food contaminated with aflatoxin causes serious illnesses and even death when consumed in large amounts.

Aflatoxin synthesis is greatly enhanced when the growing crops are subjected to stress, insufficient drying of grains and poor storage practices (Kumar *et al.,* 2021). The surveys that have been done in Kenya show that there are high amounts of aflatoxin, amounting up to 131.7 ng/g in food products which is higher than the required threshold (Knowledge Platform, 2015). More severe cases of aflatoxicosis have been observed especially in patients with HIV infection. Like other countries in Africa, Kenya lacks national aflatoxicosis surveillance and this has worsened the burden of aflatoxicosis in the country.

The use of conventional physicochemical approaches have proved to be insufficient in management of aflatoxicosis. With the challenges presented by aflatoxin, current studies are exploring the use of natural products as substitutes for managing aflatoxicosis. This study evaluated the potency of spirulina in ameliorating aflatoxin toxicity. It is known that dietary inclusion of spirulina has some beneficial effects in management of some diseases (Faraq *et al*., 2016).

Spirulina plantesis is a blue-green alga with a long history of use as a medicine by Indians for its immune-boosting properties (Rahman *et al.,* 2021). Earlier works investigated its potential in the treatment of several transmissible diseases, among them HIV, hepatitis C, and influenza (Ngo-Matip *et al.,* 2015). Its immunomodulatory property reduces the synthesis of pro-inflammatory cytokines and enhances the synthesis of anti-inflammatory cytokines, thus further directing its selection as an anti-inflammatory agent. Although this evidence is present, yet there are large lacunae still in the form of the knowledge gap as to how spirulina interacts with specific toxins like aflatoxin B1. In addition to that, the optimal dosage of spirulina has not been fully delineated and this has been the purpose of the current study. However, there is a knowledge gap on its immunologic dysfunction caused by toxic compounds like

Aflatoxin B1. The most recent scientific research shows that it has broad therapeutic effects that could protect against or be active toward preventing or treating aflatoxicosis and other infections (Gogna *et al.,* 2023; Saraswathi and Kavitha, 2023).

Today, majority of the researchers have devoted most of their time in finding the most effective and cheap products that can be used to get rid of traces of mycotoxins in feedstuffs. Some of the promising approaches that have been developed included supplementation of contaminated products with whey protein and ginseng extract (Abdel-Aziem *et al*., 2011). As such, demonstrating that *Spirulina plantesis* has some beneficial effects in preventing adverse effects of mycotoxins, provides a possible intervention strategy that can be employed in the management of exposure to permissible or high levels of aflatoxins in human health.

1.2 Statement of the Problem and Justification

Mycotoxins poses significant challenge not only to human being but also to animals. Aflatoxin B1 has been linked to impaired immune function and inflammation. The toxicity of aflatoxin B1 has been extensively studied, and it has been established that it can suppress the immune system, affect growth performance in livestock and poultry, and increase the risk of mortality. Additionally, AFB1consumption is also linked to increased risk of cancer and kidney damage in humans. More pronounced effects are likely to occur in children and people with compromised immune system due to HIV infection. On the other hand, Animals exposed to chronic aflatoxin may experience decreased weight gain, anemia, jaundice, decreased feed conversion efficiency, higher death rates, and other adverse effects. Aflatoxicosis in chickens may lead to increased fatty liver and decreased egg output (Wu *et al*., 2011).

Cases of aflatoxin contamination of food have made headlines in Kenya for many times. The highest documented quantity of aflatoxin in maize (58,000 μg/kg) has been found in Kenya. In humans, aflatoxins have led to about 500 acute illnesses and 200 deaths (Omara *et al*., 2021). In Kenya, there are expected to be 11 cases of maize illnesses out of 100,000 people in urban markets, when compared to 29 out of 100,000 in rural markets (Sirma *et al.*, 2019). In a study conducted by Omara *et al.* (2021), samples of maize grains obtained between January and May 2010 in Kenya from fields (pre-harvest), stores (post-harvest), wholesalers, merchants, and open-air sellers tested positive for aflatoxins. This, therefore, indicates that the problem of aflatoxin contamination of dry foods in Kenya has persisted, and this brought a need for intervention to stop any losses that could be incurred by humans and animals.

Although several methods beyond the use of spirulina extracts, such as binders like NovaSil Clay and other improvements in animal feed, have been used to reduce the severe health implications of aflatoxin B1, very few studies have looked at their usefulness (Phillips *et al.*, 2019). Spirulina plants has been extensively studied and shown to provide various nutritional and physiological values. The researchers believe that the capability of spirulina extracts have been found to be significantly capable of reducing the adverse health effects of AFB1 due to their anti-inflammatory and immunomodulating activities. Therefore, the primary objective of the research is to find out the potentiality of spirulina extracts to reduce inflammation and immunological dysfunction brought up by aflatoxin B1 exposure in mice. This study has consequently focused on the mechanisms of spirulina extract in preventing AFLA B1-induced toxicity by measuring changes in specific biomarkers of inflammation and immune function.

1.3 Significance of the Study

This study is essential as present management strategies of immune dysfunction and inflammation resulting from AFB1 consumption are unsuccessful. The findings of this study will have a bearing on policy and decision-making on the use of spirulina in animal feeds and human nutrition to mitigate negative AFB1-induced health consequences. The research study could have an immediate benefit directly for farm animals and for humans who may become exposed to aflatoxin B1. If spirulina therapy proves to be effective, doctors can also prescribe it to patients with various immune system and inflammation disorders. Furthermore, animal feed makers might incorporate spirulina as a supplement into their products, resulting in improved livestock health. Overall, the findings of this study have the potential to help create more natural and effective therapies for immunologic dysfunction and inflammation caused by aflatoxin B1 poisoning in a number of contexts. These findings can have important implications for human health, as aflatoxins are common food contaminants that can cause various health problems, including liver damage, cancer, and immunotoxicity.

1.4 Research Questions

i. What is the effect of *Spirulina plantesis* extract on body weight in mice following aflatoxin-induced toxicity?
ii. What is the effect of *Spirulina plantesis* extract on humoral responses following aflatoxin B1-induced toxicity?
iii. What effect does *Spirulina plantesis* extract has on cytokine production following aflatoxin induced toxicity?

iv. How does *Spirulina plantesis* extract affect mRNA levels of inflammatory cytokines following aflatoxin B1-induced toxicity?

1.5 Hypotheses

i. *Spirulina plantesis* extract treatment will result in a significant improvement in the body weight in mice following aflatoxin-induced toxicity.

ii. *Spirulina plantesis* extract treatment will lead to a significant enhancement of humoral responses following aflatoxin B1-induced toxicity.

iii. *Spirulina plantesis* extract treatment will result in a significant modulation of cytokine production following aflatoxin-induced toxicity.

iv. *Spirulina plantesis* extract treatment leads to a significant alteration in mRNA expressions of inflammatory cytokines in response to aflatoxin B1-induced toxicity.

1.6 Objectives

1.6.1 General Objective

To evaluate the effects of aqueous extracts of spirulina plantesis against aflatoxin B1 (AFB1)-induced immune dysfunction and inflammation in mice.

1.6.2 Specific Objectives

i. To determine the effect of *Spirulina plantesis* extract treatment on body weight after AFB1 induced toxicity

ii. To determine effect of *Spirulina plantesis* extract on humoral responses following aflatoxin B1-induced toxicity.

iii. To determine the effect of *Spirulina plantesis* extract on cytokine production following aflatoxin B1-induced toxicity.

iv. To determine effect of *Spirulina plantesis* on *m*RNA expressions of inflammatory cytokines in response to aflatoxin B1-induced toxicity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aflatoxins: Occurrence and Structure

Aspergillus fungus produces a class of mycotoxins known as aflatoxins, which are strong hepatotoxins and liver carcinogens. In terms of structure, all aflatoxins are composed of an unsaturated lactone backbone and a coumarin ring. Foods infected with Aspergillus, a bacteria that produces aflatoxin, and dairy products from animals given infested feed can both contain aflatoxin (Patriarca and Pinto, 2017). AFB1 and its metabolite aflatoxin M1, which were initially found in milk, are the most well-studied aflatoxin species. In some animal models, aflatoxin G1 is also extremely toxic and carcinogenic. Although they are strong hepatotoxicants, aflatoxins B2, G2, and M2 have not been shown to cause cancer, thus, different countries, through their regulatory agencies, have imposed regulations (Marroquín *et al.*, 2014). Aflatoxin, often known as acute toxicity caused by human exposure, is more common in African and Asian nations than in industrialized ones. The Food and Agriculture Organization (FAO) have set standards to on allowable limits of AFB1. For instance, FAO has set 15 ppb as a maximum limit for all types of nuts and almonds (Gong *et al.,* 2015).

Other than AFB1, there are several other types of aflatoxins that can be produced by *Aspergillus flavus, Aspergillus parasiticus,* and *Aspergillus nomius*. These include aflatoxins B2, M1, M2, G1, and G2 (Figure 2.1) (Akiyama *et al*., 2001; Jiang *et al.,* 2021). Aflatoxin B1 is certainly the most toxic among all and has been studied most extensively. Aflatoxin B2 is less potent than B1, while G1 and G2 belonging to category B2. It is worth mentioning that though aflatoxins are a serious health concern, majority of the food products are safe to eat and the risk of the ingestion of too much of these toxins is very small (Negash, 2018). Furthermore, there are also a variety of actions that can be implemented in order to curb the health effects of aflatoxin intake, which include proper food storage and handling and regularly monitoring food products for the presence of the toxins.

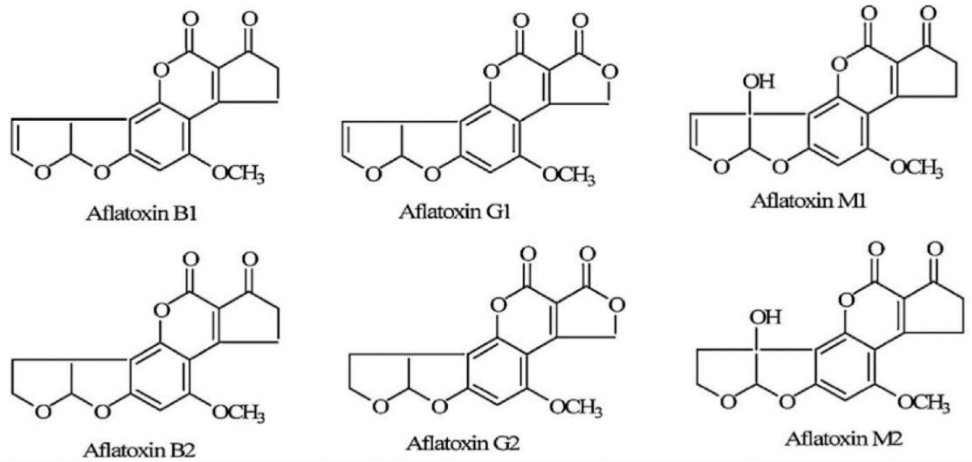


Figure 2.1 Major classes of aflatoxins (Jiang *et al.*, 2021)

2.2 Metabolism of Aflatoxin in the Liver

The liver is the principal organ where aflatoxins are mostly transformed biologically. The metabolism of aflatoxin occurs in two phases. The first phase biotransformations are often oxidative, reductive, or hydrolytic processes. This phase provides the required precursors for phase 2. Phase 2 reactions are typically conjugation reactions, where the essential chemical structure is modified. A molecule may be activated or detoxified in phase 1 modifications, but phase 2 transformations, dependent on conjugated cellular components, could result in either decontamination or the development of biochemical lacerations. The cytochrome P450 (CYP450) enzyme system plays a major role in mediating phase 1 reactions. Sulfate, glucuronide, glutathione (GSH), and amino acid conjugation processes are a part of phase 2 metabolic processes (Monosson, 2012).

In phase 1, Aflatoxin B1 is changed into different products by various forms of enzymes called CYP450 subfamilies. One of the resulting products of metabolism of AFB1 is

AFB1-exo-8,9-epoxide. Numerous studies have documented that it is the root cause of mutations (Yilmaz *et al.,* 2017). Furthermore, other products are mainly produced during the metabolism and these are detoxification products. The aflatoxin B1-exo-8,9-epoxide has been thought to be the primary precursor of aflatoxin B1 that inflicts injuries, as this substance is able to attack some atoms of cells. AFB1 exo-8,9-epoxide is metabolic product resulting from a reaction catalyzed by cytochrome P450. The epoxide itself is very toxic and reactive. It is capable of scavenging and binding to macromolecules such as DNA and cellular proteins. There are different sub-classes of cytochrome P450 and the notable ones are 1A2 and 3A4. These sub-classes play a role in metabolizing various processes AFB1 (Yilmaz et al., 2017). Specifically, AFB1 epoxide react with DNA through nitrogen atoms and this result in mutations, and eventually cause cancer (Dharumadurai *et al.,* 2011).

Cytochrome P450 3A4 has been reported to have high affinity towards AFB1. This enzyme is produced in large amounts especially in the human liver where metabolism of this toxin takes place (Saba & Seal, 2022). Other forms of the CYP450 enzyme, such as CYP450 1A2, also contribute to the activation of AFB1, but to a smaller extent, even at relatively low concentrations of aflatoxin B1. Cytochrome P450 3A4 mainly contribute to the formation of AFB-2,3-epoxide which is toxic to genes, while cytochrome P450 1A2 is a precursor to the exoisomers and nongenotoxic endoisomers. Cytochrome P450 1A2 exhibit an increased affinity for activating aflatoxin B1 even at lower levels after ingestion of contaminated food (Saba and Seal, 2022).

In Phase 2 reactions, AFB1 is detoxified by being combined with glucuronic acid, sulfate, and GSH (Lauwers *et al.,* 2019). The AFB1 products from biotransformations in the initial phase are further processed by enzymes called glutathione-S-transferases (GSTs), which mainly speed up the reactions that combine AFB1 with other molecules.

After oxidation in the first phase, AFB1 readily combine with molecules containing SH moiety in the second phase. This allows for further breakdown and removal of the toxin from the system. In most mammals, the Aflatoxin B1-8,9-epoxide easily conjugates with GSH. Such a reaction is catalyzed by glutathione-S-transferases (Yilmaz *et al.,* 2017). Figure 2.2 outlines reactions taking place in phase 2 while metabolizing AFB.

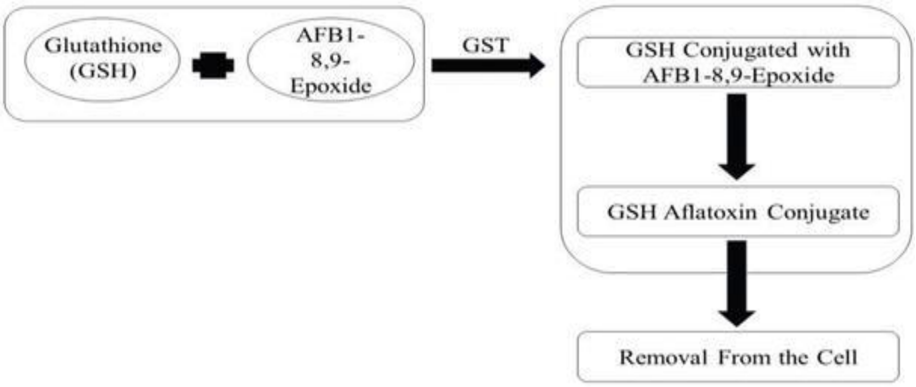


Figure 2.2 Aflatoxin B1 metabolic routes (Yilmaz *et al.*, 2017)

2.3 Mechanism of Aflatoxin B1 Toxicity

Although much about the toxicity of aflatoxins is still unknown, much research has tried to focus on the mechanisms of its action. This knowledge can be used by food safety organizations to develop strategies for preventing and controlling aflatoxin contamination, as well as to achieve regulatory goals.

In the body, specifically in the liver, aflatoxin AFB1 is bioactivated to the most reactive epoxide metabolite, AFB1-exo-8, 9-epoxide. This epoxide can form reactions with a whole bunch of biochemical components like DNA, protein and lipids. (Bbosa *et al.,* 2013; Khan *et al.,* 2021) Notably, DNA and Aflatoxin B1-exo-8,9-epoxide react to form nitrogen compounds that bring out mutations, which might induce cancer as shown in the image Figure 2.3. Among others, this process can lead to the production of a bulky compound, which may either block DNA replication or col animation of genes (Bbosa *et al.,* 2013).

Besides, AFB1-exo-8,9-epoxide can bind protein to produce some structural and functional patterns' abnormalities. So, the function of proteins can be affected to some degree by misfolding that alter their activity, transport of molecules across the membrane and inability of cells to signal to each other. Besides the possible targets, such as DNA and proteins, AFB1-exo-8,9-epoxide is more likely to produce oxidative stress (Zhang *et al.*, 2011). This releases of ROS as peroxide or free radical occurs through the interaction of epoxide with lipids. These harmful oxidative stresses may affect any cellular structures, which include cell membranes, proteins and ribonucleic acids (Benkerroum, 2020). The oxidative stress from these processes can result in the inflammation and cell death of which aflatoxins is one of the major toxins that causes damage.

The AFB1 also has an effect on the immune system through the interference of white blood cells and weakening the immune system capacity to fight off infections. This can increase susceptibility to infections and other diseases. Acute toxicity can occur when large amounts of aflatoxins are ingested (Benkerroum, 2020). Symptoms may include liver damage, vomiting, and in severe cases, death. Consumption of aflatoxins for extended period of time may heightened the likelihood of developing hepatocellular carcinoma and other ailments (Melaram, 2021).

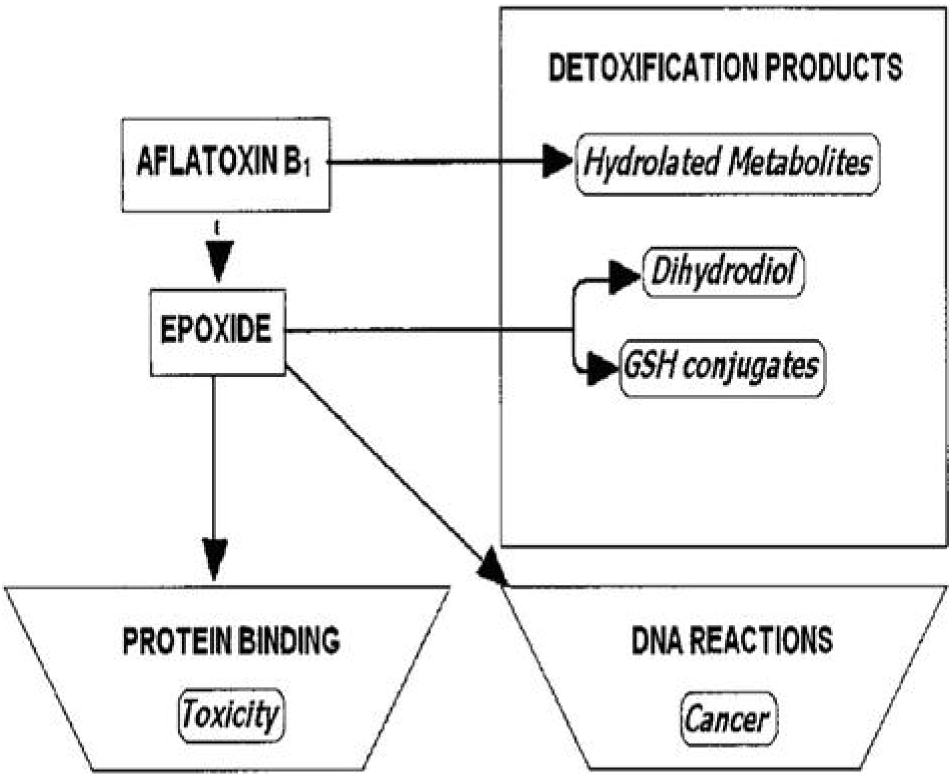


Figure 2.3 AFB1 metabolic routes (Dabuo *et al.*, 2022)

2.4 Immunomodulatory Effects of Aflatoxin B1

Cytokines are a class of proteins that mediate immune responses when the host is triggered. There are several types and inflammatory cytokines are the key molecules that are produced during inflammation. These cytokines regulate host defense against invading foreign particles or microorganisms (Zhang and An, 2007; Abdulkhaleq *et al*., 2018). It has been found that ingestion of aflatoxin B1 raises the concentrations of IFN-γ and IL-2. During pathological state, the immune organs release inflammatory cytokines to modulate inflammatory response, and thereby causing severe tissue injury (Long *et al*., 2016a; Chen *et al*., 2018). Aflatoxin B1 is one among the mycotoxins synthesized by *Aspergillus flavus* and it is known to contaminate food products. A number of scientific studies done using animal models have pointed out that Aflatoxin B1 exposure can cause immunosuppression. In animal experiments, AFB1-induced downregulation of immune system come as a result of depressed T or B cell activity, decreased immunoglobulin level and interfere with macrophage or neutrophil-effector activities (Jiang *et al.*, 2008). ROS induced inflammation and secretion of inflammatory cytokines is proposed to be a key driver of AFB1 induced hepatocellular carcinoma (HCC) by causing necrotic cell death in the liver (Maurya, 2018).

Although there is a significant amount of research on the effects of Aflatoxin B1 on immune cells, few studies have specifically looked at its impact on cytokine expression and the findings are inconsistent. Some studies have found that AFB1 can affect the secretion of IL 2 by spleen cells and IL-1 by peritoneal macrophages in mice when administered with 1mg/kg of aflatoxin B1 (Hou *et al.*, 2022). Additionally, exposing mice to AFB1 at varying levels (0.005-0.075 mg/kg) has been noted to result in a significant effect on the expression of IL 4, IFN- gamma, and TNF factor alpha in rat spleen cells (Qian *et al*., 2014). Research has also revealed that AFB1 can suppress the production of inflammatory cytokines in rats and mice that have inhaled aflatoxin B1 aerosols (Jakab *et al*., 1994). Studies in chicks have found that 0.3mg/kg of feeds contaminated with aflatoxin B1 can decrease the levels of interleukin 2 and interferon gamma in the blood (Jiang *et al*., 2015). Though, other researchers have found increasing concentrations of IL 6, IFNγ, and TNFα and mRNA expression in the blood and spleen of birds given feeds contaminated with 0.074mg/kg of Aflatoxin B1 (Li *et al*., 2014).

When food or feed contaminated with AFB1 is consumed, the gastrointestinal tract can be exposed to elevated levels of the toxin. The intestinal tract not only acts as a physical barrier, but also contribute towards cellular immunity through its active involvement in the mucosal immune system (Bondy & Pestka, 2000). Different types of T-lymphocyte subclasses can be found in the intra-epithelium and lamina propria of the intestine. These T cells are responsible for many functions in the gastro-intestinal immune system, which are modulated by cytokines produced (Wittig and Zeitz, 2003). However, there is scanty information published about the impact of aflatoxin B1 on the expression of cytokine mRNA and T cell subclasses in the intestinal mucosa that are caused by dietary AFB1.

Interaction with AFB1 is correlated with acute effects that, on a wider range, are bad for the functionality of the immune system. There are several pathological outcomes of aflatoxicosis including the weakening of immunity, enlarged immunological organs, dysfunctional liver and gut barrier damage caused by AFB1 (Lai *et al.,* 2022). The influence of AFB1 on the defensive mechanisms of HIV-1 infected patients is of great importance since these individuals differ from other people in regard to immune response. Different researches have evaluated the role aflatoxin B1 consumption plays on the outcomes of HIV-disease. A study by Jolly *et al.* (2013) demonstrated that HIV patients with AFB1 exposure had significantly higher risk of HIV viral load (Jolly *et al.,* 2013). It was revealed that people with higher AFB1-albumin (AF-ALB) were likely to have more viral loads than those who had low levels. They are, in a way, similar with the concluded results of Jolly *et al.* (2011). This means Aflatoxin B1 inhibits immune system in a way that even science is not able to underestimate these effects. Such moderate issues are likely to bring in severe complications.

2.5 ROS Induced Inflammation in Response to Aflatoxin B1

Reactive oxygen species (ROS) are molecules that are oxygen based containing a lot of reactivity towards biomolecules when the interaction takes place. They are naturally generated in cells as a metabolism by-product, but may also be overproduced due to toxins present in the environment, known to be aflatoxin B1 ones. Many studies have pin-pointed that the effects of ingesting aflatoxin B1 are a rise in inflammatory processes and generation of free radicals in a variety of cell types and animal models (Li & Liu, 2019; Yilmaz and Bag, 2022). For instance, aflatoxin B1 has been reported to provoke cytokine synthesis, which is pro-inflammatory in nature, such as TNF-alpha and IL-6, and that it stimulates the nuclear factor-kappa B (NF-kB) signaling pathway (Mehrzad et al., 2017). Such pathway has been suggested and noted to take part in inflammation control.

Aflatoxin B1 has also demonstrated the ability to affect not only DNA, proteins and lipids but also the cellular functions that create malfunction and cell death (Verma, 2004; Yilmaz and Bag, 2022). This damage thus happens via active formation of reactive oxygen species (ROS), which can lead to oxidation and biomolecules' fragmentation. Furthermore, AFB1 also contributes to occurrence of breast cancer by means of increases in inflammation and oxidative stress (Asim *et al.,* 2011; Long *et al.,* 2016b). In the same line, the empirical results obtained using animal models have demonstrated that the use of antioxidants and anti-inflammatory compounds help to significantly decrease the inflammatory response and the oxidative stress resulting from the exposure of the aflatoxin B1 (Gao *et al.*, 2021; Ahmed *et al.*, 2022).

In their study, Hou *et al*. (2022) found out that the genes coding for inflammatory cytokines TNF-α and IL-1α are upregulated in experimental groups exposed to aflatoxin B1. High levels of these inflammatory cytokines induced the cells to proliferate rapidly. The ROS and inflammation eventually drive tumor progression in experimental groups. At molecular level, ROS induces genomic damage and necrosis in cells, thus leading to generation of pro-inflammatory factors that drive tumorigenesis (Aggarwal *et al*., 2019; Singh *et al*., 2015). It has also been noted that aflatoxin B1 causes necrotic cell death in the liver to drive development of cancer in the liver (Singh *et al*., 2015). Therefore, it is postulated that aflatoxin B1 induced Hepatocellular carcinoma may involve ROS led inflammatory cascade during the development of carcinoma in liver as shown in Figure 2.4.

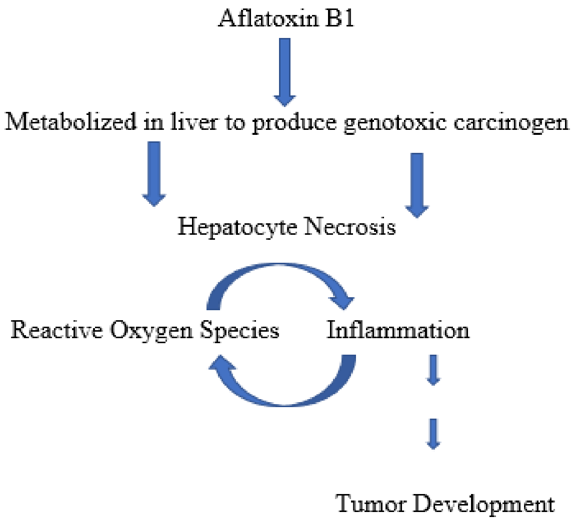


Figure 2.4 ROS & Inflammation together drive progression of AFB1induced carcinogenesis (Maurya, 2018)

2.6 *Spirulina plantesis*

Spirulina, a type of blue-green algae, has been consumed for centuries by the Aztecs and other indigenous populations in Mexico and Africa. In the 1970s, spirulina gained attention as a potential source of nutrition for malnourished populations and as a supplement for various health conditions (Siva Kiran *et al*., 2015). Geographically, spirulina is found in many parts of the world where there is a suitable environment for its growth, such as warm, alkaline lakes, and in man-made environments like ponds, tanks and open channels (Sánchez *et al*., 2015). Spirulina is gown in most parts of the world for commercial purposes, including Africa, Asia, and South America and China (Soni *et al*., 2021).

Many studies of the history of spirulina have shown that it was a very special food for Aztecs and they called it "tecuitlatl," besides it has been used for centuries as food from Africa and some other countries. Spirulina has gained a lot of popularity in the field of sustainable foods since it can be grown basically anywhere using only a minimum amount of resources and not too much space. Furthermore, spirulina has been explored for supporting long-duration space travel as it can be grown in hydroponic system and gets its nutrition from fresh water.

Owing to its nutritional and medicinal uses, spirulina has gained popularity. In a report published by FAO (2004), spirulina is rich in proteins, essential amino acids and vitamins. These nutrients are enough to recompensate the nutritional desire of people who are deprived of such nutrients. Spirulina is rich in omega-3 fatty acids which can help in the prevention of heart disease by lowering the risk factors such as total cholesterol, triglycerides, and LDL and increasing HDL. People with type 2 diabetes who take spirulina supplements and observers these found to have significant reduction in their fasting blood sugar reading. The other proof that supports these health claims is that spirulina's therapy also helps relieve the symptoms of allergic rhinitis (Bobescu *et al.*, 2020).

Therefore, spirulina is enthusiastically known for being the superfood and is widely embraced by many corners of the globe. Nutritional value and the possible health benefits make it a 'superfood' of the future. Moreover, it could be a promising substitute for traditional food sources in areas where the later are scarce.

2.7 Medicinal and Nutritional Uses of Spirulina

Spirulina has been used for either nutritional or medical purposes. Spirulina contain essential nutrients like protein, vitamins, minerals and antioxidants which have been found to have some health benefits. In addition, it contributes to the health of malnutrition people and serves as a supplement for various diseases (Suter, 2011; Kulshreshtha *et al.*, 2008).

Spirulina stands out due to its high protein among those found in nature, depending on the source (Grosshagauer *et al.,* 2020). It consists of the whole set of amino acids including essential amino acids that are much better than all other proteins of plant or animal origin such as, meat or milk. For example, compared to other protein sources, it is 85.2% more digestible (Devi *et al.*, 2018). Taking 28g of spirulina per day eliminates muscle weakness and tiredness experienced by young male soccer players. Spirulina has a very high protein efficiency ratio (PER) and Protein Digestibility Corrected Amino Acid Score (PDCAAS) which means it is a very good quality protein, with a high net protein utilization (NPU) (Wang *et al.*, 2021). This is important for vegetarians, athletes, or those recovering from an illness.

Gamma-linolenic acid (GLA) is categorized with the omega-3s, but is structurally and functionally closer to the omega-6 EFAs. Although Spirulina contains GLA, it is a controversial source (Roughan, 1989). Some scientific research suggests that GLA is not as biologically active in its non-conversion state and only in certain forms (dietary or supplemental) in combination with specific nutrient deficiencies supports its physiological activity. Based on the fat profiles of Spirulina, it is likely a beneficial source of EPA, but not DHA (Diraman *et al.,* 2009). Unfortunately, there are only a few studies that have analyzed the EFAs in Spirulina.

Research has shown that Spirulina has high amounts of vitamins, minerals and antioxidants, as well as anti-inflammatory, anti-oxidant and anticancer attributes (Asghari *et al*., 2016). It has been shown to mitigate the levels of cholesterol, blood pressure, glucose tolerance and cardiovascular diseases, (Bobescu *et al.,* 2020). It has also the bioactivity to strengthen the immune system. Studies on spirulina show that the it has also been used as a supplement for people that are suffering from diseases like allergies, diabetes, high cholesterol levels (Deng and Chow, 2010; Seyidoglu, 2017). Furthermore, it has been studied as a possible treatment of age-related macular degeneration, which is a principal reason of blindness in old-aged population (Cho *et al.,* 2022).

2.8 Immunomodulatory Effects of Spirulina Extracts

The immunomodulator function of spirulina has been the focus of the modern research. Spirulina is completely digestible and thus can be used to supplement a healthy diet. The World Health Organization (WHO) reports that consuming very small quantities of spirulina can improve the health status of the person and build strong immunity to fight any kind of disease (Alesci *et. al.*, 2022). The results of recent studies underline the claims that having spirulina will improve health, especially in immune boosting.

In the recent years, the number of Americans that are diagnosed with various autoimmune disorders has increased significantly. Approximately 2 million American citizen falls prey to these diseases which among them include rheumatoid arthritis, lupus and Crohn’s disease (Pahwa *et al.,* 2021). These conditions arise when our immune system attacks us by fighting against our own organs and tissues. Some of the diet types, as well as dietary supplements have shown as the remedies for the autoimmune diseases. In particular, the findings showed that spirulina that helps immune system of the body to prevent inflammation and may prevent autoimmune disorders (Ravi *et al.*, 2010).

Despite the fact that inflammation as an immune response is very useful and is used by bodies as they fight infection and offer protection against diseases (Scott *et al.*, 2004), it also has negative health consequences. Nonetheless, the adverse effects may arise from inappropriate interplay between inflammation and healing processes when they are not in sync. The balance which typically keeps chronic diseases in check is by and large broken down by these conditions. It has been established that spirulina minimizes the inflammation by maintaining the number of anti-oxidant enzymes in the cells and affecting the expression of those inflammatory cytokines (Wu *et al.*, 2016). For example, the article by Rasool *et al.* (2006) indicated that a spirulina extract could decrease leukotriene levels when used in *in vitro* tests. Another study revealed that the spirulina releases was able to inhibit prostaglandin E2.

The anti-inflammatory properties of spirulina stem from its ability to fine-tune the functions of proteins like transcription factors that occur naturally in the body (Pak *et al.*, 2012; Pham *et al.*, 2016). Some of these modulator proteins are known to play an essential role in preventing and reducing the inflammatory responses. In addition to these mentioned processes, spirulina may also work through its high chlorophyll content (Srivastva *et al.*, 2024).

Chlorophyll is a pigment found in plants that contains the ability to use sunlight and convert it into chemical power which is utilized by plants to perform their metabolism. Research has shown us that chlorophyll possesses great anti-inflammatory capabilities by means of switching off different immune-signaling pathways implicated in the onset of inflammatory diseases development. Chlorophyll is initially broken down extracellularly and the products that follow are taken into the cell where they exert their activity (Olofsson *et al*., 2014). Therefore, the anti-inflammatory attributes of spirulina may be mediated by its effects on the protein activity of transcription factors and other proteins in the body, as well as by its high content of chlorophyll (Subramoniam *et al*., 2012). Although further studies are required to have a full understanding of these effects, spirulina appears to be a promising natural remedy for reducing inflammation and preventing disease.

Spirulina has been widely used as food starting from Aztec civilization (Pal & Bose, 2022). It has been shown that dried biomass of spirulina is highly nutritious with about 70% protein content. In addition, it has been shown to contain vitamin B12, pro-vitamin A (beta carotene) and minerals, particularly iron. Several countries have recommended the use of spirulina because it is generally regarded as safe (GRAS). Spirulina has no toxic effects and it has been approved by FDA and ANVISA (Damessa *et al.,* 2021). Spirulina has been formulated into a form that can be easily eaten. It has been incorporated into nutritional drinks, fruit juices and frozen desserts among others.

Dietary spirulina has been shown to provide immunomodulatory properties in mice. Spirulina powdered extracts have been shown to improve production of IL-1 (Hayashi *et al*. 2007), IL-4 (Mao *et al*., 2005) and interferon gamma, increases antibody production (Hayashi *et al*. 2007) and improves macrophage function and phagocytosis (Hayashi *et al*., 2007) as well as NK cell damage activities (Akao *et al*., 2009). The use of spirulina as a food supplement and as animal feed supplement also has been associated with enhancements in animal growth, health-promoting effects and nutritional product quality (Lafarga *et al*., 2020; Spínola *et al.,* 2024).

2.9 Current Management Strategies for Aflatoxin B1 Toxicity

It is worth noting that studies on the effects of Aflatoxin B1 on human health are ongoing, and more studies are needed to develop management strategies for toxicity. Once consumed, this harmful substance is swiftly taken by the body and finally gets to the liver where it is metabolized. Researchers have researched several management methods to combat Aflatoxin B1 toxicity in humans such as dietary interventions and the use of adsorbent materials. The later has the ability to adsorb the toxins in the stomach thus preventing them from being absorbed in the body (Nava *et al.*, 2023).

As aflatoxins are highly toxic to domestic animals as well as humans, several measures are available to lower the rate of this toxin intake. From the 2006 report of WHO, the therapeutic approach is to reduce the absorption of aflatoxin through the gut and to protect against the harmful effects of aflatoxin on the body. Avoiding consumption of contaminated foods may not be feasible and there is need to develop an effective approach to reduce the exposure of AFB1 through food (Phillips *et al*., 2019). Currently, chemopreventive agents have been used to manage aflatoxin poisoning. This approach entails using natural or synthetic products to block, slow, reverse or modify harmful processes initiated by aflatoxins (Kensler *et al.,* 2004). Some of the chemopreventive agents are found in human diet and they include various phytochemicals. In China, chemoprotective agent Oltipraz has been prescribed to people who have consumed aflatoxin alongside food (Helzlsouer and Kensler, 1993). Qin *et al.* (1997) noted that green tea administration prevents the onset of hepatocarcinogenesis caused by AFB1.

According to Phillips *et al*. (2019), NovaSil Clay (NS) is one of the approaches that have been used to combat aflatoxin effects in animals when given alongside the diet. NovaSil clay adsorb AFB1 with high affinity in the GI tract thus preventing its uptake to the bloodstream. This binding causes a reduced bioavailability of aflatoxin in the body system without affecting usage of vitamins and other essential nutrients (Philips *et al*., 2019). Other studies have also found that curcumin can be used in management of liver damage caused by AFB1. Supplementing with curcumin effectively reduces oxidative stress and liver damage caused by AFB1 by suppressing the formation of reactive oxygen species and AFB1-adducts and upregulating the Nrf2/HO-1 signaling pathway (Li *et al.*, 2019).

2.10 Use of Animal Models in Aflatoxicosis Studies

Animal models have played a pivotal role in immunological studies. Numerous animals have been used to study the effects of aflatoxin B1 on various immunological parameters. With these models, there is a general agreement that the response towards aflatoxin B1 largely dependent on the concentration, animal species, gender and age (Chen *et al.*, 2013). Previous studies on birds indicate that aflatoxin at 0.95mg/kg concentration is capable of reducing nutrients uptake and daily weight gain in poultry by 11% (Chen, 2016). In contrast, the immunological parameters are also changed after AFB1 intake by broilers. Jiang *et al.* (2015) reported that a concentration of 0.6mg/kg of aflatoxin B1in broilers diet lowers the levels of T cell subclasses and expression of cytokine mRNA levels in the ileum. This infers that aflatoxin B1 interferes with the role of immunity in intestinal mucosa. The lower levels of cytokine mRNA expression in birds is due to reduced levels of T-cell sub-classes induced by aflatoxin B1.

In many studies, pigs have been used as candidates for aflatoxicosis studies. Pigs are very sensitive to aflatoxin and their contamination occurs during high consumption of cereals. AFB1 is hepatotoxic as well as carcinogenic to pigs. Studies have also documented that it damages innate and acquired immune components (Meissonnier *et al*., 2008). In their study, Chaytor *et al*. (2011) indicated that aflatoxin B1 exposed to pigs is able to change inflammatory responses. There is also a remarkable decrease in the production of pro-inflammatory cytokines and elevation of anti-inflammatory cytokines in newly weaned piggies given minimal doses of AFB1 for four weeks (Marin *et al*., 2002).

Mice are perhaps the best alternative models in the study of aflatoxicosis and various immunomodulatory effects. They are preferred for the reason that their physiology and genome are very similar to that of humans, making immunological studies particularly important in the study of human diseases. Mouse models have been widely used in studies meant to bring an understanding of the immune system (Hamilton *et al.*, 2020). Mice have been studied as models for diseases including aflatoxicosis. What makes it the best animal model for this study is their susceptibility to aflatoxins and are capable of developing diseases such as carcinoma as a result of exposure. In addition, a lot is known, just like in humans, how the disease progresses in mice and this facilitates easy design of experimental studies and interpretation of the results. Moreover, they are preferred because of low cost incurred during breeding and maintenance as compared to other animal models (Khuda *et al.,* 2022).

For this study, mice were used to study the effect of spirulina in ameliorating immune dysfunction and inflammation induced by AFB1. From the literature, mice can tolerate up to 600ug/kg of aflatoxin (Coulombe and Sharma, 1985; Mohsenzadeh *et al*., 2016). Aflatoxin B1 dose at 200ug/kg has been shown to be potent (Zheng *et al*., 2002; Masese *et al*., 2022; Masese *et al*., 2023). Mice of same gender was used because studies have shown that there are variations in the immune response in both genders, so using only one gender allows for more consistent and comparable results (Choudhry *et al*., 2007; Gregory *et al*., 2000). Besides, using only one sex decreases variability and, therefore, the complexity of the study, leaving a more significant window for the control of other possibly confounding variables.

According to the gathered information in the literature, aflatoxin is still a significant public health challenge despite interventions that the government and different researchers are citing. In addition, many studies show that aflatoxin B1 exposure leads to immunologic dysfunction and inflammation in animals, which will have devastating effects on human health. However, studies on the possible benefit of spirulina extracts in reducing these adverse effects of aflatoxin exposure are lacking. The aim of this research is to determine how spirulina can ameliorate the inflammation and immunologic dysfunction brought about by the toxicity of Aflatoxin B1 in mice. The findings of this study may fill in the existing gaps in the literature about the possible therapeutic benefits of spirulina extracts in reducing the toxic effects of aflatoxin exposure. It may also offer useful insights for the creation of novel strategies to address this risk to the public's health.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical Approval

This study was conducted upon clearance by Kenya Medical Research Institute (KEMRI) Ethical Review Committee (Approval number KEMRI/ACUC/02.06.19).

3.2 Source of Materials

3.2.1 Spirulina

Packaged *Spirulina plantesis* powder, as shown in Figure 3.1, was obtained from Masinde Muliro University of Science and Technology (MMUST). The institution has been running a project for cultivation of *Spirulina plantesis* with the community. The samples were purchased and taken to KEMRI.

Figure 3.1: Spirulina powder (Photo by author)

3.2.2 Aflatoxin

AFB1 was purchased from Fermentek Ltd. Company, located in Jerusalem, Israel (Cat. 44076.568543), with a purity level of 98% or higher as determined by HPLC analysis.

3.2.3 Animal Model

The effect of aqueous *Spirulina plantesis* extracts against aflatoxin B1 induced immunologic dysfunction and inflammation was studied in male BALB/c mice. The mice were obtained from KEMRI animal house where they are kept under normal conditions- 12 hours of light and 12 hour of night, room temperature and pressure, and 40-60 humidity.

3.3 Study Design

Randomized controlled trial (RCT) study design was used. This study design allows for evaluation of different interventions to determine their effectiveness. The method entails randomly selecting and putting study participants into different groups where they are treated and evaluation done to determine whether the outcomes from all the groups differ significantly. In this study design, the subjects of study (mice) are randomly selected and put into groups to study the effects of Spirulina extract treatments on immunologic dysfunction and inflammation brought about by AFB1.

3.4 Sample Size Determination

The sample size determination was done using Resource Equation Method (Mead, 1988) as shown below:

$$E = \text{Total No. of mice} - \text{No. of groups}$$

The value of E denotes the degree of freedom of ANOVA. The value of E ranges from 10 to 20. If E is less than 10, then it implies that recruiting more mice increases the probability of obtaining significant results. Conversely, a value beyond 20 implies that recruiting more animals in the experiment will not improve the probability of obtaining significant results.

For maximum acceptable limit (E = 20), the number of mice was calculated as follows:

E = Total No. of mice – No. of groups

E = 20, number of groups = 6

Let y be the total No. of mice;

y = E + No. of groups

y = 20 + 6

y = 26 mice

Therefore, each group had a minimum of 4 mice. Five mice per group were selected during the study. However, as stated by Mead (1988), recruiting more mice (E>20) will not improve the probability of obtaining significant results.

3.5 Experimental Approach

Thirty male BALB/c mice of 8 weeks old and average body weight of 30-33g were selected from KEMRI’s Animal House. The animals were reared under regulated laboratory conditions of normal light. Food and water were given to mice *ad libitum*.

After 4 days of acclimation period, the mice were randomly selected and placed in six groups with 5 mice per group. For positive control, activated charcoal was used. Activated charcoal has a high affinity for many toxins, preventing absorption in the GIT. This makes it an effective control in studies related to toxin-binding (Dalvi & McGowan, 1984; Abd *et al.*, 2014). The rationale for the selection of activated charcoal as the positive control is based on its proven effectiveness in adsorbing AFB1, and it is generally used as a reference product in combating toxic effects. Product was selected because of overall ameliorative effect. Despite different mechanisms of action, activated charcoal works by physical adsorption and *Spirulina platensis* works through immunomodulation and antioxidants, the selection was aimed at comparing it with a well known detoxifying agent. This enables a complete analysis of the mechanism of Spirulina beyond just its role of toxin sequestration, showing how the extract is able to play this role in reducing AFB1-induced toxicity.

Negative control was only given 200µg/kg of aflatoxin B1 and the dose was informed by other relevant studies (Masese *et al.*, 2022). The titrated dosage of spirulina extract was influenced by previous studies on spirulina's potential effects on immune system (Chu *et al.*, 2013).

The mice in the groups were given subacute oral dose of the treatments as shown in Table 3.1 for 14 days. To monitor growth performance, the body weight was measured on the 1st day, 4th day, 7th day, 10th day and the last day of the experiment (14th day) thereafter mice sacrificed following animal care and use standards.

Table 3.1: Experimental Design

Group	Status	Treatment
1	Normal Control	Standard food and water
2	Negative Control	200µg/kg AFB1
3	Positive Control	200µg/kg AFB1 + Activated charcoal
4	Experimental Group A	50mg/kg Spirulina extract + 200µg/kg AFB1
5	Experimental Group B	100mg/kg Spirulina extract+200µg/kg AFB1
6	Experimental Group C	150mg/kg Spirulina extract+200µg/kg AFB1

3.6 Laboratory Procedures

3.6.1 Preparation of AFB1 Stock Solution

Ten milligrams of Aflatoxin B1 was dissolved in 7% Dimethyl sulfoxide (DMSO) as outlined by Shen *et al.* (1996). DMSO is the preferred solvent because of its high solvating abilities. The stock solution was prepared in dark bottle and stored -4^{o}C. Working solution of AFB1 was prepared by dissolving the stock solution sterile PBS.

3.6.2 Extraction of Spirulina

A hundred and ten grams (110g) of dried spirulina was weighed and further pulverized using a mortar and pestle to form fine particles. Grinding was done to increase surface area for extraction. The powder was transferred to a clean and sterile conical flask and 750ml of distilled water added to completely submerge it. The mixture was thoroughly mixed to form a slurry then heated to 65°C for 25 minutes to facilitate extraction. Subsequently, the mixture was passed through a fine mesh strainer to isolate the liquid extract from the solid biomass. The extract was freeze-dried using freeze drier.

The percentage yield of the extract was calculated as follows:

Weight of extract = 48.6g

Weight of dried spirulina = 110g

% yield = (48.6/110) x 100 = 44.18%

Figure 3.2 shows some of the steps involved in the extraction of process.

Figure 3.2 (a) Weighing of Spirulina (b) Coating round bottom flask with spirulina extract using dry ice put in acetone (c) Freeze drying of spirulina extract (Photos by author)

3.6.3 Preparation of Aqueous Solution of Extract

A stock solution of Spirulina extract was prepared by dissolving 48.6g of the extract in 200ml of distilled water in a sterile bottle. The mixture was thoroughly mixed using a magnetic stirrer for 10 minutes. This yielded a stock concentration of 0.243g/ml (243mg/ml). The stock solution was diluted accordingly to yield dosages that can be administered to mice.

3.6.4 Sample collection and preparation

At the last day of experimental period (one day after administration of the last aflatoxin dose), the mice were sacrificed. The blood was drawn from the mice through cardiac puncture under anesthesia then placed in 2ml Eppendorf tubes. Serum was prepared from the collected blood samples by allowing it to clot at room temperature. The blood was spun for 20 minutes at 1800g and supernatant (serum) collected. The serum collected was stored at -20 °C and later used for immunological studies.

The abdomen was opened and liver carefully removed from the mice through laparotomy. The liver tissues were collected as quickly as possible to minimize any degradation. The liver tissues were prepared and put in sterile tubes and then stored at -80 °C for RNA extraction later.

3.6.5 Measurement of IgA, IgG and IgM Response

Antibody responses to aflatoxin B1 intake was determined according to the manufacturer's protocol (Beijing Solarbio Science & Technology Co., Ltd.). All the reagents and standards were prepared before the experiment. The assay employed the quantitative sandwich ELISA method where monoclonal antibody specific for IgA has been pre-coated onto a 96-well plate. The pre-coated plate was opened and washed twice with wash buffer then 100 µl of the IgA standard and samples added to designated wells of the plate. The plate and then incubated for two hours at 25 °C. The sample and standard were removed from the wells and plate washed four times with wash buffer. A 100µl volume of biotin-conjugated antibody specific for IgA was added to every well to detect the captured IgA protein in the sample and then the plate put in an incubator set at 25 °C for 1 hour. The biotin-conjugated detection antibody was aspirated and the plate washed four times and then then 100µl of substrate solution added to every well which had sample and then plate incubated for 30 minutes at 25 °C. Fifty microliters of stop solution (sulfuric acid) was added to each well and the plate read at 450 nanometers within 5 minutes.

The concentrations of IgG in serum was determined using ELISA. The ELISA kit and protocol was developed by Beijing Solarbio Science & Technology Co., Ltd. To begin, the ELISA plate was washed three times using the wash buffer provided then 100 microliters of the standard IgG and serum samples added to respective well in the plate. The plate was then incubated at room temperature for 2 hours. The samples and the standard were aspirated from the wells and the plate washed four times using the wash buffer before adding 100 microliters of working solution of Biotin-conjugate anti-mouse IgG antibody. The plate was incubated at room temperature for an hour. The contents were aspirated and the plate washed 5 times with wash buffer and then 100 microliters of working solution of Streptavidin-HRP added to every well with standard and sample. The plate was incubated at room temperature for 30 minutes. The contents were aspirated and plate washed for 5 times then 100 microliters of substrate solution was added to each well with standard and sample. The plate was incubated for 15 minutes in the dark thereafter 50 microliters of stop solution was added to each well. The plate was read at 450nm within 5 minutes after addition of stop solution. The optical density readings were recorded in Microsoft Excel and later analyzed.

Similarly, determination of levels of IgM was done using ELISA protocol developed by Beijing Solarbio Science & Technology Co., Ltd. Briefly, the plate with IgM capture antibody was washed twice with wash buffer before addition of 100 microliters of IgM standard and serum samples. Plate was incubated for 2 hours at room temperature thereafter contents aspirated and washed 4 times with wash buffer. A hundred microliters of detection antibody was added to each of the wells and the plate incubated for an hour at room temperature. The content of the plate was aspirated and the plate washed 4 times before addition of a hundred microliters of substrate solution then incubated in the dark for 15 minutes at room temperature. Fifty microliters of stop solution was added to every well and the optical density readings taken at 450nm within 5 minutes of addition of stop solution. The optical density readings were recorded in Microsoft Excel and used for determination of IgM concentrations.

3.6.6 Measurement of IL2, IFN-gamma and IL-4 production in serum

Serum levels of the cytokines were measured using ELISA kit following the protocol provided by Beijing Solarbio Science & Technology Co., Ltd. All the reagents and standards were prepared before the experiment. The assay used the quantitative sandwich ELISA method where monoclonal antibody specific for IFN-gamma has been pre-coated onto a 96-well plate. The pre-coated plate was opened and washed 3 times with wash buffer then 100 µl of the IFN-gamma standard and samples added to designated wells of the plate. The plate was covered with a paper and then put in an incubator for 90 minutes at 37 °C. The sample and standard were removed from the plate and washed four times with wash buffer. A 100µl volume of biotin-conjugated anti-mouse interferon gamma antibody was added to every well to detect the captured interferon -gamma protein in the sample and then the plate incubated for one hour at 37°C. The biotin-conjugated antibody was aspirated and the plate washed four times and then then 100 µl of working solution of Streptavidin-HRP added to every well which had standard and sample and the plate put in an incubator for 30 mins at 37°C. Streptavidin-HRP working solution was aspirated and the plate washed 5 times before adding 100 µl of substrate solution to each well. The plate was incubated for 15 minutes at 37°C. Fifty microliters of stop solution (sulfuric acid) was added to each well and the plate read at 450 nanometers.

The above method was used in the determination of serum levels of IL-2 and IL-4.

3.6.7 Measurement of mRNA levels of inflammatory cytokines

3.6.7.1 mRNA Extraction

Expression of IL 6 and TNFα in liver tissues was determined by RT-PCR method as outlined by Amsen *et al*. (2009). First, RNA was extracted from liver tissue samples. The first step involved homogenizing 50mg of liver tissue with 1ml of PRImeZOL™ reagent (Canvax, Córdoba, Spain) and incubating the samples for 5 minutes. Next, 0.2ml of chloroform was added and the samples are thoroughly mixed for 15 seconds to mix. The samples were then incubated for an additional three minutes at 25 °C before being centrifuged at 12000xg for 15 minutes at 4 degrees Celsius. The samples separated into three layers, and the topmost clear phase transferred to sterile tube. One volume of 70% ethanol was added to the aqueous phase and the samples vortexed. 700 µl of the sample was then put in a spin column inserted into a 2ml collection tube and spun for 15 seconds at 800xg. The flow through was poured off, and the spin column washed with 700µl of 1X wash buffer 1, spun for 15 seconds at 800xg, and the flow through poured off again. The spin column was then washed with 500µl of wash buffer 2, spun for 15 seconds at 800xg, and the process repeated one more time. The spin column was spun to dry and remove remaining buffer and then transferred to a new Eppendorf tube. The RNA trapped in the membrane of the spin column was eluted with 50µl of RNase-free water. The purity and amount of the isolated RNA was measured using NanoDrop Microvolume Spectrophotometer then RNA analyzed by gel electrophoresis to check integrity.

The RNA was stored at -20 °C for use in measurement of mRNA levels of inflammatory cytokines.

3.6.7.2 cDNA Synthesis

The cDNA was prepared using Solis Biodyne FIREScript RT cDNA Synthesis Kit. A cDNA master mix was reconstituted as shown in Table 3.2. The primer sequences for targeted genes are provided in Table 3.3. After the cDNA master mix, cDNA was synthesized using manufacturer’s program as outlined in Table 3.4.

Table 3.2: cDNA master mix components

Component	Volume in µl
Random primers	1
dNTP Mix (20mM of each)	0.5
RT Reaction Buffer with DTT	2
FIREScript RT	1
RNase Inhibitor	0.5
Nuclease-free water	13
cDNA Template	2
Total	**20**

Table 3.3: Primer sequence for RT-PCR

Gene	Primer Sequence (Amsen *et al*., 2009)	Length	Product Size
Hprt	F:5′-CTGGTGAAAAGGACCTCTCG-3′ R:5′-TGAAGTACTCATTATAGTCAAGGGCA-3′	20 26	109
IL-4	F:5′-AGATCATCGGCATTTTGAACG-3′ R:5′-TTTGGCACATCCATCTCCG-3′	21 19	67
TNF	F:5′-CTCCAGGCGGTGCCTATGT-3′ R: 5′-GAAGAGCGTGGTGGCCC-3′	19 17	67
IFN-γ	F:5′-GGATGCATTCATGAGTATTGC-3′ R:s5′-CCTTTTCCGCTTCCTGAGG-3′	21 19	127

The following program was used for cDNA synthesis

Table 3.4: Conditions for cDNA synthesis as per manufacturer’s protocol (Solis Biodyne FIREScript RT cDNA Synthesis Kit)

Step	Temp (°C)	Time
Primer annealing step	25°C	10 min
Reverse transcription step	50°C	30 min
Enzyme inactivation step	85°C	5 min

3.7 Data Management and Analysis

The results from ELISA, RT-PCR, and body weight measurements were tabulated on a spreadsheet using Microsoft Excel. Descriptive statistics was done on Microsoft Excel and the results presented as $\bar{x}$ ± standard error mean and statistically analyzed using GraphPad Software Version 8.0.2. Statistical significance of differences among the study groups were evaluated using one-way ANOVA followed by Tukey's test. $p \leq 0.05$ was considered significant. The results were presented in tables and graphs.

CHAPTER FOUR

RESULTS

4.1 RNA Quality Assessment

Figure 4.1 shows gel electrophoresis of the RNA samples. The samples exhibited distinct and well-defined bands of 28S and 18S ribosomal RNA (rRNA) with a notable difference in intensity as shown in Figure 4.1. Specifically, the 28S rRNA band appeared roughly twice as strong as the 18S rRNA band, which is a characteristic feature of RNA that has not undergone degradation.

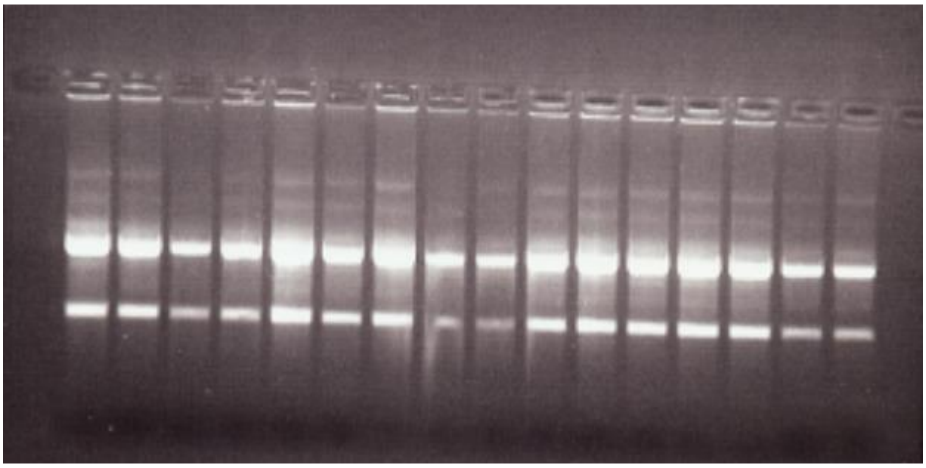


Figure 4.1: Gel electrophoresis of the RNA samples (Photo by author)

4.2 Effects of treatments on body weight

The body weight of mice were regularly measured to determine growth performance. Measurements were done on the 1st day, 4th day, 7th day, 10th day and the last day of the experiment (14th day). The results are shown in Table 4.1.

Table 4.1: Body weight monitoring

Groups	Mice	Weight in Grams (g) 1st Day	4th Day	7th Day	10th Day	14th Day
Group I	M1	32	33	33	34	35
	M2	33	33	34	35	36
	M3	31	34	34	36	37
	M4	30	31	31	33	35
	M5	32	33	33	35	37
Group II	M1	34	33	30	28	25
	M2	31	30	28	27	25
	M3	32	32	30	28	26
	M4	34	31	31	30	29
	M5	30	28	27	26	25
Group III	M1	34	32	33	32	30
	M2	33	33	32	30	31
	M3	30	30	31	31	33
	M4	33	32	33	33	34
	M5	29	31	31	32	32
Group IV	M1	33	30	29	29	27
	M2	30	29	29	27	26
	M3	34	30	28	29	29
	M4	31	30	30	29	28
	M5	33	32	30	29	30
Group V	M1	29	29	30	31	31
	M2	34	33	31	32	32
	M3	32	31	30	30	29
	M4	28	29	29	30	30
	M5	34	33	31	32	33
Group VI	M1	30	32	33	35	36
	M2	29	30	30	31	31
	M3	32	33	35	35	36
	M4	29	30	31	33	34
	M5	30	31	32	32	33

The body weight changes over time were analyzed using repeated measures ANOVA. Figure 4.2 shows body weigh changes in mice over different time intervals. From the analysis output, it was noted that the interaction effect across all the treatment groups over time is significantly high ($p < 0.0001$). This indicates that different treatments result in significant changes in terms of weight as the time goes. Further, it was noted that the main effect of column factor is highly significant ($p = 0.0001$). The column factor here denotes the different treatment groups. This implies that there is significant differences in body weight among the treatment groups overall. However, it is worth noting that the measurable effect on body weight varies across the group, with some treatment groups showing greater effects than others. It was also noted that the main effect of time is not significant (p = 0.2571). This means that there is no significant change in body weight across the different time intervals.

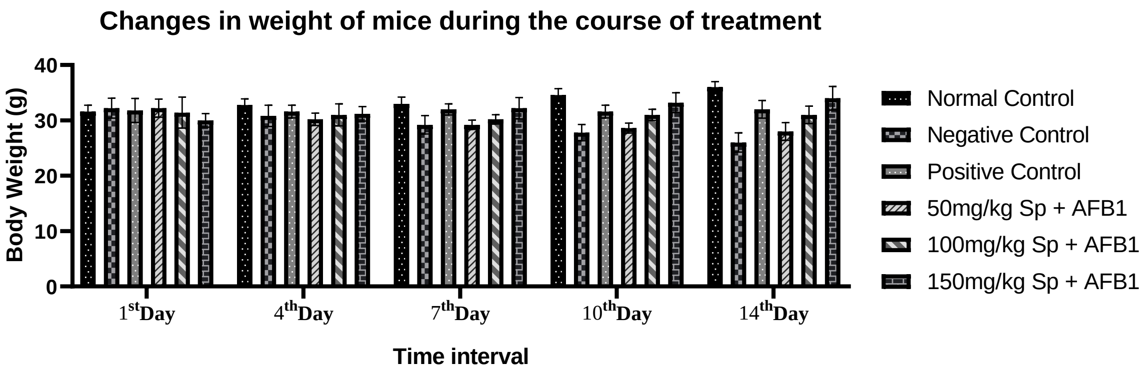


Figure 4.2: Graphical representation of weights of the mice treated with aflatoxin B1 alone and co-treatments during time intervals 1st, 4th, 7th, 10th and 14th day. The data was analyzed using Repeated Measures ANOVA. Values presented as the x̄ ±SEM. Sample per group n=5.

4.3 Effects of treatments on serum levels of IFNγ, IL 2, and IL 4

Figure 4.3 depicts the effects of spirulina extract on serum concentrations of IFN, IL-2, and IL-4 in mice administered aflatoxin B1. Cytokines IFN and IL-2 are considerably upregulated in the aflatoxin B1 alone-treated group as compared to the group that received no treatment ($p < 0.05$). As opposed to the group that received no treatment, the group that received AFB1 had a considerably lower blood concentrations of IL 4 ($p < 0.05$). There is spirulina dose dependent inhibition of IL-4 production. The results shows that spirulina extract dosages of 100mg/kg and 150 mg/kg body weight had significant impact on IL 4 concentrations ($p < 0.05$). The results also indicated that activated charcoal (positive control) had no effect appreciable effect on IL-4 levels. There was no significant difference in blood levels of IL 2 between the dosages of 50 mg/kg and 100 mg/kg Spirulina ($p > 0.05$). However, a significant difference was observed when compared to the normal control ($p<0.0001$). IFNγ and IL 2 blood concentrations were found to be significantly reduced in the group that received spirulina extract at doses of 100mg/kg and 150 mg/kg in comparison to the AFB1-treated group ($p < 0.05$).

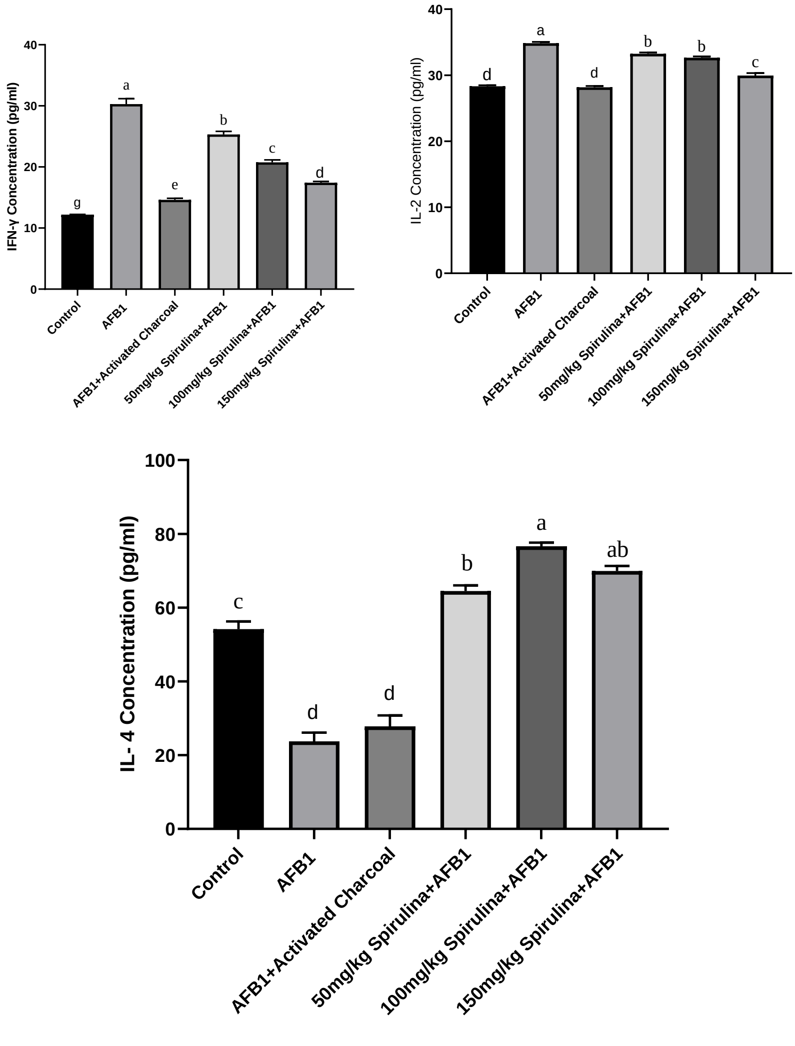


Figure 4.3: Serum concentrations of cytokines (a) IFN, (b) IL-2, and (c) IL-4 after treatments for 14 days. The mean values are presented as the $\bar{x}$ ±S.E.M, number of samples per group=5. The letters denote level of statistical significance, where different letters show statistical significance ($p < 0.05$). Bars labeled with distinct letters exhibit a statistically significant variance ($p < 0.05$).

4.4 Effects of treatments on serum levels of immunoglobulins

As shown in Table 4.2, the blood concentrations of Immunoglobulin A and Immunoglobulin M in the group given AFB1 did not differ considerably from the untreated group at the final day of the study (p >0.05). This suggests that dietary intake of AFB1 at a dosage of 200µg/kg for 2 weeks does not significantly affect antibody-mediated defense. Conversely, in comparison to the untreated group, the AFB1-treated group's blood levels of IgG considerably decreased (p<0.05). This also correlates to decreased serum levels of IL-4. Additionally, oral administration of spirulina extract at various dosages showed no appreciable impact on the levels of IgA, IgG, and IgM in the blood.

Table 4.2: Effects of treatments on serum titers of immunoglobulins A, G and M in mice

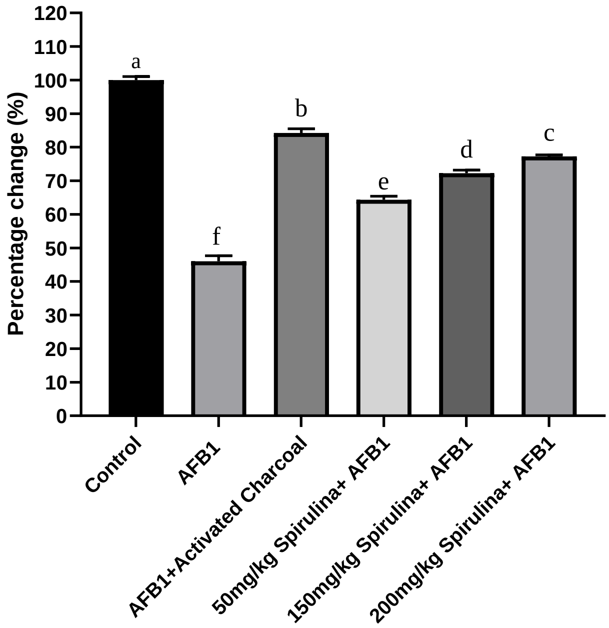
Group	IgG (ng/ml)	IgA (ng/ml)	IgM (ng/ml)
Normal Control	15.51±0.11^{a}	0.730±0.004^{a}	2.168±0.004^{a}
Aflatoxin B1 (200 µg/kg)	14.54±0.04^{b}	0.729±0.005^{a}	2.167±0.004^{a}
Activated charcoal 1g/kg+ AFB1 200µg/kg	15.36±0.06^{a}	0.731±0.004^{a}	2.175±0.009^{a}
Spirulina 50mg/kg + AFB1 200 µg/kg	15.7±0.12^{a}	0.737±0.004^{a}	2.162±0.002^{a}
Spirulina 100mg/kg+AFB1 200 µg/kg	14.59±0.06^{b}	0.737±0.004^{a}	2.166±0.006^{a}
Spirulina 150mg/kg + AFB1 200 µg/kg	15.58±0.17^{a}	0.735±0.004^{a}	2.169±0.006^{a}

The values indicate mean ± SEM of 5 mice per group. Means with superscript letter "a" are not significantly different (*p*>0.05)

4.5 Effect of Treatments on mRNA Expression of Cytokines

The gene expressions of inflammatory cytokines TNF, IL 4 and IFN gamma was determined after the experiment. In comparison to the untreated group, the mRNA expression of IL 4 was suppressed in all the groups as shown in Figure 4.4(a) (p<0.05). Tumor necrosis factor α and IFN mRNA levels were upregulated in groups treated with activated charcoal, AFB1, and spirulina treated in comparison to the untreated group. Spirulina extract treatment decreased (p< 0.05) the increase in TNF and IFN mRNA levels caused by Aflatoxin B1 (Figure 4.4(b).

A



B

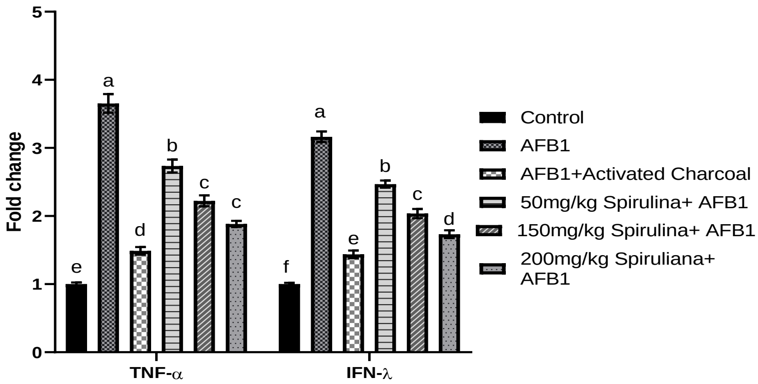


Figure 4.4: The effects of treatments on mRNA expressions of (A) Interleukin 4 (B) TNF and IFN. Different lowercase letters are significantly different ($p<0.0001$)

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Body Weight Response to AFB1 and Spirulina Extract

It has been demonstrated that mice exposed to AFB1 lose weight and this can cause other negative health consequences (Huang *et al.*, 2020). The potential of AFB1 to cause oxidative stress, inflammation, and DNA damage, which result in cellular death and disruption of regular metabolic processes, is assumed to be the origin of these effects.

Another finding is that those mice treated with 200 µg/kg body weight of AFB1 underwent an acute weight loss. This was in concurrence with the findings of Lai *et al.* (2022), which reported that mice lose weight acutely two to three days after Aflatoxin B1 treatment. The studies further reported that Aflatoxin B1 weakens the integrity of the gut wall. Aflatoxin B1 will express its action in the gut, altering the absorption of vital nutrients, thereby calling for a retarded development and a decrease in weight (Pu *et al.*, 2021). Simultaneously, it is also considered that the AFB1-induced low activity of numerous enzymes in the GI tract and disturbance in gluconeogenesis and fatty acid synthesis are responsible for body weight loss in the drug-treated group (Feng *et al.*, 2017; Hassaan *et al.*, 2020).

Lai et al. (2022) reported that AFB1 causes weight loss in mice. Excessive doses of spirulina from this study appear to have a dose-dependent effect on the body weight change. Small doses of spirulina extract, which is at 50 mg/kg of body weight, do not significantly contribute to the reversal of weight losses in mice induced by AFB1. However, it was found that a higher dosing of 150 mg/kg of body weight can prevent weight loss due to AFB1 ingestion. That means spirulina prevented damages caused by AFB1 ingestion. Ingesting spirulina leads to weight gain (Jamil *et al.,* 2015). Cho *et al.* (2020) discovered that the administration of spirulina increases the level of parathyroid hormone (PTH) and growth hormone, which further promotes growth and increase bone strength. Spirulina contains phycocyanin, an antioxidant substance. It removes or scavenges free radicals, preventing oxidative stress in the body by not allowing its occurrence in the body (Romay *et al.,* 2003; Brito *et al.,* 2020).

Spirulina also provides amino acids and minerals, which also add to its antioxidant nature (Babadzhanov *et al.,* 2004). It is well recognized that activated charcoal can be used to manage aflatoxin (Decker and Corby, 1980). Based on the case studies, supplementing mice with activated charcoal and aflatoxin B1 reduces AFB1's effect on body weight (Jindal *et al.,* 1994). This is because activated charcoal absorbs the Aflatoxin very well, and this is evidenced in in vitro and in vivo experiments as well. Although the *Spirulina plantesis* extract provides a similar result with a different mode of action, it can also be applied to alleviating weight loss initiated by AFB1 ingestion.

5.1.2 Inflammatory Response to AFB1 and Spirulina Extract

To determine the health status of the immune system, measuring the amounts of inflammatory cytokines in the blood and splenic tissue was carried out (Prassana *et al*., 2010). It has been observed that inflammatory cytokine expressions are minimal under normal physiological conditions but shoot up when stimulated by alien particles. The results of this study demonstrated that mice fed with aflatoxin B1 resulted in enhanced levels of TNF and IFN mRNA expression. These mRNA expressions for the said cytokines are substantially lesser when treated with Spirulina extract. This is probably because Spirulina contains anti-inflammatory compounds.

The anti-inflammatory impact of spirulina enables the downregulation of inflammatory cytokines. Abundantly present antioxidants, such as phycocyanin, act efficiently against free radicals by reducing oxidative stress. Two other substances within spirulina, gamma-linolenic acid, and polysaccharides, have also been shown to have an anti-inflammatory effect. This would lead to a decreased inflammation of the body caused by the decrease in cytokine synthesis. Cytokines are the proteins that participate in an inflammatory response; thus, they cause inflammation of the body (Shih *et al.,* 2009).

This study also found that mice exposed to AFB1, without any other treatment, showed downregulation in their expression of IL-4 mRNA. As also demonstrated by Qian *et al.* (2014), such results suggest that the expressional level of IL-4 was decreased during exposure to aflatoxin B1.

5.1.3 Serum levels of inflammatory cytokines

Several research investigations have documented that Aflatoxin B1 increases the amount of cytokines in the blood. In their study, Li *et al.* (2014) discovered that birds given feed infected with Aflatoxin B1 produced more cytokines, notably IFNγ. In mice given Aflatoxin B1, there was a considerable drop in blood levels of IL 4 compared to the control group. An anti-inflammatory cytokine called IL 4 is released when in an inflammatory reaction. Several studies have shown that Aflatoxins inhibits IL 4 mRNA and protein expression (Dugyala and Sharma, 1996). Spirulina extract supplementation at dosages of 100mg/kg body weight and 150 mg/kg body weight had discernible impact on IL-4 concentrations. It was also noted that activated charcoal had no significant impact on the levels of IL-4 as expected (being a positive control). This implies limitations and complexities when it comes to finding a positive control in aflatoxicosis studies.

The spirulina extract decreases the expression of Interleukin-4, a cytokine indispensable for the control of inflammatory response and one of the principal actors in allergies and asthma. The ability of spirulina extract to reduce the levels of both mRNA and protein of IL-4 reduces the amplitude of the inflammatory reactions, according to Marin *et al.* (2002). This is most likely because spirulina contains anti-inflammatory and antioxidant constituents, which support the regulation of the immune system and the prevention of oxidative stress (Bruneau *et al.*, 2012).

Likely, the doses are indeed not high enough to cause significant fluctuations in IL 4 concentration. The findings also revealed that subjects administered spirulina extract and activated charcoal after the Aflatoxin B1 injection also resulted in much lower IFN and IL2 in the blood compared to the control group. This means that the inflammatory reaction caused by the oral intake of Aflatoxin B1 by the mice can be mitigated by oral administration of spirulina extract. The same results on the anti-inflammatory effects of spirulina extract were published by Pham *et al*. (2016). The results of this study suggests that dosing with spirulina may help to lessen the inflammation that AFB1 causes in animal model.

This study determined the level of cytokines both at protein level (objective iii) and molecular level (objective iv). While this might have similar outcomes in terms of interpretation, the two levels address distinct facets of the immune response pathway. It is important to note that while genes can be expressed or activated to produce proteins, not all genes are translated into proteins and similar case might happen in this study. This study noted that mRNA expression of cytokines lead to translation of cytokines in serum.

5.1.4 Effects of treatments on Humoral Immune System

The immune system's humoral responses are facilitated by antibodies, which are proteins made by the B lymphocytes. These particular proteins are capable of identifying and attaching to complementary antigens (Tomar and De, 2014). A method used to test the humoral immunity when activated by Aflatoxin B1 is by determining the blood levels of Immunoglobulins A, G, and M. (Meissonnier *et al*., 2008). From the findings of this study, the concentrations of IgA and IgM in the group administered 200µg/kg of Aflatoxin B1 were decreased, but not considerably, in comparisons to the control group. This suggests that mice exposed to AFB1 at a dosage of 200 µg/kg body weight do not show any detectable immunosuppressive effects.

According to Pierron *et al*. (2016), IgG, IgA, and IgM levels in pigs fed with Aflatoxin B1-contaminated diets did not significantly change. However, it was shown in this stuidy that IgG levels were considerably lower in the AFB1-treated group supports those obtained by Giambrone *et al*. (1978). After treatment with spirulina extract and AFB1 an hour later, IgA, IgG, and IgM blood levels were not significantly affected after treatment for 14 days. This can be due to the treatment's dose, length, and animal model's responsiveness.

5.2 Conclusions

i. The study revealed that the treatments given led to changes in weight gain among the various groups of mice. The results point clearly to the fact that, although some of the treatment groups achieved higher levels of body weight alterations, these were not sustained over time. This provides a strong argument to the effect that the kind of treatment offered, rather than the period, has a significant impact on body weight. Hence, it can be deduced that the aqueous extract of Spirulina platensis has a particular benefit on the body weight of mice as compared to other treatments and reveals influence of growth rate during the course of the study period.

ii. The results revealed that the Spirulina platensis extract affects the cytokine concentrations in serum of the mice fed with aflatoxin B1 substantially. Furthermore, the extract inhibits the high levels of IFNγ and IL-2 produced due to the action of aflatoxin B1 and brings them to near-normal levels. Moreover, antagonistic effects of Spirulina extract on the synthesis of II-4 prove that it can exert immunomodulating effects. In contrast, activated charcoal did not produce any significant change in the IL- 4 levels. These findings indicate that Spirulina platensis can mitigate the effects of aflatoxin B1 on immunologic anomalies in mice.

iii. The results show that dietary intake of aflatoxin B1 does not alter the blood concentration of Immunoglobulins A and M, pointing out that the antibody defense mechanism can still be robust. But it was observed that aflatoxin B1 caused a significant reduction in Immunoglobulin G levels and this correlates to lower serum levels of IL-4. The treatment with Spirulina platensis extract did not alter the serum immunoglobulin levels of IgA, IgG and IgM significantly, thus can be concluded that Spirulina does not affect the humoral immune system when mice are exposed to aflatoxin B1.

iv. According to the results presented, AFB1 suppresses the mRNA level of IL-4 while increasing the mRNA levels of TNF and IFNγ in mice. The results also show that the treatment of Spirulina platensis extract has the ability to eliminate the effects of aflatoxin B1 in that it lowers expressed TNF and IFNγ mRNA. These results indicate that, the consumption of Spirulina platensis reduces the inflammation brought by aflatoxin B1 and regulates inflammation cytokine genes.

5.3 Recommendations

5.3.1 Recommendations for future research

i. Spirulina has demonstrated a potential to prevent weight loss resulting from AFB1 ingestion. It is therefore recommended that it should be incorporated as a dietary supplement in human and farm animal feeds, particularly in areas where aflatoxin contamination of food is very prevalent.

ii. Based on the findings of humoral immune response, longer treatment period should be considered. Longer treatment period should be done to understand whether there is effect of treatments in humoral mediated immune system. The results on the effects of AFB1 on humoral immune components is not conclusive as there were no significant effect on the levels of IgA, IgG, and IgM.

iii. Even though spirulina has demonstrated ameliorative effect on aflatoxicosis models, further research is necessary to determine the optimal dosages and the mechanisms of action of spirulina extract in reducing inflammation. Despite numerous studies have documented probable mechanism of action of spirulina, future studies should focus on elucidating the exact mechanism of action and standard dose. Dosage of 150mg/kg body weight was shown to be effective but further studies should be done to confirm if higher dosages could effectively be used to counter the inflammatory effects of AFB1.

iv. Studies should be done on the effect of spirulina and development of hepatocarcinoma in cancer research models. From the findings, spirulina supplementation of food should be promoted as a protective approach against hepatocellular carcinoma caused by AFB1. The findings of this study has shown that spirulina has a potential to regulate mRNA expression of key inflammatory cytokines that are involved during the pathogenesis of hepatocellular carcinomas.

v. Future studies should extrapolate the same findings in human subjects or farm animals to check applicability. The studies that were carried out in mice models are promising and future studies should focus on human, who also bear direct consequences of AFB1 effects. Different animal models respond differently to aflatoxins and extrapolating the same study on human subjects would bring confidence in the management of aflatoxicosis using spirulina.

vi. Future studies should analyze more panels of cytokines to understand the effect spirulina intervention in aflatoxicosis. There are several other important inflammatory cytokines that mediate inflammatory responses.

5.4 Limitations of the Study

i. The results on the effects of AFB1 on humoral immune components is not conclusive as the study was conducted within shorter period (14 days). This might have resulted in no significant effect on the levels of IgA, IgG, and IgM.

ii. This study only analyzed few of the key inflammatory cytokines involved during AFB1-induced toxicity thus no knowledge is generated with regard to the effect on other important cytokines that play a significant role in mediating inflammatory responses.

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APPENDICES

Appendix I: Animal Use Approval from KEMRI Animal Care and Use Committee (ACUC)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/ACUC/ 02.06.19

20th June 2019

Prof. Charles Mutai
CTMDR, KEMRI

Prof. Mutai,

RE: Animal use approval for "Evaluation of protective effects of Spirulina platensis and Spirulina platensis (Var Lonar) against aflatoxin B1 induced toxicities" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that the use of laboratory mice is justified in achieving the study objectives and issues raised earlier have been adequately addressed.

Approval is granted for a period of two years starting from when the final SERU approval will be obtained. If you still intend to handle mice after the period covered by this initial approval, you are required to submit an application for continuing approval to the ACUC 1 month prior to the expiry of the SERU approval. In addition, the committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

The committee expects you to adhere to all the laboratory animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

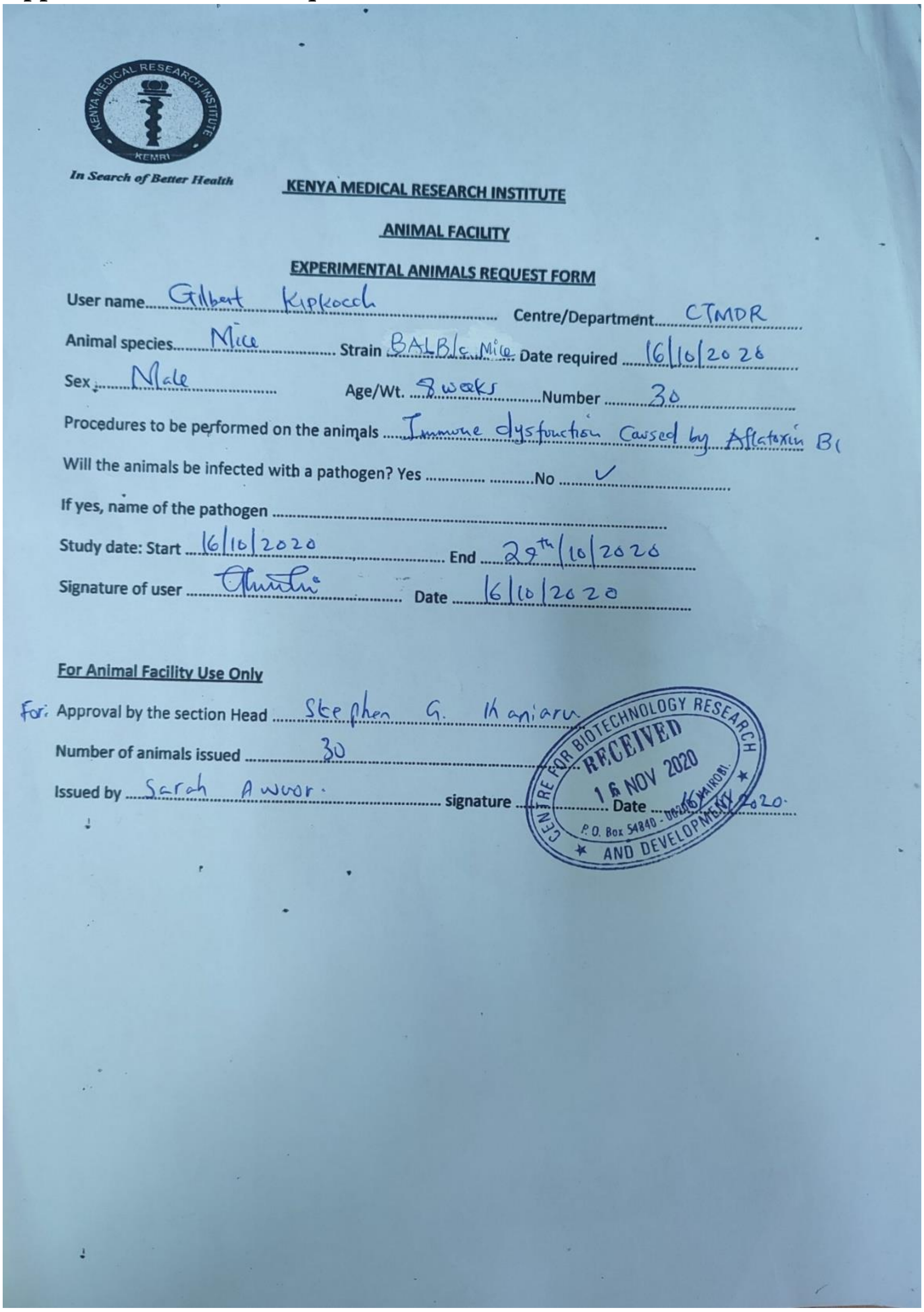
Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 28 JUN 2019 ★
ANIMAL CARE AND USE COMMITTEE
Signature: ....

In Search of Better Health

Appendix II: Animal Request Form



In Search of Better Health

KENYA MEDICAL RESEARCH INSTITUTE

ANIMAL FACILITY

EXPERIMENTAL ANIMALS REQUEST FORM

User name: Gilbert Kipkoech Centre/Department: CTMDR

Animal species: Mice Strain: BALB/c Mice Date required: 16/10/2020

Sex: Male Age/Wt.: 8 weeks Number: 30

Procedures to be performed on the animals: Immune dysfunction Caused by Aflatoxin B1

Will the animals be infected with a pathogen? Yes .... No ✓

If yes, name of the pathogen ....

Study date: Start 16/10/2020 End 29th/10/2020

Signature of user [signature] Date 16/10/2020

For Animal Facility Use Only

For: Approval by the section Head: Stephen G. Kaniaru

Number of animals issued: 30

Issued by: Sarah Awuor. signature .... Date 16/11/2020.

CENTRE FOR BIOTECHNOLOGY RESEARCH AND DEVELOPMENT
RECEIVED
16 NOV 2020
P.O. Box 54840 - 00200 NAIROBI

Appendix III: RNA Concentrations and Purity

RNA Quantification & Purity | 2nd Feb 2022

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	Water (Blank)	OFFICE	2/3/2022 11:41:00 AM	-0.2	ng/µl	-0.006	-0.023	0.27	0.15	RNA	40.00
2	11	OFFICE	2/3/2022 11:42:42 AM	339.7	ng/µl	8.492	4.037	2.10	1.90	RNA	40.00
3	15	OFFICE	2/3/2022 11:45:21 AM	250.2	ng/µl	6.254	2.977	2.10	1.94	RNA	40.00
4	12	OFFICE	2/3/2022 11:47:51 AM	283.7	ng/µl	7.094	3.359	2.11	1.99	RNA	40.00
5	14	OFFICE	2/3/2022 11:52:14 AM	213.8	ng/µl	5.345	2.532	2.11	0.83	RNA	40.00
6	13	OFFICE	2/3/2022 11:53:16 AM	219.8	ng/µl	5.496	2.592	2.12	0.85	RNA	40.00
7	21	OFFICE	2/3/2022 11:54:08 AM	144.2	ng/µl	3.605	1.702	2.12	1.04	RNA	40.00
8	22	OFFICE	2/3/2022 11:55:04 AM	177.4	ng/µl	4.434	2.103	2.11	1.81	RNA	40.00
9	23	OFFICE	2/3/2022 11:55:52 AM	264.1	ng/µl	6.603	3.147	2.10	1.99	RNA	40.00
10	24	OFFICE	2/3/2022 11:56:51 AM	199.8	ng/µl	4.995	2.376	2.10	1.81	RNA	40.00
11	25	OFFICE	2/3/2022 11:57:31 AM	269.3	ng/µl	6.733	3.180	2.12	1.30	RNA	40.00
12	31	OFFICE	2/3/2022 11:58:22 AM	185.7	ng/µl	4.642	2.206	2.10	1.32	RNA	40.00
13	32	OFFICE	2/3/2022 11:59:21 AM	132.4	ng/µl	3.310	1.565	2.11	0.67	RNA	40.00
14	34	OFFICE	2/3/2022 12:00:09 PM	348.2	ng/µl	8.705	4.131	2.11	1.50	RNA	40.00
15	35	OFFICE	2/3/2022 12:00:58 PM	241.8	ng/µl	6.046	2.882	2.10	1.73	RNA	40.00
16	41	OFFICE	2/3/2022 12:01:40 PM	221.8	ng/µl	5.545	2.634	2.11	1.71	RNA	40.00
17	42	OFFICE	2/3/2022 12:02:15 PM	363.4	ng/µl	9.084	4.367	2.08	2.09	RNA	40.00
18	43	OFFICE	2/3/2022 12:02:51 PM	467.7	ng/µl	11.693	5.674	2.06	2.21	RNA	40.00
19	44	OFFICE	2/3/2022 12:03:36 PM	224.7	ng/µl	5.618	2.657	2.11	1.31	RNA	40.00
20	45	OFFICE	2/3/2022 12:04:20 PM	298.6	ng/µl	7.465	3.539	2.11	2.04	RNA	40.00
21	51	OFFICE	2/3/2022 12:05:00 PM	303.8	ng/µl	7.595	3.606	2.11	2.13	RNA	40.00
22	52	OFFICE	2/3/2022 12:06:07 PM	260.8	ng/µl	6.520	3.074	2.12	1.50	RNA	40.00
23	53	OFFICE	2/3/2022 12:06:52 PM	208.5	ng/µl	5.213	2.472	2.11	1.77	RNA	40.00
24	54	OFFICE	2/3/2022 12:08:02 PM	152.1	ng/µl	3.803	1.783	2.13	0.56	RNA	40.00
25	55	OFFICE	2/3/2022 12:08:58 PM	403.7	ng/µl	10.093	4.794	2.11	1.60	RNA	40.00
26	61	OFFICE	2/3/2022 12:09:47 PM	159.8	ng/µl	3.995	1.905	2.10	1.60	RNA	40.00
27	62	OFFICE	2/3/2022 12:10:40 PM	206.5	ng/µl	5.161	2.434	2.12	1.88	RNA	40.00
28	63	OFFICE	2/3/2022 12:11:24 PM	161.7	ng/µl	4.043	1.896	2.13	1.91	RNA	40.00
29	64	OFFICE	2/3/2022 12:12:03 PM	362.2	ng/µl	9.054	4.299	2.11	2.02	RNA	40.00
30	65	OFFICE	2/3/2022 12:12:41 PM	133.5	ng/µl	3.337	1.557	2.14	0.59	RNA	40.00

Appendix IV: Temperature Monitoring Tool

Date	Temperature at 9 am (^0C)	Temperature at 3 pm (^0C)
16th Oct 2020	24	25
17th Oct 2020	25	26
18th Oct 2020	25	25
19th Oct 2020	25	24
20th Oct 2020	24	24
21st Oct 2020	25	26
22nd Oct 2020	25	25
23rd Oct 2020	25	26
24th Oct 2020	24	25
25th Oct 2020	24	24
26th Oct 2020	24	25
27th Oct 2020	24	25
28h Oct 2020	24	25
29th Oct 2020	25	24

Appendix V: Screenshot of Published Paper

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Evaluation of Effects of Spirulina Extracts on Immunologic Dysfunction and Inflammation Associated with Aflatoxin B1 Induced Toxicity in Mice

Kipkoech Gilbert, Musembi Susan, Masese Johnson, Mburu David, Jepkorir Mercy, Kuria James, Mwitari Peter, Mutai Charles

Abstract

The contamination of foods by various mycotoxins has been reported as a major public health concern across the world. The most predominant type of fungal toxins are aflatoxins which are synthesized by certain fungi that contaminate agricultural crops or produce. The main aflatoxin producing fungi are Aspergillus flavus and Aspergillus parasiticus which contaminate food crops in the farm and after harvesting. Out of all the types of aflatoxins, the most potent type is aflatoxin B1. The mechanism of toxicity and health effects of aflatoxins have

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