EVALUATION OF PROTECTIVE EFFECTS OF *SPIRULINA PLATENSIS* AGAINST AFLATOXIN B_1 INDUCED TOXICITIES IN SWISS ALBINO MICE

JOHNSON ONGERI MASESE

A Thesis Submitted in Partial Fulfilment for the Requirements for the Award of Doctorate Degree in Pharmacology and Therapeutics of Masinde Muliro University of Science and Technology

August 2024

DECLARATION

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

Signature: ------------------------------------ Date: ---------------------------------------

Johnson Ongeri Masese **REG No: HPT/LH/01-55588/2016**

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a thesis entitled "**Evaluation of protective effects of *Spirulina platensis* against aflatoxin B_1 induced toxicities in Swiss albino mice**"

Signature: ------------------------------------ Date: ----------------------------

Prof. Charles Mutai

Department of Medical Laboratory Sciences

Masinde Muliro University of Science and Technology

Signature: ------------------------------------ Date: ----------------------------

Prof. Gordon Nguka

Department of Nutritions and Dietectics

Masinde Muliro University of Science and Technology

COPYRIGHT

This thesis is copyright materials protected under the Berne Convention, the copyright Act 1999 and other international and national enactments in that behalf, on intellectual property. It may not be reproduced by any means in full or in part except for short extracts in fair dealing so for research or private study, critical scholarly review or discourse with acknowledgment, with written permission of the Director School of Graduate Studies on behalf of both the author and Masinde Muliro University of Science and Technology.

DEDICATION

This work is dedicated to my beloved parents the late Mr. Jeremiah Masese Onyangore and Mrs Yunuke Moraa Masese, my dear wife Lydiah Kerubo Onchomba and my children Roy, Chloe, Joy and Ryan. I am also grateful for the support I received from my brothers Justus, Peter, Nelson, Benard and Fred. I also dedicate this thesis to my sisters Gladys and Stela.

ACKNOWLEDGEMENTS

First of all, I give all thanks to the almighty God. I wish to express my appreciation to my supervisors Prof. Charles Mutai and Prof. Gordon Nguka for their guidance, time, dedication and encouragement throughout the course of this thesis. Their advice, corrections and suggestions greatly assisted me to complete this work. I am forever greatly indebted to them.

Special thanks to the School of Public Health, Biomedical Sciences and Technology of Masinde Muliro University, for granting me permission to study, provision of great mentorship and guidance throughout my academic advancement.

Special thanks to the Centre for Traditional Medicine and Drug Research of Kenya Medical Research Institute, for providing valuable resources including laboratories that enabled me to carry out the research work successfully. I appreciate the invaluable support and mentorship I received from Dr. Peter Mwitari of Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute. His availability, provision of access to their laboratories, equipment and reagents significantly helped me to complete this thesis.

In addition to these, I am grateful for the technical assistance I received from Pathologists Lancet Kenya Limited Laboratories, Nairobi during the analysis of biochemistry samples. I am also grateful to the Faculty of Veterinary Medicine, department of Veterinary Pathology, Microbiology and Parasitology of University of Nairobi for the great support and technical assistance during the analysis of histopathological samples. Special thanks to Dr. Nichodemus Kamuti who assisted in histopathological analysis of the samples. I also thank my colleagues for their encouragement during my studies.

TABLE OF CONTENTS

TITLE PAGE .... **I**

DECLARATION .... **II**

COPYRIGHT .... **III**

DEDICATION .... **IV**

ACKNOWLEDGEMENTS .... **V**

TABLE OF CONTENTS .... **VI**

LIST OF TABLES .... **XII**

LIST OF FIGURES .... **XIV**

LIST OF PLATES .... **XV**

LIST OF APPENDICES .... **XVI**

ACRONYMS AND ABBREVIATIONS .... **XVII**

OPERATIONAL TERMS .... **XXI**

ABSTRACT .... **XXII**

CHAPTER ONE: INTRODUCTION **1**

1.1. Background information 1

1.2. Problem Statement 4

1.3. Study objectives 5

1.3.1. Broad objective 5

1.3.2. Specific objectives 5

1.4. Research questions 6

1.5. Justification 6

1.6. Significance of the Study 7

1.7. Scope of the study 8

1.8. Study Limitations 8

1.9. Study delimitations 9

CHAPTER TWO: LITERATURE REVIEW 10

2.1. Introduction 10

2.2. History Of Aflatoxins 10

2.3. Geographical Distribution 11

2.4. Aflatoxin Contaminations 12

2.4.1. Aflatoxins occurrence in animal feeds and human food 12

2.4.2 Aflatoxin Limits........ 13

2.4.3. Global impact of aflatoxins 15

2.4.4. Prevalence of aflatoxins in Kenya 17

2.5. Aflatoxin Chemistry 24

2.6. Toxicokinetics Of Aflatoxins 26

2.6.1. Exposure to aflatoxins........ 26

2.6.2. Distribution of aflatoxins in the human body and animals 26

2.6.3. Aflatoxins metabolism 27

2.7. Toxicity And Mechanism Of Action 28

2.7.1. Aflatoxins toxicity 28

2.7.2. Acute aflatoxicosis........ 31

2.7.3. Mechanism of hepatotoxicity 33

2.7.4. Chronic aflatoxicosis 36

2.7.6 Aflatoxin B_1 induced hepatocellular carcinoma........ 38

2.7.7. Aflatoxin B_1 induced immune suppression........ 39

2.7.8. Histopathological changes associated with aflatoxins........ 43

2.8. Mitigation against Aflatoxins........ 44

2.8.1. Introduction 44
2.8.2. Physical Methods 44
2.8.2.1. Cleaning 45
2.8.2.2. Thermal applications 45
2.8.2.3. Irradiation 46
2.8.2.4. Adsorbents 47
2.8.3. Chemical Methods 47
2.8.4. Biological Methods 48
2.8.4. 1. Bacteria 49
2.8.4.2. Yeast 49
2.9. Novel Methods Of Mitigation 50
2.9.1. Genetically modified (GM) Bt corn 50
2.9.2. Biocontrol 50
2.10. Spirulina Platensis 51
2.10.1. Introduction 51
2.10.2. Historical use 53
2.10.3. Renewed interest on Spirulina 53
2.10.4. Spirulina species 54
2.10.5. Health benefits of spirulina 55
2.10.6. Probable mechanism of action of Spirulina platensis 56
2.10.7. Spirulina platensis safety 59
2.10.8. Commercialization of Spirulina 61
2.11. Aflatoxins Studies Using Experimental Animal Models 61

CHAPTER THREE: MATERIALS AND METHODS 65

3.1. Introduction 65

3.2. Study Site 65

3.3. Materials And Equipment 65

3.3.1. Chemicals 65

3.3.2. Preparation Of Afb_1 Stock Solution.. 66

3.3.3. Extraction Of Spirulina Platensis Powder 66

3.4. Sampling Design.. 67

3.5. Sample Size Calculation 67

3.6. Animal Husbandry.. 68

3.7. Aflatoxin Analysis Of Experimental Diet... 69

3.8. Experimental Design.. 70

3.9. Mice Feeding Behaviour 71

3.10. Preparation Of Serum 72

3.11. Evaluation Of Protective Effect Of Spirulina Platensis Extract On Biochemical Changes Induced On Liver By Afb_1.. 72

3.12. Evaluation Of Protective Effect Of Spirulina Platensis Extract On Histopathological Changes Induced By Afb_1. 72

3.13. Investigation Of Protective Effect Of Spirulina Platensis Extract Against Afb_1 Induced Immune Depression. 74

3.13.1. Assay Procedure For Quantitative Determination Of Igg 75

3.13.2 Assay Procedure For Quantitative Determination Of Igm & Iga 76

3.14. Investigation Of The Probable Mechanism Of Action Of The Protective Effect Spirulina Platensis Extract Against Aflatoxin B_1 Induced Toxicity. 77

3.14.1. Preparation Of 0.15m Kcl 77

3.14.2. Preparation Of 50% Trichloroacetic Acid (Tca) 78

3.14.3. Assay Procedure For Thiobarbituric Acid Reactive Substances (Tbars)........ 78

3.15. Laboratory Safety And Precautions.. 79

3.16. Handling And Transportation Of Biological Specimen.................................. 80

3.17. Handling And Disposal Of Waste 80

3.18. Data And Information Collection 81

3.19. Data Analysis And Presentation 81

3.20. Ethical Considerations 82

CHAPTER FOUR RESULTS 83

4.1. Introduction 83

4.2. Weight Changes.. 83

4.4. Assessment Of The Protective Effect Of Spirulina Platensis Extract On Histopathological Changes Induced By Afb_1. 94

4.4.1.Histopathological Changes Induced By Afb_1 On The Liver…………………...94

4.4.2. Semi-Quantitative Grading Of Liver Tissues... 96

4.4.3. Histopathological Changes Induced By Afb_1 On The Kidney........................ 98

4.4.4. Semi-Quantitative Grading Of Kidney Tissues Using Ordinal Four Step Scale System... 100

4.5. Evaluation Of Protective Effect Of Spirulina Platensis Extract Against Afb_1 Induced Immune Dysfunction.. 102

4.6. Investigation On The Probable Mechanism Of Action Of Protective Effect Spirulina Platensis Extract Against Afb_1 Induced Toxicity.................................... 109

CHAPTER FIVE: DISCUSSION 113

5.1. Introduction 113

5.2. Body Weight Changes 113

5.3. Evaluation Of The Protective Effect Of Spirulina Platensis Extract On Biochemical Changes Induced By Afb1 114

5.4. Evaluation Of The Protective Effect Of Spirulina Platensis Extract On Histopathological Changes Induced By Afb1 117

5.4.1. Histopathological Changes In Liver 117

5.4.2. Histopathological Changes In The Kidney 119

5.5. Evaluation Of The Protective Effect Of Spirulina Platensis Extract On Immune Dysfunction Induced By Afb_1 121

5.6. Investigation On The Probable Mechanism Of Action Of Protective Effect Of Spirulina Platensis Extract Against Afb_1 Induced Toxicity 125

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS 129

6.1. Introduction 129

6.2. Conclusions 129

6.3. Recommendations 130

6.4. Suggestions For Further Research 131

REFERENCES 132

APPENDICES 160

LIST OF TABLES

Table 2. 1: Kenyan aflatoxicosis reported cases - Ministry of Public Health and Sanitation, 2012......21

Table 2. 2: Normal reference ranges for biochemical parameters in Swiss albino mice (Serfilippi *et al*., 2003, Otto *et al*., 2016)......33

Table 2. 3: Toxicity of Spirulina......60

Table 2. 4: Summary of studies conducted using chemoprotective substances against aflatoxicosis using different species of mice and rats......63

Table 4. 1: The mean weight in grams of male Swiss albino mice during the experiment period......83

Table 4. 2: Post-hoc comparison of mean weight using Tukey's Honestly Significantly Differenced (HSD)......85

Table 4. 3: Biochemical changes in the serum of mice after 28 days exposure period. 87

Table 4. 4: Post-hoc comparison of ALT using Tukey's Honestly Significantly Differenced (HSD)......89

Table 4. 5: Post-hoc comparison of AST using Tukey's Honestly Significantly Differenced (HSD)......91

Table 4. 6: Post-hoc comparison of Alkaline phosphatase using Tukey's Honestly Significantly Differenced (HSD)......93

Table 4. 7: Semi-quantitative grading of liver tissues......96

Table 4. 8: Post-hoc comparison of semi-quantitative data of liver tissues using Dunnet's test......98

Table 4. 9: Semi-quantitative grading of kidney tissues......101

Table 4. 10: Post-hoc comparison of semi-quantitative data of kidney tissues using Dunnet's test 102

Table 4. 11: Mean values for IgG, IgM and IgA in mice 103

Table 4. 12: Post-hoc comparison of IgG mean values using Tukey's Honestly Significantly Differenced (HSD) 104

Table 4. 13: Post-hoc comparison of IgM mean values using Tukey's Honestly Significantly Differenced (HSD) 106

Table 4. 14: Post-hoc comparison of IgA mean values using Tukey's Honestly Significantly Differenced (HSD) 108

Table 4. 15: Mean concentration of MDA equivalents (μmol) in the liver and kidney samples...109

Table 4. 16: Post-hoc comparison using Tukey's Honestly Significantly Differenced (HSD) of the mean concentration of MDA equivalents (μmol) in the kidney samples 111

LIST OF FIGURES

Figure 2. 1: Geographical distribution of aflatoxins media reports across Kenya from 1960 to 2018 (adopted from Mutegi *et al*., 2018). 22

Figure 2. 2: Chemical structure of aflatoxins..25

Figure 3. 1: Flow diagram showing procedure for quantitative determination of IgG75

Figure 3. 2: Flow diagram showing procedure for quantitative determination of IgM and IgA. 76

LIST OF PLATES

Plate 4. 1: Photomicrographs of the liver tissues (X40; Hematoxylin and Eosin staining) 96

Plate 4. 2: Photomicrographs of kidney tissues (X400; Hematoxylin and Eosin staining) 99

LIST OF APPENDICES

Appendix I: Weight Monitoring Tool 160

Appendix II: Tool For Monitoring Health Status of Mice during the Study 161

Appendix III: Study approval by Directorate of Postgraduate Studies - MMUST ... 162

Appendix IV: Study approval by Institutional Ethics Review Committee (IERC) .. 163

Appendix V: Research authorization by NACOSTI 164

Appendix VI: Research authorization by Ministry of Interior & Co-ordination of National Government 166

Appendix VII: Research authorization by the State Department of Early Learning and Basic Education 167

Appendix VIII: Study approval by KEMRI Animal Use and Care committee 168

Appendix IX: Experimental Animals Request Form 169

Appendix X: A 96 well plate template used to record the layout of the experimental specimen before reading of absorbance using Multiskan GO® equipment 170

Appendix XI: Kemri Waste Tracking document 171

Appendix XII: Study Photographs 172

Appendix XIII: List of Study Publications 177

ACRONYMS AND ABBREVIATIONS

AF	Aflatoxins
AF-alb	Aflatoxin albumin
A. *flavu*s	*Aflatoxin flavus*
ALP	Alkaline phosphatase
A. parasiticus	*Aflatoxin parasiticus*
AFB1	Aflatoxin B_1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M_1
AFB-Lys	AFB1-lysine adducts
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
BW	Body Weight
CCP	Critical Control Point
CDC	Centre for Disease Control
CFR	Case fatality rate
CTMDR	Centre for Traditional Medicine and Drug Research
CPP	Critical Control Point
CYP	Cytochrome P450
DF	Degrees of freedom
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNP	Dinitro phenyl
DNPH	Dinitro phenyl hydrazine
DON	Deoxynivalenol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
EPA	The US Environmental Protection Agency
FAO	Food and Agriculture Organization
FDA	US Food and Drug Authority
FB1	Fumosin B1
GM	Genetically modified
GSTA	Glutathione S-transferase
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HRP	Horseradish peroxidase
HSCAS	Hydrated sodium calcium aluminosilicate
HSD	Honestly Significantly Differenced
H&E	Hematoxylin and eosin
γ-GT	Gamma glutamyl transferase
GIT	Gastrointestinal tract
GM	Genetically modified
GST	Glutathione S transferase
GSTs	Glutathione-S-transferases
IARC	International Agency for Research on Cancer
Ig	Immunoglobulin

IERC	Institutional Ethics Review Committee
KAIS	Kenya AIDS Indicator survey
KEBS	Kenya Bureau of Standards
KEMRI	Kenya Medical Research Institute
KCL	Potassium Chloride
L	Litre
LAB	Lactic acid bacteria
LD_{50}	Median lethal dose
LDH	Lactate dehydrogenase
LHP	Lipid hydroperoxides
M	Molar
MBAR	Millibar
MDA	Malondialdehyde
MMUST	Masinde Muliro University of Science and Technology
MS Excel	Microsoft Excel® 2013 software
NACOSTI	National Commission for Science, Technology and Innovation
NaOH	Sodium hydroxide
NEMA	National Environment Management Authority
NRF	National Research Fund
ng/ml	nanogram / millilitre
O_3	Ozone gas
OD	Optical density
OT	Ochratoxins
PCPB	Pest Control Products Board
pg/mg	picogram / milligram

pg/ml	picogram /millilitre
PPB	Parts per billion
PPM	Parts per million
ROS	Reactive Oxygen Species
SD	Standard deviation
SERU	Scientific Ethics and Review Unit
SDGs	Sustainable Development Goals
SGPT	Serum glutamate-pyruvate transaminase
SGOT	Serum glutamic oxaloacetic transaminase
SST	Serum Separator tube
SOD	Superoxide dismutase
SP	*Spirulina platensis*
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
µg/kg	Microgram per Kilogram
UN	United Nations
UV	Ultra violet
WFP	World Food Program
WHO	World Health Organization
ZEA	Zearalenone

OPERATIONAL TERMS

Aflatoxins: Aflatoxins are toxic secondary metabolites produced by certain Aspergillus species that can infect and thrive on various crops in the field and during storage. In this study, only aflatoxin B_1 was investigated.

Aflatoxin B_1 induced toxicities: In this study, biochemical changes, immunosuppression and histopathological changes on the liver and kidney caused by aflatoxin B_1 were investigated.

Spirulina Platensis: Extracted *Spirulina platensis* powder (MMUSTMUG SPIRULINA®) that was purchased from MMUST botanical garden shop.

Protective effects: Ameliorative effects of *Spirulina platensis* extract against biochemical changes, immunosuppression and histopathological changes on the liver and kidney caused by aflatoxin B_1.

ABSTRACT

Aflatoxins are toxic metabolites produced by Aspergillus species principally by *Aspergillus flavus and Aspergillus parasiticus*. Aflatoxins are teratogenic, mutagenic, carcinogenic, immunosuppressive and have been associated with various diseases conditions. Chemoprotective strategies are required to reduce both exposure to and the adverse health effects of aflatoxins, hence the basis for the present study. The main objective of this study was evaluation of protective effects of *Spirulina platensis* (SP) extract against aflatoxin B_1 (AFB_1) induced toxicities in male Swiss albino mice. These were evaluated by biochemical changes, histopathological changes, immune changes and probable mechanism of action. Randomly, 25 male Swiss albino mice were allocated into 5 groups. Group I (Control group); mice received normal diet. Group II mice received SP 100 mg/kg/day. Group III mice received 200 µg/kg/day of AFB_1. Group IV mice received 200 µg/kg/day of AFB_1 and SP 100 mg/kg/day. Group V mice received 200 µg/kg/day of AFB_1 and SP 100 mg/kg/day. The treatments were administered orally for 28 days. One-way ANOVA statistical test was used to compare group means. Data was statistically significant if ($P<0.05$). If statistically significant differences were found ($P<0.05$), post-hoc comparisons between multiple groups were done using Tukey's Honestly Significantly Differenced (HSD). Python® 3.0 with statistical libraries data analysis software was used. Results showed that compared to group I (control), group III (200 µg/Kg/day AFB_1) had increased levels of alanine aminotransaminase (ALT); (44.0±6.83 IU/L vs. 61.0±8.19 IU/L; p=0.054) and aspartate aminotransferase (AST (176.75±44.34 IU/L vs. 256±115.99 IU/L; p=0.0195). Mice that were co-treated with 200 µg/Kg/day of AFB_1 and 200 mg/Kg/day of SP extract exhibited lower levels compared to mice treated with only 200 mg/Kg/day of AFB_1; ALT (49.8±7.9 IU/L vs. 61.5±8.19 IU/L; p=0.039) and AST (229.8±95 IU/L vs. 256±11.15 IU/L; p=0.04819). These findings were furthered by histology photomicrographs of liver and kidney tissues samples. With regard to the immune changes, comparison of group I (control) with group III; IgA reduced (AFB_1 200 µg/Kg/day) (0.7147 ± 0.001 vs. 0.7075 ± 0.010); IgM levels were also reduced (0.0916 ± 0.003 vs. 0.0866 ± 0.019); IgG levels were elevated (0.1746 ± 0.001 vs. 0.2808 ± 0.243). Administration of AFB_1 200 µg/Kg/day followed by supplementation of *S. platensis* extract 200 mg/Kg/day as seen in group V compared to group III (AFB_1 200 µg/Kg/day) reversed depression of IgA levels (0.7124 ± 0.005 vs. 0.7075 ± 0.010; P=0.05437); IgM (0.1005 ± 0.004 vs. 0.0866 ± 0.019; P=0.0178); IgG levels were reduced (0.1749±0.001 vs. 0.2808± 0.243; P=0.0155). In regard to MDA equivalents, co-administration of (AFB_1 200 µg/Kg/day + SP 100 mg/Kg/day) reduced the the mean concentration of MDA equivalents (µmol) in the liver and kidney compared to group III (AFB_1 200 µg/Kg/day). Also, co-administration of (AFB_1 200 µg/Kg/day + SP 200 mg/Kg/day) further reduced the the mean concentration of MDA equivalents (µmol) in the liver and kidney compared to group III (AFB_1 200 µg/Kg/day). In conclusion, co-treatment of *S. platensis* extract in doses ranging from 100 mg/Kg/day to 200 mg/Kg/day inhibits biochemical changes, immune changes, histopathological changes in liver and kidney and it lowers MDA equivalent concentration caused by 200 µg/Kg/day of AFB_1 in male Swiss albino mice. This study recommends clinical trials on *Spirulina platensis* to evaluate its protective effect against aflatoxin induced toxicity in human beings.

CHAPTER ONE

INTRODUCTION

1.1. Background information

Aspergillus species primarily *A. parasiticus* and *A. flavus* produce aflatoxins as secondary metabolic products. *Aspergillus flavus* produces aflatoxin B_1 (AFB_1). Aflatoxin B_1 is more active biologically than all the other known aflatoxins (Ghada *et al.,* 2012; Oskoueian, 2015; Mutegi *et al*., 2018).

Aflatoxins (AF) commonly occur in regions that lie between latitudes 40ºS and 40ºN of the equator (WHO 2005 and the U.S. Centers for Disease Control and Prevention, 2005). Aflatoxins frequently contaminate foodstuffs in hot (24^0C to 35^0C) and humid climatic conditions of above 7% and 10%; without and with ventilation respectively. Insect infestation is one of the risk factors that has been identified (Williams *et al.,* 2004, Gugliandolo *et al*., 2020; Alperen *et al*., 2021). Fungal invasion often begins before harvest time. Secondly, poor crop harvesting and storage practices also lead to this contamination (Bbosa, 2013; Yaman *et al.,* 2016, Gugliandolo *et al*., 2020).

Aflatoxins contamination is a perennial challenge in both developed and undeveloped countries. The main foodstuffs likely to be contaminated by aflatoxins are pistachio nuts, peanuts, cottonseed, maize and spices among others (Carvajal, 2015; Lijuan *et al.,* 2016). Foods obtained from animals are also significantly affected (Milad, 2011; Abeer, 2015; Carvajal., 2015).

Estimations have shown that about a quarter of the world's animal and human food crops contain aflatoxins (Ellen *et al*., 2013; Abeer, 2015; Carvajal, 2015). Four (4) billion people in poor nations are estimated to be vulnerable to aflatoxins (Carvajal, 2015; Obade *et al.,* 2015; Sriwattanapong *et al*., 2017). About 50% of Sub-Saharan

Africa, population is exposed to aflatoxins (Kew, 2013). The US Food and Drug Administration (FDA) recognizes mycotoxins to be inevitable food impurities. Subsequently, its main objective is to ensure reduction of aflatoxins contamination (Williams *et al*., 2004).

Furthermore, there is inadequate enforcement of aflatoxins regulatory limits in many developing countries thus exposing a high population to their harmful health effects (Farombi *et al.,* 2005; Ellen *et al*., 2013, Yaman *et al.,* 2016). Aflatoxins regulatory levels ranging from 4 to 30 ppm have been set on human foods depending on the country of regulation (MMWR, 2004). In the European Union substantially lower limits of 4.0 ppb and 2.0 ppb for total aflatoxins and AFB_1 respectively have been set (Mishra and Chitrangada, 2003; Lijuan *et al.,* 2016).

Aflatoxin exposure remains a significant hazard to both man and animals' health. Ingestion of meals contaminated by aflatoxins has been documented to cause: immune dysfunction, hepatotoxicity, teratogenicity, mutagenicity, growth retardation and hepatic cancers (Verma, 2001; Oskoueian, 2015; Yaman, *et al.,*2016).

Hepatitis B and C and AFB_1 have been linked with hepatocellular carcinoma, which has a poor 5-year survival prognosis. It accounts for about 15% of all cancer deaths worldwide (Farombi, 2005; Costa *et al.,* 2007; Maryam, 2015). In addition to the above, AFB_1 causes immunosuppression, changes haematological profiles, gastrointestinal morphology and internal organ damages in many animals. Furthermore, prevalence of endemic diseases in Africa, Asia and Europe; inclusive of Reye's syndrome and kwashiorkor have been attributed to aflatoxins (Lijuan, 2016).

It has been well established that aflatoxins toxicity primarily targets the liver. AFB_1 toxicity varies depending on the age, duration of exposure, nutritional status, extent and animal species (Carvajal, 2015; Miao, 2016; Lijuan, 2016). Since complete elimination of aflatoxins contamination is not possible, chemo preventive strategies are important to mitigate their adverse effects. Extensive studies aimed at mitigating adverse effects of aflatoxins have been done using nutritional, biological, physical and chemical binders to mitigate the effects of aflatoxins. However, each of these methods have their merits and demerits that limit their large-scale application.

Spirulina platensis (SP) exists as blue green algae found in freshwater. It contains vital amino acids, vitamins, minerals and important fatty acids. It contains carotenoids that gives it significant antioxidant properties (Arpita *et al.,* 2014; Mona, 2015). For numerous years it has been used in food supplementation. Animal studies have demonstrated potential ameliorative effects of spirulina against toxic agents, for instance against: nephrotoxicity, hepatotoxicity and cardiotoxicity (Arpita *et al.,* 2014). Globally, there is renewed interest in *Spirulina* species that has been described as “a wonder drug” (Makhuvele *et al*., 2020; Dinicolantonio *et al.*, 2020).

Conclusively, chemoprevention methods that employ effective techniques and the use of conventional drugs in combating aflatoxin menace have however been faced with several challenges. The demand for new agents, preferably with diverse mechanism of action are therefore required. In these circumstances, several natural products used traditionally for reversing the effects of aflatoxins have been evaluated for anti-aflatoxin activity. Supported by National Research Fund (NRF) and Kenya Medical Research Institute (KEMRI) internal grants; this study has evaluated the efficacy of *Spirulina platensis* plant used traditionally for treatment of liver and kidney diseases.

There is renewed emphasis on the natural product extract as an active principle because of the synergistic or additive effects between various classes of compounds (Makhuvele *et al*., 2020; Dinicolantonio *et al.*, 2020; Moneera *et al*., 2021) . Consequently, *Spirulina platensis* extract can be considered as botanical drug possessing pharmacological effects against aflatoxins. Therefore, it could be formulated as phytomedicine for further evaluation in clinical trials. Economically sustainable newer approaches as food additives are essential to minimise AFB_1 adverse effects on man and animals.

1.2. Problem Statement

It is highly desirable that feeds and food for both animal and human consumption should not contain aflatoxins. However, chronic intake of aflatoxin-contaminated food is a worldwide challenge especially in sub-Saharan Africa. Several countries have introduced recommended aflatoxin maximum limits for food for human consumption. The limits range between 1 μg/kg to 30 μg/kg. Twenty (20) parts per billion (ppb) aflatoxin concentration limit has been adopted in Kenya from the United States of America (Mishra and Chitrangada, 2003; MMWR, 2004; Alakonya *et al.,* 2009).

However, it is well documented that aflatoxins in available food and feeds often exceed the recommended regulatory standards. The variability and actual range in aflatoxin levels in human food in resource limited countries is hard to quantify due to lack of routine and standardised aflatoxin testing. Furthermore, even in cases where the maximum aflatoxin levels are met; daily intake of 1 μg/kg to 5 μg/kg is likely to accumulate in the body causing adverse effects of aflatoxin (Alakonya *et al.,* 2009).

Several mitigation measures to minimise aflatoxin adverse effects have been evaluated. Despite of these, each of these measures have their limitations: unsustainability, adverse effects on nutritional value of food, adverse reactions of chemical methods and some are environmentally unsustainable.

Spirulina platensis is rich in nutritional value and antioxidants; its production is sustainable and environmentally friendly. This study was conducted to evaluate *Spirulina platensis* as a potential alternative natural product to ameliorate against AFB_1 induced toxicities.

1.3. Study objectives

1.3.1. Broad objective

This study sought to evaluate protective effects of *Spirulina platensis* extract against AFB_1 induced toxicities in male Swiss albino mice.

1.3.2. Specific objectives

In this study the following were the specific objectives:

i. To evaluate the protective effect of *Spirulina platensis* extract on biochemical changes induced by AFB_1 in male Swiss albino mice.

ii. To assess the protective effect of *Spirulina platensis* extract on histopathological changes induced by AFB_1 on the kidney and liver of male Swiss albino mice.

iii. To investigate the protective effect of *Spirulina platensis* extract against AFB_1 induced immune dysfunction in male Swiss albino mice.

iv. To investigate the probable mechanism of action of protective effect of *Spirulina platensis* extract against AFB_1 induced toxicity in male Swiss albino mice.

1.4. Research questions

i. Does *Spirulina platensis* extract have protective effects on biochemical changes induced by AFB_1 in male Swiss albino mice?
ii. Does *Spirulina platensis* extract have protective effects on histopathological changes induced by AFB_1 on the kidney and liver of male Swiss albino mice?
iii. Does *Spirulina platensis* extract have protective effects AFB_1 induced immune dysfunction in male Swiss albino mice?
iv. What are the probable mechanisms of action of protective effect *Spirulina platensis* extract against AFB_1 induced toxicity in male Swiss albino mice?

1.5. Justification

Aflatoxin contamination of foodstuffs is a common challenge worldwide. It is well documented that aflatoxin contaminates various foodstuffs in Sub-Saharan Africa including Kenya. The adverse effects of consuming aflatoxin contaminated foodstuffs are well known. In 1993, World Health Organization (WHO) classified aflatoxin as Class 1 carcinogen (Bbosa, 2013).

Several studies have been done on aflatoxin mitigation using various substances to mitigate the effects of aflatoxins. Some of these substances includes: quercetin, sylimarin, phenolics, rosmarinic acid, catechins, carnosic acid, thymol and hesperidin have been discovered to possess significant cell protective effects. Nonetheless, none

of these substances has been processed and marketed as AFB_1 cell protective product due to their inadequate supply (Oskoueian *et al.,* 2015).

Presently, there is no antidote for the AFB_1 toxicity management. Subsequently, easily available and sustainable plant sources should be considered as suitable alternative sources. Hence the basis for this study assessing *Spirulina platensis'* extract protective effects against AFB_1 induced toxicities.

Lastly, it is morally and ethically unacceptable to carry out exploratory investigative toxicological studies in human subjects using well established hepatotoxic and carcinogenic agents. Consequently, the basis for the current study using experimental mice animal models. The study findings might then be extrapolated to humans.

1.6. Significance of the Study

Evidence-based data on the ameliorative effects of *S. platensis* against aflatoxin B_1 induced hepatotoxicity, renal toxicity, immune suppression and its probable mechanism of action in mice has been generated from this study. Subsequently, extrapolation of this study findings to humans is important.

Secondly, this study fits into the food security issue of the Big Four agenda of the economic blueprint of Kenya. Moreover, this study lies within the health sector of the social pillar of Kenya's Vision 2030. Furthermore, this study falls under goal number 2 (Zero Hunger) of the United Nations' (UN), Sustainable Development Goals (SDGs).

In addition to these, peer-reviewed scientific publications have been generated from this work. The capacity for scientific research on medicinal plants has been enhanced.

Significant information to policy makers, regulatory bodies and other relevant stakeholders in the veterinary and human healthcare industries has been documented.

Lastly, identification and recognition of *S. platensis* as locally available AFB_1 toxicities modulators that can be supplemented in both human food and animal feeds at minimal cost is important. This is attractive and relevant in poor resource countries where the majority of the population can use is as a prophylactic agent. Eventual commercialization of *Spirulina platensis* in mitigation against toxic effects of aflatoxins has been enhanced.

1.7. Scope of the study

Spirulina platensis extract's protective effect against biochemical changes, histopathological changes and immune changes induced by AFB_1 were evaluated. The study also, investigated *Spirulina platensis* extract's probable mechanism of action against AFB_1 induced toxicities.

1.8. Study Limitations

These findings may not be applicable to all mycotoxins in natural existence. Secondly, this study did not assess the impact of toxicological interactions of AFB_1 with other potential contaminants like heavy metals, other mycotoxins and pesticides (herbicides, insecticides and fungicides) that are likely to occur concurrently. Thirdly, this study was restricted to evaluation of *Spirulina platensis* extract's protective effects against AFB_1 induced toxicities. Thus, the study findings may not be extrapolated to other pharmacological effects of this plant species.

In addition to these, only two dosing levels of *Spirulina platensis* (100 mg/Kg/day and 200 mg/Kg/day) were evaluated. Lastly, renal function tests were not carried out; the

study relied on histopathological findings as basis for protective effect of *Spirulina platensis* on histopathological changes induced by AFB_1 on liver and kidney. The microscope used for histopathological evaluation did not have provision for insertion of scale bars.

1.9. Study delimitations

This study was delimited to the evaluation of biochemical changes, histopathological changes, immune effects and probable mechanism of action of *S. platensis* extract against AFB_1 activated toxicities in male Swiss albino mice. Secondly, the study was delimited to an experimental animal model carried out for 28 days. Thirdly, the study was delimited to the evaluation of ameliorative effects of *Spirulina platensis*. Data analysis was delimited to One way ANOVA statistical test that was used in comparison group means.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

This chapter covers literature review relevant to aflatoxins' impact of on livelihood vulnerability in Kenya and its global burden. A review of previous similar studies and their findings on impact of aflatoxins is presented below.

2.2. History of aflatoxins

Aflatoxins are fungal secondary metabolic substances associated with contaminated food products. Ingestion of aflatoxin contaminated food leads to serious health effects and even death. Gradual deterioration of health has been observed after long periods of exposure to aflatoxins. Once it contaminates feeds and foodstuffs for both animal and human consumption, it causes aflatoxicosis.

The term "aflatoxin" is derived from three (3) words (Lijuan *et al.,* 2016). The "a" stands for the *Aspergillus* genus; the "fla" stands for the species flavus; and the "toxin" stands for the poison. Aflatoxins thrive well in dry, hot climatic conditions; (+/- 30 to 40^{0}C). Prevalence of aflatoxins is promoted by pests, drought and poor harvesting techniques. Since aflatoxins discovery, more than six decades have passed (Pickova *et al*., 2021).

In the spring and summer of 1960 about 100,000 turkey poultry deaths were reported in South East England (John, 2008; Noreddine 2019; Pickova *et al*., 2021). The main characteristics of this disease that were reported included: poor appetite, weakness of wings, acute hepatic necrosis with bile hyperplasia, lethargy, altered neck and head posture at the time of death. The disease was later named ''turkey X disease'' (Mishra and Chitrangada, 2003; Bennett and Klich, 2003; John, 2008).

Subsequently, aflatoxins were identified and confirmed to cause ‘‘turkey X disease’’**.** A toxic factor was later isolated in Brazilian groundnuts that were found to be contaminated by *Aspergillus flavus* strain metabolites. Aflatoxins were also associated with cancer in rainbow trout following their intake of feeds consisting of cottonseed meals and peanuts (Williams *et al*., 2004; Noreddine, 2019). In addition to these, aflatoxins were subsequently identified and confirmed to be present in cottonseed meal and maize (Williams *et al*., 2004; Bbosa, 2013; Pickova *et al*., 2021).

Interestingly, 1960 to 1975 have been named “mycotoxin gold rush years” due to numerous scientists who participated in aflatoxins related studies that were well funded (Bennett and Klich, 2003).

Four (4) aflatoxin forms: B1, B2, G1, and G2 have been identified in plant-based food. In animal-based food; M1 (metabolic product of AFB_1) and M2 have been confirmed (Hamid *et al*., 2013; Mutegi *et al*., 2018). Aflatoxin B_1 has been linked with hepatocellular carcinoma; it’s the most harmful aflatoxin form. Visual detection of aflatoxins is difficult (Hamid *et al*., 2013; Mutegi *et al*., 2018).

2.3. Geographical distribution

Aflatoxins occur between equator latitudes of 40° S and 40° N. However, poor countries in these areas are of the main public health concern. Their staple foods consist mainly of the foods contaminated by aflatoxins (Eva *et al*., 2011). Above all, because of poor food security systems in many developing countries; a large population is at risk of consuming aflatoxin contaminated food even after knowing it may be contaminated. This is due to lack of alternative foods (Williams, 2004).

It is well established that AFB_1 is found in Africa and Southeast Asia (Hamid *et al*., 2013). These regions have favourable climatic conditions especially temperature above 28^0 C and high humidity that promotes rapid growth of mycotoxin producing species primarily *A. flavus* and *A. parasiticus* that are the principal AFB_1 sources (Gong *et al*., 2004; Hamid *et al*., 2013; Mutegi *et al*., 2018).

2.4. Aflatoxin contaminations

2.4.1. Aflatoxins occurrence in animal feeds and human food

Aflatoxins contaminate food and feeds: in the fields, when processing food and during its storage. Numerous studies have confirmed their unpredictable concentrations (Gordon, 2008; Bbosa *et al.,* 2013). They have great seasonal variations. They also exhibit differences in different growing geographical locations. Variations associated with storage conditions have been documented (Gordon, 2008; Bbosa *et al.,* 2013; Nduati *et al*., 2017).

Contamination by aflatoxins affects various cereals including: maize, rice, sorghum, oats and barley. Secondly, oilseeds affected comprises: cottonseed, peanuts, nuts, almonds, pistachios, hazelnuts, coconut and cacao. Thirdly, dry fruits affected are: dates, raisins and figs. Lastly, spices that are contaminated by aflatoxins include: hot pepper, cumin and black pepper. These primarily occur before, during and after harvest (Carvajal, 2015; Obade, 2015; Maryam, 2015).

It has been estimated that globally about 25% of grain production contains aflatoxins, hence signifying the prevalence of this challenge. Drought-stressed field conditions promote formation of aflatoxins. (Carvajal, 2015; Maryam 2015). Some of these foods are the staple diets and chief cash crops especially in poor resource countries (Kew, 2013; Craig, 2013). Overall, in sub-Saharan Africa consumption of maize

remains to be the primary aflatoxin exposure source (Gershim, *et al*., 2014; Lijuan *et al*., 2016; Nduati *et al*., 2017).

Aflatoxins also contaminate milk, cheese, eggs, and meat products. This occurs when animals consume aflatoxin contaminated feeds (Dhanasekaran *et al.,* 2011; Kew, 2013). Lastly, aflatoxin contamination may occur in populations having poor food drying, storage capacity and absence of regular routine monitoring (Kew, 2013).

2.4.2 Aflatoxin Limits

Maximum levels of AFB_1 and total aflatoxins (inclusive of AFB_1, AFB_2, AFG_1 and AFG_2) are the indicators presently used to minimise aflatoxins in most animal feed and human foods (Carvajal, 2015; Lijuan *et al.,* 2016). The maximum levels for total aflatoxins and AFB_1 in food varies between 10 and 5 mg/kg respectively in about 75 countries (Lijuan *et al.,* 2016). Human foods are allowed aflatoxin contamination levels of 4 to 30 ppm depending on the country of regulation (MMWR, 2004). Moreover, there is variation in these regulations determined by whether the country setting the limits exports or imports the commodities (Carvajal, 2015).

The European Economic Community (EEC) has set markedly lower levels for aflatoxins in food of 4.0 and 2.0 ppb for total aflatoxins and AFB_1 respectively (Mishra and Chitrangada, 2003). A standard for total aflatoxins in animal feed of 15 to 20 mg/kg has been set by FDA (Lijuan et al*.,* 2016). Thus, it is clear from the foregoing that economic pressures have created double standards for human food and animal feed (Williams *et al.,* 2004; Lijuan *et al.,* 2016).

In China, the maximal limit for AFB_1 has been set between 5 mg/kg to 20 mg/kg (Lijuan *et al*., 2016). In Korea, 10 mg/kg has been set as the maximal aflatoxin level

for AFB_1 in all human food. In the Netherlands, Switzerland and USA the AFB_1 limits ranges from 1 mg/kg to 20 mg/kg (Lijuan *et al.*, 2016).

In Kenya, total aflatoxin was previously set at 20 parts per billion (ppb), a limit adopted from the United States of America (Mishra and Chitrangada, 2003; MMWR, 2004; Alakonya *et al*., 2009). However, in 2009, total aflatoxin limit in human foodstuff was reduced to 10 µg/kg. Kenya Bureau of Standards (KEBS) is mandated to verify and confirm food quality (Chebon *et al.,* 2016, Sirma *et al*., 2016). This corresponds to the standards set by the World Food Program (WFP) of 1 mg/kg (1 ppm) for fumonisin at and 10 µg/kg (10 ppb) for Aflatoxins (Mutiga *et al.,* 2015). In addition to these, in Kenya, AFB_1 levels have been limited up to 5 ppb (Sirma et al., 2016).

Despite these regulations, studies on aflatoxin food contamination in Africa in the last 2 decades have revealed high aflatoxin levels of 355 ppb and 500 ppb (Knipstein, 2015). The high costs incurred in aflatoxin analysis have limited testing for aflatoxin among maize stakeholders (Chebon *et al.,* 2016). Secondly, these regulations may not be applicable to many developing countries where consumption of commercially produced foods is low. Many families rely on their home grown and stored foods that are rarely analysed for aflatoxin because it's economically unsustainable and inaccessible (Williams, 2004).

In conclusion, although legislation remains to be an effective tool in the developed nations, the situation among rural communities in resource limited countries remains more complex and often not addressed. Therefore, risk assessment may be a key tool in aflatoxin exposure in such resource limited countries (Gordon, 2008).

2.4.3. Global impact of aflatoxins

Aflatoxin remains a considerable global food safety and public health problem as pinpointed (Gong *et. al*., 2016). Globally, about 5 billion people residing in poor countries remain vulnerable to chronic aflatoxin toxicity (Deabes *et al*., 2012; Sriwattanapong *et al*., 2017; Kaiming, 2020). Secondly, for numerous years it has been documented that about 25% of farm produce are contaminated by aflatoxins (Abeer, 2015; Maryam, 2015; Carvajal, 2015). However, this has been challenged. It has not been possible to trace back the authenticity of the statement even by Food and Agriculture Organization (FAO) experts (Pickova *et al*., 2021). Overall, many reports pinpoint that grains often exceed the set standard of 20 µg/kg in feeds worldwide.

An aflatoxicosis outbreak in poultry was documented in India in 1968. In 1974, an estimated 106 people died of an aflatoxin outbreak in two (2) Indian states of Rajasthan and Gujrat. The two-month outbreak was mainly limited to households' predominantly consuming maize as their staple food. It was confirmed after analysis to be caused by *A. flavus* (Kumar *et al*., 2017). In addition to these, another outbreak affecting dogs and humans was reported in 1974 in north-west India (Kumar *et al*., 2017).

Aflatoxin contaminated milk was reported in 2013 in Romania, Serbia and Croatia (Kumar *et al*., 2017). They were also detected in breast milk and food used for weaning indicating that exposure of human infants to aflatoxins may start during breast feeding and it may continue throughout one's lifetime in some African countries (Gordon, 2008).

Use of contaminated animal feed results in production of milk contaminated with aflatoxins M_1 and M_2. Aflatoxin M_1 (a marker of AFB_1 intake) and AFB_1 have been documented in Nigerian and Ghanian breast milk samples (Obade *et al.,* 2015).

Detectable levels of urine aflatoxin or serum aflatoxin albumin adduct were reported in 85% of children in studies done in Kenya (Obade *et al.,* 2015).

Aflatoxins have been reported in blood obtained from the umbilical cord at birth (Gordon 2008). Average daily dietary aflatoxin exposure in developed countries has been estimated to be under 1 ng/kg body weight (bw). This is in contrast to estimations in sub-Saharan African nations where it is more than 100 ng/kg bw per day. It is crucial to note that inadequate data was used in the calculation of these estimates (WHO, 2018). Furthermore, it has been estimated that in countries having high dietary aflatoxin intake; individuals have aflatoxin daily exposure of as high as 1.7 µg/kg body weight. However, this estimate may be more than 1 mg/day during the year (Faridha *et al*., 2006).

Even though mycotoxins remain a universal concern, their noxious effect on social, economic and health life is more pronounced in poor nations within the tropics. In developing countries, rural subsistence farming population has been identified to be at higher risk of mycotoxin exposure (Gong *et al*., 2016). Agricultural products in Kenya, Gambia, Uganda, Tanzania and South Eastern Asia nations like Vietnam, Thailand, Indonesia and China have been attributed to the high incidence of aflatoxins. This is due to inadequate quality control and poor food storage practices (Sriwattanapong *et al*., 2017).

Moreover, this runs parallel to the high incidence of hepatocellular carcinoma and acute aflatoxicosis in these regions (Noreddine, 2020). Consequently, these nations have remained to be the chief destination for investigators studying epidemiological association between exposure to aflatoxins and hepatic cancer. Subsequently, this has led to the identification of aflatoxins as a chief hepatic cancer cause in animals and humans (Noreddine, 2020).

2.4.4. Prevalence of aflatoxins in Kenya

There is limited data on aflatoxin prevalence in Kenya (Mutegi *et al*., 2018). Pioneer investigations revealed a high proportion of contamination of aflatoxin in the traditional brews and main meals among families in Murang'a District, Nyeri Province. Millet, maize, yams, pigeon peas and sorghum consisted of the meals (Mutegi *et al*., 2018).

Numerous 21st century studies have confirmed aflatoxin presence in various sampled foodstuffs. Of interest among these studies is the alarmingly high number of food commodities which exceeded the set AF limit for total AF and $AFB_{1;}$ 10 and 5 μg/kg respectively (Mutegi *et al*., 2018).

Kenya has documented about 500 acute AF cases and 200 AF related deaths (Omara *et al*., 2021). These include documented epidemics of fatally acute aflatoxicosis from 1960 to 2008; Table 2.1 (CDC, 2004; MWWR, 2004; Chebon *et al.,* 2016; Mutegi et al 2018).

In 1981, the first aflatoxicosis outbreak was documented. A Case fatality rate (CFR) of 60% was reported on 20 cases in Makueni district, Eastern Province. Most affected were patients aggregated in family households that consumed aflatoxin contaminated maize meals (1,600–12,000 ppb) (MWWR, 2004; Lewis *et al*, 2005; Ellen *et al*., 2013).

Overall, maize remains the staple Kenyan food (Lewis *et al*., 2005). In Southern and East Africa, consumption per capita annually of maize has been estimated to be 76 kg and 97 kg respectively (Kangethe *et al*., 2017). Furthermore, the aflatoxin maize poisoning is worsened by lack of dietary diversity. In addition to these, consumption of maize diets in large quantities is estimated to be about 400–500 g of maize meal

daily per adult person (Kiama *et al*., 2016; Omara *et al*., 2021). Moreover, multi-year Kenyan studies have revealed high incidences of AF contaminated maize (Mutegi *et al*., 2018).

A descriptive epidemiological investigation done in Eastern Kenya in 2004 reported an AF outbreak with one of the highest numbers of casualties ever reported. Three hundred and seventeen (317) acute hepatic failure cases were identified between January to June 2004. One hundred and twenty-five (125) persons subsequently died during the illness (Obura, 2013, Qian *et al*., 2013; Kaiming, 2020). Majority of the outbreak reported cases were in the Eastern Province following home grown maize consumption (MWWR, 2004).

Analysis of maize samples obtained from Eastern province revealed high aflatoxin levels of 4,400 ppb, which is more than 200 times above set limit of the 20-ppb (Azziz *et al.,* 2005; Kangethe *et al.,* 2009). Furthermore, about 40% of Kenyan meals contain maize and maize products in both rural and urban population (Mwihia *et al*., 2008, Mutegi *et al*., 2018). During this outbreak AFB_1 was identified to be the most commonly found type in Kenya (MWWR 2004; Azziz *et al*., 2005). Moreover, an aflatoxin exposure biomarker; aflatoxin albumin adducts (AF-alb) levels were found to be approximately 67,000 pg/mg albumin, the highest ever reported (Gong *et al*., 2012).

Other minor aflatoxin outbreaks in Eastern province were reported in 2005 and 2006 associated with the death of thirty (30) and nine (9) people respectively (Muthomi *et al.,* 2009). During the 2010 Kenyan outbreak levels; of serum AFB_1 were among the highest ever documented globally (Gong *et al*., 2012).

A South Nyanza survey revealed peanuts grown in the area; had more than 350 times above the accepted aflatoxin levels regulatory limit (Obade *et al*., 2015). Animal feed

study done in Nairobi, Kenya, revealed that of the 72 samples collected, only 5% met the set AF level limit of 10 ppb; aflatoxin concentrations were found to be between 5.13 to 1123 ppb. (Obade *et al.,* 2015).

A cross-sectional survey conducted in 2007 revealed widespread aflatoxin exposure across Kenya (Yard, 2013). However, studies have reported mean aflatoxin levels of 37 ppb and 54 ppb in maize sampled from Homa Bay and Rongo respectively. This can be compared to 44 ppb, 25 ppb and 21 ppb, in Mbooni East, Mbeere North and Makueni respectively (Obade *et al.,* 2015). Therefore, this poses significant adverse health effects on consumers of such contaminated maize.

Aflatoxins in Kenya have been found in maize and maize based commodities (uji, ugali, irio, muthokoi, busaa, githeri, chang'aa); sorghum, peanuts, rice, cassava, yams, millet, beers, dairy, herbal products, dried fish and animal feeds (Omara *et al*., 2021). Currently, Kenyan maize millers are required to carry out aflatoxin testing on incoming consignments before milling. This is a Critical Control Point (CCP) in the maize milling process (Nduati *et al*., 2017).
In addition to the above, population studies have revealed presence of aflatoxins in Kenya even in periods of absence of an active outbreak. In 2011 CDC conducted AFB_1- lysine data analysis using stored serum samples obtained from Kenya Aids Indicator Survey (KAIS), population survey. It demonstrated that there was a high prevalence of aflatoxin exposure throughout Kenya, about 80% of KAIS participants had detectable levels (Obura, 2013).

Consequently, population survey findings reported that a large population was exposed to aflatoxicosis in Coastal, Eastern, Nairobi and Central Provinces.

Furthermore, the population survey identified children under 15 years to be the most vulnerable (Obura, 2013).

Alarming data of 100% exposure among expectant and breastfeeding mothers residing in Eastern Kenya has been documented. Aflatoxin exposure begins during breastfeeding as confirmed by large number of breastfeeding women who had a positive test for aflatoxin M_1 (Mutegi *et al*., 2018). A study performed among children of Kisumu County demonstrated an association between aflatoxin levels in maize-based diet and child wasting (Adhiambo and Ohingo, 2004). However, the study (Adhiambo and Ohingo, 2004) did not confirm whether child wasting was due to aflatoxin direct effect or due to inadequate calories intake (Kiama *et al*., 2016; Kiarie *et al*., 2016; Mutegi *et al*., 2018).

Aflatoxicosis in Kenya and India often involves staple foods containing aflatoxin levels of up to 5000 ppb and above which have been associated with fatality. Secondly, daily ingestion of diets containing aflatoxin levels of 1000 ppb have been reported to cause aflatoxicosis. The cumulative daily aflatoxin intake that has been identified to cause fatality has been estimated in adults to be more than 1 mg/day or above 20 µg/kg bw. Although the time from aflatoxin ingestion to clinical aflatoxicosis with or without mortality is difficult to determine, it has been estimated to vary between 1 to 3 weeks (Christopher *et al*., 2010).

Sampled milk from different rural and urban areas of Kenya revealed high aflatoxin M_1 levels. It has been documented in both ultra-heat-treated and pasteurised milk. More than 40% of sampled unprocessed milk had Aflatoxin M_1 levels of more than 0.05 µg/kg. Consequently, these aflatoxin levels may be associated with prevalence of

high aflatoxin levels in the animal feed in the country. Furthermore, grains that visually appears contaminated are frequently given to animals due to limited disposal options and ignorance (Mutegi *et al*., 2018; Lalah *et al*., 2019).

Table 2. 1: Kenyan aflatoxicosis reported cases - Ministry of Public Health and Sanitation, 2012

(adopted from Mutegi *et al*., 2018).

Occurrence Year	Occurrence documented
1960	16,000 ducklings died in Rift Valley Province farms after being fed of groundnut contaminated by aflatoxins
1977	Many dogs and chicken died in Nairobi, Eldoret and Mombasa due to poor storage of their feeds.
1984-1985	AF contaminated maize that was imported caused death of many poultry
1998	3 people died in Meru after consumption of contaminated maize
2001	26 people in Maua had hepatotoxicity due to consumption of AF contaminated maize
2003	6 people died in Thika after eating AF contaminated maize
2004	331 people had different grades of aflatoxicosis after eating AF contaminated meals. 125 people died in Machakos, Makueni and Kitui
2006	19 people died in Makueni due to consumption of AF contaminated maize
2007	2 people died in Makindu
2008	Two people died in Kibwezi. Three people were admitted in Mutomo

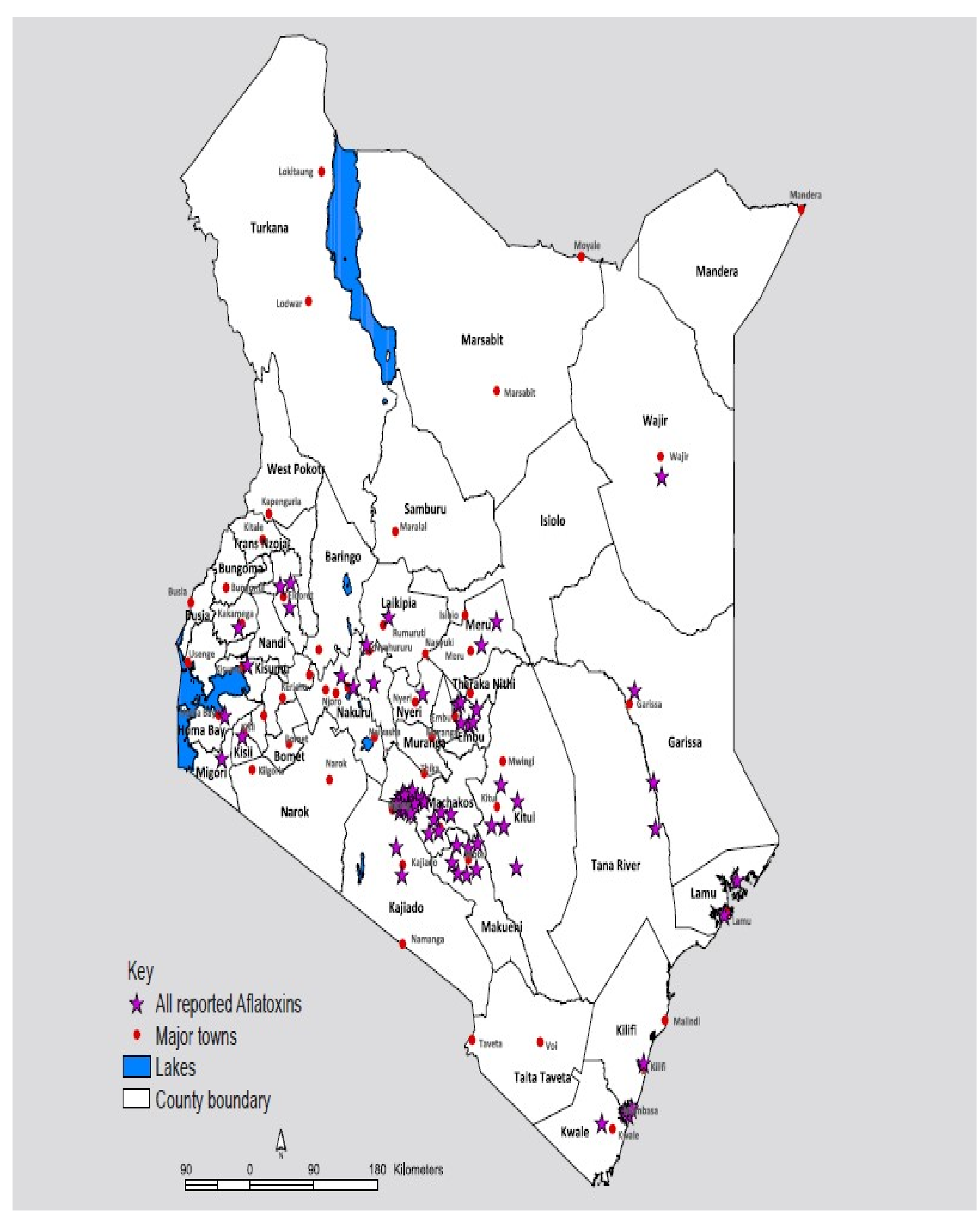


Figure 2. 1: Geographical distribution of aflatoxins media reports across Kenya from 1960 to 2018 (adopted from Mutegi *et al*., 2018).

Documented data sources reveal long periods of information gaps; there are no unique factors to signify lack of aflatoxin contamination. It has been suggested that human activities and environmental factors show likelihood of increased levels of aflatoxin contamination over these periods of information gaps.

Moreover, there are chances of misreporting since the symptoms of aflatoxicosis which include: kidney failure, hepatotoxicity, oedema and jaundice (Sharma *et al*., 2018) mimic those manifested by other diseases.

In addition to these, there are chances of under-reporting and misdiagnosis when the cases are isolated, hence inadequate to be reported as an epidemic (WHO and CDC, 2005; Mutegi *et al*., 2018). From the foregoing, it is apparent that prevalence of aflatoxin contamination is high in both human food and animal feeds in Kenya.

In conclusion, like many nations, Kenya lacks a regular national aflatoxin monitoring system. Subsequently, information on aflatoxin burden across various geographical regions and populations is scarce. Consequently, it was difficult to determine the total aflatoxicosis attributable to aflatoxins (Yard, 2013). Overall, caution is recommended when drawing conclusions about comparisons among countries with regard to the levels of aflatoxin exposure. This is because of limited data from the studies performed, incomparability of study designs and the high likelihood of sporadic seasonal findings (Gershim *et al*., 2014).

2.5. Aflatoxin Chemistry

Aflatoxins are heterocyclic products formed by *Aspergillus* species. *Aspergillus parasiticus* and *Aspergillus flavus* are the main sources of aflatoxin that contaminate agricultural commodities (Milad, 2011; Bbosa, 2013). There are over 20 aflatoxin derivatives produced by different fungi species.

It has also been found that some *A. tamarii starins* and *A. nominus* can produce aflatoxins. *A. flavus* is phenotypically similar to *A. nominus.* Lastly*, A. bombycis, A. pseudotamarii* and *A. ochraceoroseus* can also form aflatoxins. *A. flavus* mainly forms AFB_1 and AFB_2 while *A. parasiticus* forms AFG_1, AFG_2, AFB_1 and AFB_2 (Mishra and Chitrangada, 2003; Abeer, 2015; Maryam, 2015). However, in developing countries foodborne mycotoxins of clinical importance are aflatoxins and fumonisins (Muthomi *et al.,* 2009).

All these fungi are classified under the *Hyphomycetes* class, *Deuteromycotina* subdivision and *Aspergillaceae* family. Various foodstuffs are contaminated by them. The *Aspergillus* species thrives on various substrates and under diverse environmental conditions. Cumulatively, 20 different aflatoxins have been reported which are chemically classified into two groups; the difurocoumarocyclopentenone series (the difurocoumarolactone series (AFG_1, AFG_2 and AFB_1, AFB_2, AFB_2A, AFM_1, AFM_2, AFM_2A) and aflatoxicol (Mishra and Chitrangada, 2003; Abeer, 2015).

The “G” and “B” stands for green and blue colours seen when AF are subjected to UV radiation. The subscript numbers 1 and 2 stand for primary and secondary products, respectively. Metabolic substances of AFB_1 and AFB_2 are M_1 and M_2 respectively. AFM_1 and AFM_2 are found in body fluids after metabolism (Bbosa, 2013; Lalah *et al*., 2019; Noreddine 2019).

Aflatoxins are difurocoumarin derivatives. Difurocoumarin chemical structure has bifuran unit merged to coumarin unit that has a pentenone aromatic ring in B and M aflatoxin forms. G aflatoxins consist of 6 membered lactone aromatic ring. The aflatoxins of public health importance are AFB_1, AFB_2, AFG_1 and AFG_2 (Bbosa, 2013; Lalah *et al.*, 2019; Noreddine 2019).

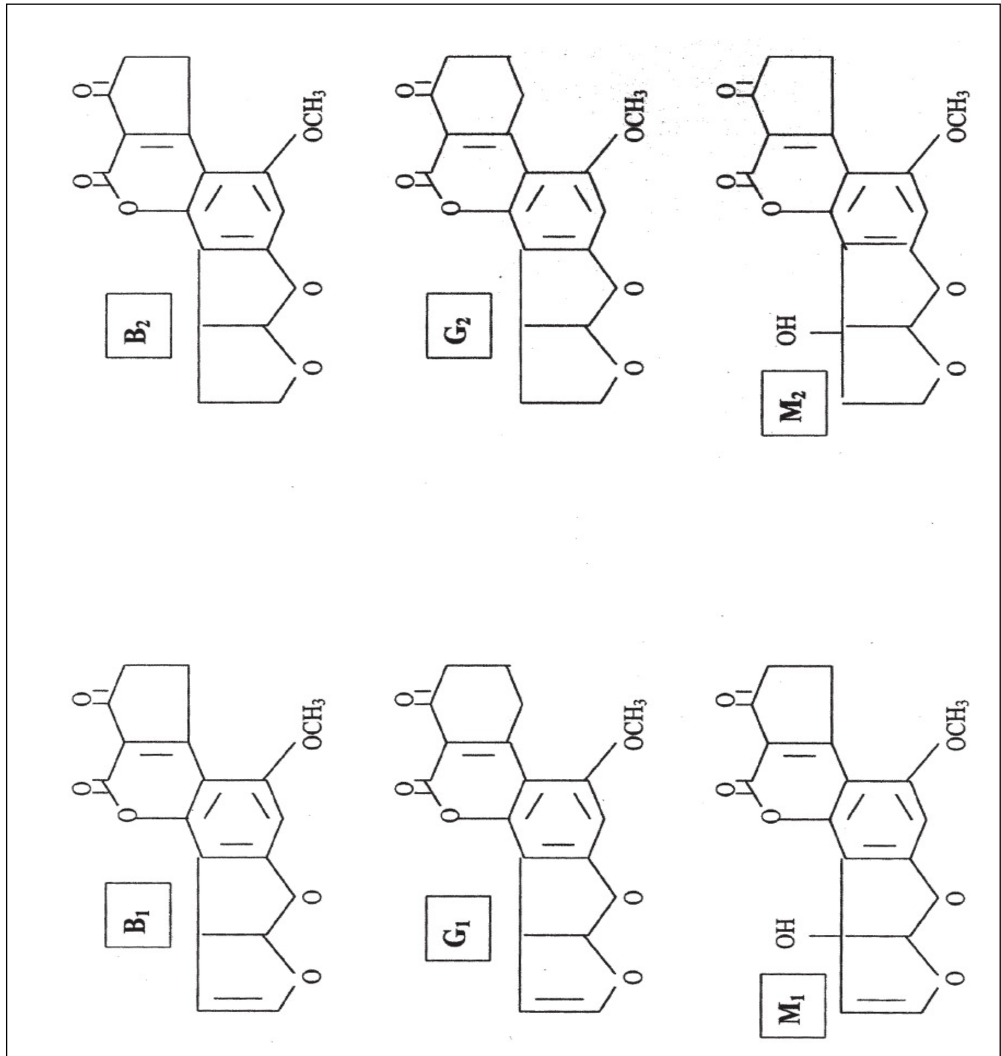


Figure 2. 2: Chemical structure of aflatoxins

(Mishra and Chitrangada, 2003; Maryam, 2015; Lalah *et al.*, 2019)

2.6. Toxicokinetics of Aflatoxins

2.6.1. Exposure to aflatoxins

Exposure to aflatoxins occurs mainly through oral intake, it can also occur through dermal and inhalation. It may also occur by ingestion of milk and eggs (Hamid *et al.,* 2013; Carvajal, 2015; Iqbal *et al*., 2019). Exposure to aflatoxins in the previous 3 months can be done using aflatoxin biomarkers like serum aflatoxin-albumin (Af-alb) adducts (Gong *et al*., 2004).

Even though aflatoxins many times occur in minute concentrations, its prevalence, potency, easily spreads in farming and storage areas making them a public health hazard. Furthermore, aflatoxins cannot be smelt, seen, tasted or felt in grains. Consequently, their detection requires laboratory testing (Nduati *et al*., 2017).

2.6.2. Distribution of aflatoxins in the human body and animals

Aflatoxins are well absorbed from the gastrointestinal tract, lungs and skin. Aflatoxins are then distributed once they reach the bloodstream; this leads to their accumulation in most tissues and body fats (Bbosa, 2013).

It has been reported that aflatoxins may get distributed to the breast milk. However, it has been suggested that (AFM_1) is considerably less toxic metabolite in comparison to the parent toxins. Thus, breastfeeding is considered to be a low aflatoxin exposure risk especially among infants whose chief weaning diet is maize based in regions of high aflatoxin contamination rates (Gong *et al*., 2004).

2.6.3. Aflatoxins metabolism

Metabolism of aflatoxins is essential in the breakdown and excretion of aflatoxins from the body. Metabolism plays a key role in aflatoxin breakdown and toxicological activities. Aflatoxins bioactivation is an essential step in their carcinogenic and toxic activities. The metabolic reactions of aflatoxins involve phase 1 and phase 2, before their elimination. Metabolism of AFB_1 occurs through cytochrome P-450 (CYP) resulting in several hydrophilic metabolites including AFM_1. AFM_1 is rapidly excreted through stool and urine (Dohnal *et al*., 2014; Carvajal, 2015; Sharma *et al*., 2018).

Human urine samples having AFM_1 indicate aflatoxin exposure over the preceding 24 hours. It has been reported to occur in about 33% of the sample population. The presence of aflatoxin-albumin adduct, confirms chronic exposure, it is approximated to be present in about 90% of the sample populations (Williams *et al*., 2004; Lalah *et al*., 2019).

Cytochromes and isoenzymes roles vary in the two (2) metabolic phases. Phase 1 metabolic reactions of oxidation-reduction are the primary CYP enzyme activity in aflatoxins activation. Liver is the primary aflatoxin metabolism site. Aflatoxins are metabolised in the body by CYP1A1 and CYP1A2, which is make up of 10% CYP isoforms (Guyonnet *et al*., 2002; Iqbal *et al*., 2019; Oladeji *et al*., 2020). AFB_1 too is metabolised by CYP 3A4, 3A5 and 3A7 (Kew, 2013).

AFB_1-aldehyde reductase and Glutathione S-transferase (GST) (Guyonnet *et al*., 2002) are also involved in AFB_1 metabolism. They lead to reactive metabolites formation. These reactive metabolites have been assessed as AFB_1 exposure biomarkers. CYP1A1 and CYP1A2 metabolize and cause activation of

procarcinogens as midway metabolic substances that cause linkage to the DNA. They are also involved in AFB_1 activation (Williams *et al.,* 2004; Hamid *et al.,* 2013; Carvajal, 2015).

Aflatoxins at low concentrations are primarily metabolised by CYP1A2 enzyme isoform. CYP3A4 isoform is the chief metabolizer for high aflatoxin concentrations (Mishra and Chitrangada, 2003; Carvajal, 2015).

An increase of AFB_1 and its metabolic products in serum, leads to reduced glutathione (GSH). This is because of increased formation of reactive oxygen species and epoxides (Williams *et al.,* 2004; Carvajal, 2015). Epoxides formed undergo reaction with serum albumin leading to production of lysine adducts or with DNA leading to formation of guanine adducts which can be detected in blood. They are key biomarkers on the extent of aflatoxin exposure in both animal and human investigations (Oladeji *et al*., 2020).

2.7. Toxicity and Mechanism of Action

2.7.1. Aflatoxins toxicity

It is well established that mycotoxins are harmful to animal health and humans as confirmed by several animal models and human epidemiological data (Khalil *et al*., 2019; Lalah *et al*., 2019). Aflatoxins have been confirmed to be very toxic to mammals. The AFB_1 LD_{50} of some animals have been documented as follows: LD_{50} 4.8 mg/kg body weight (rat), 0.62 (pigs), 0.5 (dogs), 6.3 mg/kg (chicken), 2 (guinea pigs) (Lalah *et al*., 2019). Dietary ingestion of AFB_1 contaminated food causes dose-dependent severe manifestations in both livestock and humans (Aniket *et al*., 2018).

The main aflatoxin toxic effects include: hepatotoxic, immunotoxic, carcinogenicity, mutagenicity, and teratogenicity (Nduati *et al*., 2017; Makhuvele *et al*., 2020; Alperen *et al*., 2021). Numerous factors that influence these adverse effects have been reported: age, species variation, sex, other toxicants present and nutritional status (Iqbal *et al*., 2019). It is also well documented that duration of exposure of the organism and AF concentration are chief determinants in the manifestation of the adverse effects. The harmful effects of AFB_1 have been found to occur after bioactivation of AFB_1 by CYP450 enzymes. These enzyme reactions are involved in both metabolism and detoxification (Mishra and Chitrangada, 2003; Yaman, 2016).

Currently, AFB_1 is the most researched and defined of the known aflatoxin (Alperen *et al*., 2021). It has been shown to be highly biologically active and more toxic in comparison to others (Milad, 2011; Jalila *et al*., 2016; An *et al*., 2017). Moreover, it has been categorized as a potent natural carcinogenic agent (Lijuan *et al*., 2016; Jalila *et al*., 2016). AFB_1 has been confirmed to be a potent hepatotoxic and hepatic carcinogenic compound (Ghada *et al*., 2012; Oskoueian, 2015).
Epidemiological investigations have shown that hepatitis B together with AFB_1 cause human hepatocellular carcinoma. Human hepatocellular carcinoma has a poor 5-year prognosis. Its mortality contributes to about 15% of reported cancer deaths (Cheng-Feng *et al*., 2000; Stefano *et al*., 2007, Jalila *et al*., 2016). Among resource limited countries, a positive association has been identified between aflatoxin contaminated food consumption and cancer incidence (Dhanasekaran *et al*., 2011). Carcinogenicity is regarded as one of the key impacts of aflatoxins on public health (Pickova *et al*., 2021).

Aflatoxins are immunosuppressive, carcinogenic, mutagenic, genotoxic as well as teratogenic. Their potency is shown in the order: $AFG_2 < AFB_2 < AFG_1 < AFM_1 < AFB_1$ (Carvajal, 2015; Omara *et al*., 2021; Zamir-Nasta *et al*., 2021).

Aflatoxin B_1 is a highly toxic substance; it has a Median Lethal dose (LD_{50}) that ranges from (LD_{50} = 1 to 50 mg/kg) for nearly all animals (Gerardo, 2011). Nevertheless, for highly susceptible animals like dogs, cats, pigs and ducklings, it is highly poisonous ($LD_{50} < 1$ mg/kg). AFB_1 toxic effects depend on both duration (time) and concentration (dose) (Lalah *et al*., 2019).

Aflatoxicosis can be grouped into acute and chronic; which can be differentiated depending on duration of exposure and dose of aflatoxin (Gerardo, 2011; Yaman, 2016; Lalah *et al*., 2019). The risk associated with aflatoxin is determined by the dose, animal species, aflatoxin type, animal's nutritional status, other toxins present and their interactions with aflatoxins (Yaman, 2016; Pickova *et al*., 2021).

Acute and chronic manifestations of aflatoxin exposure in laboratory, farm animals and human subjects are well known. Epidemiological studies involving humans and experimental investigations using laboratory animals have revealed that aflatoxins at acute or chronic concentrations are highly potent mutagens and hepatocarcinogens (Faridha *et al*., 2006).

At lower doses occurring during sub-chronic toxicity, as would be the case in dietary exposure, aflatoxins may cause a milder effect known as aflatoxicosis. This is manifested as poor feeding, reduced growth, lowered feed utilisation, decreased milk production and poor reproductive function (Faridha *et al*., 2006). However, the minimum aflatoxin concentration required to cause aflatoxicosis has not been established in human subjects (Nduati *et al*., 2017).

2.7.2. Acute aflatoxicosis

Acute aflatoxicosis is usually associated with intake of large doses within a short time period. This gives rise to hepatoxicity followed by morbidity that may result in mortality. Hepatic system is the primary target for aflatoxin toxicity. Upon reaching the liver, they enter inter-hepatocytes causing hepatic necrosis and (or) cell death. This results because aflatoxins metabolites cause negative reactions with various cell proteins.

Subsequently, there occurs lipid and carbohydrate metabolism inhibition. Also, synthesis of proteins is hindered. Furthermore, there is reduced hepatic function, jaundice, altered blood clotting mechanism and reduced serum proteins production by the liver. Lastly, aflatoxicosis may also be manifested as but not limited to lower limb edema, jaundice, vomiting and abdominal pain (Dhanasekaran *et al*., 2011; Nduati *et al*., 2017; Lalah *et al*., 2019).

Acute aflatoxin hepatotoxicity leads to raised concentration of serum enzymes namely: alkaline phosphatase, glutamate dehydrogenase, lactate dehydrogenase, gamma-glutamyl transferase and aspartate aminotransferase. These changes often indicate hepatotoxicity and other biochemical changes like ketonuria, glycosuria, proteinuria, and haematuria (Bbosa *et al*., 2013).

Hepatotoxicity is indicated by the elevation of the concentration of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), gamma glutamyl transferase (γ-GT) and alkaline phosphatase (ALP). This occurs because of necrotic destruction of cell membranes, allowing enzymes to leak into blood stream. Consequently, it has been confirmed that AF primarily causes

increased concentration of AST, creatinine, ALT and uric acid in AF fed mice blood serum (Juma *et al*., 2015).

Liver function tests performed by serum assays provide vital information concerning the liver physiological status. Synthetic function of the liver is revealed by albumin levels. Cellular structure and integrity are revealed by ALT and AST enzyme levels. ALP gives useful information regarding the connection of liver to the biliary tract (Farombi *et al*., 2005; Tanuja *et al*., 2016).

Alanine aminotransferase (ALT) that was formerly named as serum glutamic-pyruvic transaminase (SGPT) or serum glutamate-pyruvate transaminase (SGPT) has been routinely used as a major diagnostic biochemical indicator of liver dysfunction. The levels also rise when there is increased cell membrane permeability (Nili-Ahmadabadi *et al*., 2011; Ghada *et al*., 2012; Tanuja *et al*., 2016).

Aspartate aminotransferase (AST) was formerly named as serum glutamic oxaloacetic transaminase (SGOT). It is primarily present in the liver. It is also present in red corpuscle, cardiac cells, muscles, pancreas and kidney (Xing-Jiu *et al*., 2006). Similar to ALT, AST is also a major diagnostic biochemical indicator of liver injury.

Alkaline phosphatase (ALP) is made up of a group of enzymes that in a basic environment hydrolyse phosphate esters. The end products of this hydrolysis are the formation of an inorganic phosphate and an organic radical (Tanuja *et al*., 2016).

Bilirubin is a metabolic substance obtained from heme breakdown. The serum bilirubin increase can be used as a prognostic tool in chronic liver injuries. However, it cannot be used in mild hepatic injuries (Tanuja *et al*., 2016).

The normal reference ranges for these biochemical parameters in Swiss albino mice are shown in Table 2.2. It should be noted that they are highly susceptible to diet fed

to the mice, age and sex of mice investigated, housing conditions, sampling processes, pre-analysis sample handling, methods and equipment used for analysis. Moreover, sample size used, advances in analytical methods and processes used may cause significant variation in their reference ranges. Lastly, the reference value will vary depending on whether the mice used are inbred or outbred **(**Serfilippi *et al*., 2003, Otto *et al*., 2016**)**. From the foregoing, most studies include a control group in their investigation to act as a benchmark.

Table 2. 2: Normal reference ranges for biochemical parameters in Swiss albino mice (**Serfilippi *et al*., 2003, Otto *et al*., 2016**)

	Parameter	**Male**	**Female**
1.	ALT, IU/L	30 (24-40)	26 (22-37)
2.	AST, IU/L	48 (40-200)	50 (44-200)
3.	ALP, IU/L	90 (80-100)	135 (122-148)
4.	Albumin, g/L	27 (24.2-31.0)	28.0 (26.0-29.0)
5.	Total protein, g/L	50.0 (48-53.3)	49.8 (47.5-52.0)
6.	Globulin, g/L	24.5 (17-32)	23 (16-33)
7.	Total bilirubin, µmol/L	2.3 (2.1 – 2.4)	2.3 (2.1 – 2.5)

Subsequently, determination of the levels of these enzymes was used to assess the ameliorative effect of *Spirulina platensis* extract on biochemical changes caused by AFB_1 in mice.

2.7.3. Mechanism of hepatotoxicity

Aflatoxins causes diverse toxicological effects with various documented mechanisms, a number of them are not yet fully understood. Commendable investigations have

been done to assess aflatoxins mechanisms of toxicity thereby leading to a scientific basis for the currently available prevention and control strategies (Noreddine, 2020).

AFB_1 can cause gene mutation, DNA damage and chromosomal anomalies. AFB_1 is a pro-toxic chemical (Nili-Ahmadabadi *et al*., 2011, Qian *et al*., 2013; Ishida *et al*., 2020). 8, 9-epoxide a metabolic product of AFB_1 is highly potent with capability to bind to DNA and proteins leading to production of 'adducts' (Stefano *et al*., 2007, Ghada *et al.,* 2012., Kovesi *et al*., 2021). This metabolite is then converted to AFB_1-8, 9-diol which gets attached to albumin's lysine leading to AFB_1-lysine adducts formation.

Consequently, AFB_1-lysine adducts have been determined and confirmed as a viable human aflatoxin exposure biomarker (Nili-Ahmadabadi *et al*., 2011; Qian *et al*., 2013). Subsequently, any adducts produced during these activities have been associated with disruption of the normal cell function. Furthermore, the adduct formed above and biomarkers of AFB_1 like AFB_1-N_7-guanine adduct are valuable biomarkers in evaluation of chemo preventive substances in both human and animal investigations (Qian *et al*., 2013).

It should be noted that 8, 9-epoxide formation is a critical step for AFB_1 hepatotoxic and carcinogenic activity. It is metabolised by glutathione-S-transferases (GSTs) through glutathione conjugation (Ishida *et al*., 2020). Subsequently, the extent of hepatotoxicity is influenced by the difference between formation and detoxification of reactive metabolites by the liver (Cheng-Feng *et al*., 2000, Ishida *et al*., 2020).

Breakdown of AFB_1 to AFB_1-N7- guanine adduct is the main metabolic pathway in humans. The rate of breakdown is comparable to other species affected by AF - induced hepatic carcinogenicity (Vincent *et al*., 2000). However, both animals and

humans have protective enzyme systems that reverse cell and DNA damage induced by 8, 9- epoxide metabolite. For instance, glutathione S-transferase (GSTA) converts 8, 9- epoxide to endogenous compounds. This greatly reduces its toxic effects (Cheng-Feng *et al*., 2000; Stefano *et al*., 2007, Ghada *et al.,* 2012).

Some studies have reported that human beings have minimal GSTA activity for breakdown 8, 9-epoxide in comparison to mice and rats. Subsequently, this suggests that humans have reduced capability of 8, 9-epoxide detoxification (Vincent *et al.,* 2000; Ghada *et al.,* 2012).

Interestingly, there is significant variation observed in rodents with regard to resistance to AFB_1 toxicity. Mice have been found to be more resistant to AFB_1 hepatotoxicity. This has been attributed to their high GSTA toward AFB_1 metabolic activity. This is in contrast to rats that are regarded to be among the highly sensitive animals to carcinogenicity and AFB_1-induced hepatotoxicity (Ishida *et al*., 2020). Subsequently, these interspecies variations in their sensitivity to hepatotoxicity induced by AFB_1 may limit the application and extrapolation of experimental animal models data to human beings (Ishida *et al*., 2020).

It has also been confirmed that AFB_1 elevates free radicals' formation, peroxidation of lipids and oxidative damage promotion. Thus, this causes severe cell damage and death (Cheng-Feng *et al*., 2000, Stefano *et al*., 2007). It should be noted that oxidative damage will occur when the formation of free radicals (hydrogen peroxide, hydroxyl radical and superoxide radical) exceeds antioxidant capacity in the tissue. The antioxidants involved include non-enzymes vitamin C and glutathione.

Enzyme antioxidants includes; glutathione peroxidase, superoxide dismutase (SOD) and catalase (CAT). Both enzyme and non-enzymatic antioxidants are the primary

determinants of cell's defensive mechanisms. Consequently, oxidative stress is a critical determinant in AFB_1 toxicity mechanism (Nili-Ahmadabadi *et al*., 2011; Vaziriyan *et al*., 2018; Shahid *et al*., 2019).

However, its not clear whether aflatoxins increase peroxidation of lipid directly through increased formation of ROS or through enhanced tissue sensitivity to peroxidation. It has been suggested that the two processes may occur concurrently (Kovesi *et al*., 2021). Furthermore, it has been reported that the long plasma half-life of lipid peroxidation products allows them to pass through lipid bilayer membranes leading to oxidative damage in both adjacent and far away cells (Kovesi *et al*., 2021).

2.7.4. Chronic aflatoxicosis

Chronic intake of aflatoxins causes childhood cirrhosis, proliferation of bile duct, osteosclerosis of bone, necrosis of liver, liver veno-occlusive lesions and immune suppression (Mishra and Chitrangada, 2003; Williams, 2004; Bbosa, 2013). Noteworthy, it has been documented by a Kenyan study that babies born by AF exposed mothers have considerably lower birth weights (Shan, 2019). A Gambian study on association between aflatoxin levels and birth weights also reported a negative correlation (Shan, 2019).

Aflatoxins have been associated with child stunting though not proven. It has also been suggested that aflatoxin intake levels may influence HIV/AIDS and malaria. However, scientific data on this is largely indeterminate (Asiki *et al*., 2014; Negash, 2018). Furthermore, several studies have identified a close association among the following: childhood growth stunting, kwashiorkor disease, marasmus, Reye's syndrome and aflatoxin exposure (Adhiambo *et al*., 2004; Iqbal *et al*., 2019; Shan 2019). Moreover, aflatoxins have been identified in tissue assays of children who

have Reye's syndrome and kwashiorkor. Subsequently, aflatoxins have been associated with these two diseases (Zain, 2011).

Moreover, considering that children in Sub-Saharan Africa are often weaned on to cow's milk and grain porridge thus exposing them to possible aflatoxicosis. Furthermore, during this age paediatrics are not immune competent. Consequently, ingestion of AFM_1 and AFB_1 may further suppress their immune system thus making them vulnerable to opportunistic diseases (Kangete and Langat, 2009; Asiki *et al*., 2014). Aflatoxin have also been associated with reduced food conversion, low growth rates and reduced micronutrient absorption (Ndung'u *et al*., 2013; Oladeji *et al*., 2020).

2.7.5. Inter and intra species AFB_1 response variation

There is no animal species which has resistance against acute or chronic AFB_1 toxicity (Shivender *et al*., 2012). Secondly, it has also been revealed that there is variation in response among different animal species to aflatoxin toxic effects of AFB_1. Hence, that is the basis for performing this study on male Swiss albino mice: their resistance against AFB_1 is more than other animals e.g., rats (Mogilnaya *et al*., 2010).

However, liver cancer is not manifested in all aflatoxin exposed animals, some of the effects are limited to particular species. For example, among the poultry species only the duck is affected by aflatoxin induced hepatocellular carcinoma (Gerardo, 2011; Yaman, 2016). Among animals highly susceptible to aflatoxins include: piglet, duckling, dog, rabbit and cat. Among animals found to be relatively resistant include: chicken, hamsters, mice and chinchillas. In addition to these, older animals have been reported to exhibit more resistance to AFB_1 compared to younger ones. Lastly, female animals exhibit more resistance than males (Gerardo, 2011; Yaman, 2016).

Other parameters that have been suggested to cause variation in response to aflatoxins include: exposure level, age, exposure duration, health, environmental factors and nutritional status (Shivender *et al*., 2012).

2.7.6 Aflatoxin B_1 induced hepatocellular carcinoma

The liver has been confirmed to be the key organ affected by AFB_1 toxicity (Lijuan *et al*., 2016). Over 750,000 annual mortality cases associated with hepatocellular carcinoma (HCC) have been reported. Subsequently, HCC is globally ranked second among the leading aetiologies of cancer mortality (Sriwattanapong *et al*., 2017).

Development of HCC may take up to twenty (20) years after AF exposure. This duration may be reduced in the presence of hepatitis B or C (Nathan *et al*., 2021). A synergistic relationship has been reported between Hepatitis B or C and AFB_1 in hepatic cancer aetiology (Guyonnet *et al*., 2002; Gong *et al*., 2004, Khan *et al*., 2021). Subsequently, it remains difficult to ascribe aetiology of all hepatic cancer cases solely to AFB_1 (Zain 2011; Lalah *et al*., 2019; Oladeji *et al*., 2020). Other identified risk factors include chronic alcohol intake and iron overload (Cardwell, 2000; Hamid *et al*., 2013).

International Agency for Research on Cancer (IARC), in 1993, categorized AFB_1 as carcinogenic agent. AFB_1 was classified under Group I. Subsequently, it has been confirmed to be a cancer inducing agent (Makun *et al*., 2012, Jalila *et al*., 2015; Lalah *et al*., 2019). AFG_1, AFG_2 and AFB_2 were also identified as possible carcinogens (Lijuan *et al.,* 2016).

Liver is the primary cancer site in rats, mice, salmon, hamsters, trout, monkeys and ducks. Tumours in other parts have been reported to be uncommon. AFM_1 carcinogenic potency has been found to be ten (10) times less than that of AFB_1

(Christopher *et al*., 2010). AFB_1 has been documented as a powerful hepatotoxic and hepatic carcinogenic agent (Nili-Ahmadabadi *et al*., 2011; Nathan *et al*., 2021).

One of the primary adverse effects of AFB_1 on the hepatic system is decreased total protein levels. Subsequently, protein synthesis inhibition occurs by formation of RNA, DNA and proteins adducts. This results in inhibition of RNA and DNA synthesis (Bbosa *et al*., 2013). AFB_1-DNA adducts have been identified as AFB_1 exposure biomarkers. AFB1-DNA adducts can also act as HCC in humans risk predictors (Sriwattanapong *et al*., 2017).

Although the connection between aflatoxin exposure and hepatic cancer is well documented and established; concerns have emerged concerning the role of other health parameters like growth retardation and immune suppression. These are often seen in veterinary studies, which are most often not considered in human studies. Such factors may have a major role in community disease prevalence (Gordon, 2008).

2.7.7. Aflatoxin B_1 induced immune suppression

Immunotoxicity in both animals and man caused by naturally occurring compounds in food and environmental contaminants has remained to be a subject of increasing interest for many years (Genevieve *et al*., 2000).

Chronic ingestion of aflatoxin contaminated meals causes immune depression (Rania *et al*., 2015; Jalila *et al*., 2016; Rushing *et al*., 2019). Immune system is altered either in a synergistic or additive manner (Cardwell, 2000). Aflatoxins affects both acquired and natural immunity leading to reduced infection resistance. Subsequently, the individual becomes more susceptible to opportunistic infections (Zain, 2011; Kobra *et al*., 2018).

Aflatoxins affect both the cellular and humoral immunity hence reducing the host resistance to infections (Patrick *et al*., 2006; Yi Jiang *et al*., 2008; Zain, 2011). Epidemiological and experimental investigations have shown that acute and chronic AFB_1 exposure leads to several immune system effects including alterations in expression of cytokine in various animals (Alix *et al*., 2016; Feibo *et al*., 2019; Nour *et al*., 2020).

Immunosuppression by AFB_1 has been confirmed in various animals including rats, cattle, trout and poultry. Contrasting reports have been reported depending on age, species and gender. The association between ingestion of aflatoxin contaminated food and health condition of the affected individuals has been reported by epidemiological investigations (Silvia *et al*., 2018).

An experimental animal model using birds, has documented that aflatoxins reduce the levels of immunoglobulins IgA, IgG and IgM. Also, they lower the complement activity in chicken (Bbosa *et al*., 2013). Significantly, animal studies performed using rats, poultry and pigs revealed that exposure to aflatoxins contaminated food causes immune depression (Williams *et al*., 2004). However, this should be interpreted with caution as some studies have documented an elevation in the levels of IgM and IgA. This may occur depending on the dose level used in the investigation (Samir *et al*., 2010).

Farm animals' studies have shown that aflatoxins ingestion leads to hypertrophy of bursal and thymus in chickens and immunosuppression in turkeys. Impaired bovine lymphocyte mitogenesis has been reported. In addition to these, a reduced antibody response to *Salmonella* and a depressed cell-mediated immunity in chickens have

been reported. Lastly, delayed-type hypersensitivity in pigs has also been revealed (Patrick *et al*., 2006).

Experimental animal model studies in poultry, rats and pigs have revealed that exposure to aflatoxins causes immune depression (Williams *et al*., 2004; Rushing *et al*., 2019). Phagocytosis cell activity by white blood cells may also be affected by aflatoxins (Williams *et al*., 2004).

Generally, it seems that toxic responses to fumosin B_1 (FB_1) and AFB_1 regarding the induction and progression rate of resulting pathologies depends on dosage level, cell proliferation rate and exposure duration (Samir *et al*., 2016). Immune system sensitivity to aflatoxins enhanced immunosuppression occurs because of susceptibility of immune moderated activities and regulation of the cellular and humoral functions. Among the manifestations of aflatoxins mediated immunosuppression include: a suppressed T or B lymphocyte function, antibody production reduction and suppressed macrophage-neutrophil activity (Tomkova *et al*., 2001).

There are conflicting reports concerning immune depression caused by aflatoxins. This may partially be explained by variations in experimental designs, the specific immune response investigated and other secondary effects of the administered aflatoxins dose. Moreover, aflatoxins as other cytotoxic molecules may reveal toxic manifestations at different doses from those that cause immunosuppression. Furthermore, it has been documented that aflatoxins may affect release of cytokines depending on the time, dosing schedule and dose administered (Samir *et al*., 2016; Jalila *et al*., 2016). Lastly, dissimilar doses of AFB_1 can suppress or stimulate the immune activity (Mogilnaya *et al*., 2010; Rushing *et al*., 2019).

Although most of the available studies that investigated such mechanisms were done using experimental animal models, immunosuppression by AFB_1 has also been investigated and replicated in vitro using human cells. Furthermore, it has also been investigated and replicated in Ghanian case–control studies among the highly exposed. However, there is limited data on aflatoxin immunotoxicity of the other aflatoxins besides AFB_1. Lastly, data on immunotoxicity of AFB_1 when in combination with other aflatoxins is limited (Noreddine, 2020).

Moreover, it has been documented that aflatoxins may reduce efficacy of vaccines and increase susceptibility of an organism to infections (Kimanya *et al*., 2010). Also, ingestion of AFB_1 may enhance colonisation of the gastrointestinal tract by *Escherichia coli* (Kimanya *et al*., 2010).

Overall, there is limited, inconclusive and inconsistent evidence of *in vivo* aflatoxin immunosuppression in humans. Consequently, such data largely remains inconclusive (Williams *et al*., 2004, Christopher *et al*., 2010). Furthermore, previous researchers investigated the aflatoxin-albumin adduct concentration in humans. Subsequently, this signifies chronic aflatoxin exposure. However, it has been documented that cells exposed to aflatoxin usually recover a few days post exposure.

Therefore, aflatoxin-albumin adduct measurement may not be the best assay method of exposure determination for immunological investigations (Williams *et al*., 2004). Also, the documented studies have been cross-sectional, involving a small number of participants; they have not been investigated and replicated in large and diverse populations. Nevertheless, the studies suggest that the aflatoxins induced immunotoxicity could occur in chronically exposed persons. Subsequently, these merits further investigation in human subjects (Christopher *et al*., 2010). Overall,

experimental animal and human studies have shown that AFB_1 causes immune depression.

2.7.8. Histopathological changes associated with aflatoxins

Toxicological investigations should include both biochemical studies and histological studies. This gives evidence of both anatomical localization of toxin action and the respective biochemical changes.

Previous researchers have reported significant histopathological lesions in rats exposed to aflatoxins. Examination of liver tissues have revealed marked histopathological changes. Among the histopathological changes reported include: fibrosis, necrotic changes, cloudy swelling, fatty changes and hydropic degeneration (Knipstein *et al*., 2015; Yaman *et al*., 2016).

Earlier investigators likewise indicated that aflatoxicosis causes proliferation and epithelial hyperplasia in bile ducts of liver bile ducts. The histopathological severity of the changes is determined by the duration and dose of AFB_1 administered. Furthermore, aflatoxicosis causes liver necrotic changes (Yaman *et al*., 2016).

Several studies have revealed that AFB_1 causes severe kidney damage. Necrotic and degenerative changes in the cells of nephron's proximal tubule have been confirmed. Hyaline casts have also been reported in tubular lumen. Moreover, epithelial nuclei enlargement has been reported in an experimental study (Devendran *et al*, 2011; Abeer, 2015; Yaman *et al*., 2016). In addition to these, glomeruli enlargement along with capillaries lumina dilation have been documented. Atrophy of glomeruli and thickening of its walls has also been reported (Devendran *et al*, 2011; Abeer, 2015).

2.8. Mitigation against Aflatoxins

2.8.1. Introduction

Globally there have been numerous efforts to reduce aflatoxins contamination in both animal feeds and human food (Khalil *et al*., 2019; Shan, 2019). Strategies that are aimed at production of safe procedures that are cost effective and sustainable have been used to lower aflatoxins levels in feedstuffs.

Even though several strategies are available for the eradication of mycotoxin contamination, they have numerous limitations. These include efficacy limitations, practical considerations, likelihood of nutritive value loss and cost limitations in the decontamination process (Khalil *et al*., 2019).

There is no known antidote developed for the management of aflatoxins poisoning including for the most potent AFB_1 (Juma *et al*., 2015; Shan, 2019). L-methionine has been used at doses of 200 mg/kg bw as drug of choice in AFB_1 management. It is intraperitoneally (IP) given every eight hours till all the clinical manifestations are reduced. Other indications of L-methionine include management of hepatotoxicity induced by paracetamol poisoning. Likewise, sodium thiosulfate is given at 50 mg/kg in the management of aflatoxicosis. It should be given at an 8 hours interval. It has been suggested to have potential beneficial therapeutic effects (Juma *et al*., 2015). The following are some of the other approaches documented.

2.8.2. Physical Methods

Mycotoxins (ochratoxin, aflatoxins, citrinin and T-2 toxin) are readily miscible in organic solvents. A mixture of solvents or several solvents can be used in the extraction of aflatoxin from feedstuffs (Mishra and Chitrangada, 2003).

2.8.2.1. Cleaning

Multi step processes involving the removal of husks, dust and mould colonisation products. This can be followed by mechanical sorting and washing. Hulling of coffee has been reported to reduce mycotoxins (Mishra and Chitrangada, 2003; Maryam, 2015).

Contaminated foods have been found to have a different colour or density compared to uncontaminated foods. Therefore, sorting of kernels to remove discoloured pods based on appearance is recommended to reduce aflatoxin levels (Maryam, 2015).

However, aflatoxin have low water solubility, thus it's not feasible to wash the aflatoxins using water (Maryam, 2015). Cleaning method is commonly used as a preliminary mycotoxin decontamination method. Also, its application on a large scale is limited.

2.8.2.2. Thermal applications

Aflatoxin decomposition occurs at temperatures of 237 °C to 300 °C. Aflatoxin B_1 solids remains unaffected by dry heat at temperatures of up to 267 °C. Therefore, aflatoxins are not affected by temperatures during boiling food or cooking, ultra-pasteurization of milk and fermentation processes (Carvajal, 2015). However, some studies have reported reduction of aflatoxin concentration in foodstuffs after heat treatments of roasting, boiling, steaming and baking (Maryam, 2015).

The extent and efficacy of the reduction method is determined by: contamination source, pH, ionic strength, aflatoxin binding extent, aflatoxin level, food composition, moisture content and heat penetration (Maryam, 2015). This method has limited application on large scale commercial application. Furthermore, the high temperatures required will not be economical and sustainable for such a purpose.

2.8.2.3. Irradiation

Radiation can be divided into two groups: non-ionizing and ionizing radiation. Ionizing radiation (e.g., gamma, ultraviolet and x-rays) may induce significant changes in irradiated objects' composition. It may be accompanied with minimal or without thermal changes. Often it is without any production of toxic molecular changes (Maryam, 2015).

Non-ionizing radiations (includes: visible light, radio, infrared and microwaves) in optimal wavelength causes temperature elevation. The molecular changes caused are nontoxic to humans.

Gamma radiation is regarded as a cold temperature process. It has been used to prolong shelf life of certain foods by reduction of contaminations by microorganisms (Maryam, 2015). The application of gamma radiation for aflatoxin inactivation has been reported. However, reports have been conflicting and inconclusive. Some studies have reported that the gamma radiation may not be effective in reducing levels of aflatoxins. Moreover, other studies have reported diverse levels of decontamination in different foods when using gamma irradiation. The efficiency of gamma radiation in aflatoxin decontamination significantly depends on radiation dose used (Maryam, 2015; Silvia *et al*., 2018).

Furthermore, sensitivity of aflatoxins to gamma radiation varies, AFG_1 and AFB_1 exhibit more sensitivity compared to AFG_2 and AFB_2 (Maryam, 2015). In addition to the above, feedstuffs irradiation may significantly reduce aflatoxin content. Sunlight exposure of contaminated feeds has also been reported to be effective. Overall, irradiation methods have little significance especially in practical large-scale

applications. Secondly, it is of little significance in presence of highly contaminated human foods and animal feeds.

2.8.2.4. Adsorbents

The use of aflatoxin-binding adsorbents that do not get absorbed from gastrointestinal. They attach physically to the mycotoxin. This is the most commonly used physical method in the prevention of mycotoxin toxic effects by contaminated feed. This method is currently popular. Effectiveness of this process depends on the chemical composition of the adsorbent(s). Prior to application of this method for routine use, it should be confirmed that the adsorbent does not lead to reduction of vital nutrients from the food or feedstuff (Maryam, 2015).

Bentonite can remove up to 100% of aflatoxins from liquid solution by getting bound to aflatoxins in ingested feed and also minimises toxicity. Bentonite is available worldwide as deposits consisting of expandable smectite minerals. It has been documented that modified zeolites are among the most efficient adsorbents in mycotoxin food decontamination (Maryam, 2015).

Overall, physical methods have several limitations including low detoxification effect, poor product detoxification status and limited application (Guan *et al*., 2021).

2.8.3. Chemical Methods

Aflatoxins reacts with many chemicals including alkali, acids and oxidising agents. They alter them to non-poisonous or less poisonous substances (Maryam, 2015; Lalah e*t al*., 2019). Among the chemicals tested for their effectiveness in AF detoxification include citric acid, hydrochloric acid, calcium hydroxide, ammonium per-sulphate, formaldehyde, sodium potassium carbonate, sodium hypochlorite, sodium bisulfite, hydrogen peroxide and ozone gas (O3) (Maryam, 2015; Lalah *et al*., 2019).

Acidic and alkaline conditions may lead to the lactone ring in the aflatoxin opening forming beta keto acids which are water soluble. They can be eliminated by water washing (Maryam, 2015). Secondly, alkaline and acidic conditions can cause lactone ring structure hydrolysis (Maryam, 2015).

However, most of the chemical processes documented cannot be easily applied. They require drastic conditions of pressure and temperature. Some lead to the formation of toxic residues. Moreover, chemical processes may lead to decomposition of the sensory, nutritional and other vital product characteristics. Moreover, some of the products obtained may be unstable (Lalah *et al.*, 2019; Makhuvele *et al.*, 2020; Guan *et al.*, 2021). Consequently, these shortcomings often limit wide scale application of chemical methods in mycotoxin decontamination.

2.8.4. Biological Methods

Globally, there has been growing interest in biological detoxification of AFB_1 in the recent past (Jalila *et al.*, 2016). Microorganisms have competitive activity on nutrients, space and interactions among others. This is a potential method for aflatoxin decontamination in both pre- and post-harvesting (Maryam, 2015). Furthermore, biological detoxification methods have high specificity and often harmless products are formed. Lastly, it has been reported that it can lead to complete detoxification of samples under ideal conditions (Guan *et al.*, 2021).

Among the main challenges of biological methods include: the microorganisms may utilise the foods for their metabolic needs and they may also release undesirable compounds (Mishra and Chitrangada, 2003).

2.8.4. 1. *Bacteria*

Bacterial species like *Lactobacilli spp., Lactococcus, Bacillus subtilis, Bifidobacterium, Propionibacterium, Ralstonia spp., Pseudomonas spp. and Burkholderia spp*., have been reported to be effective in the inhibition of aflatoxins (Mishra and Chitrangada, 2003; Maryam, 2015; Jalila *et al*., 2016). Lactic acid bacteria (LAB) consist of genetically modified bacteria with characteristic inhibition of bacterial growth. They inhibit growth of microorganisms responsible for degradation of products or those that could be harmful to human beings (Maryam, 2015).

Among some of the constraints include: bacteria AFB_1 detoxification was found to be due to cell binding instead of metabolism. Subsequently, it was suggested to be reversible, hence, AFB_1 might not be completely eradicated from contaminated foodstuff (Jalila *et al*., 2016).

2.8.4.2. Yeast

Yeast species namely *Pichia anomala* and *Candida krusei* possess significant activity against *A. flavus*. Also, these yeast strains have been found to have considerable activity against *Aspergillus* growth in laboratory experimental conditions (Maryam, 2015). However, aflatoxins attachment to yeast strains occurs rapidly and it's a reversible process. Their attachment ability is generally less effective compared to that of bacterial strains. Secondly, it has also been shown to be strain specific (Maryam, 2015).

Overall, there are practical challenges in the applications of these fungi including the long period of time needed for aflatoxins detoxification. Secondly, it involves a

complicated process in order to get the active principles (Jalila *et al*., 2016). In addition to these, enzymatic breakdown by-products also limit their widespread application (Makhuvele *et al*., 2020).

2.9. Novel Methods of Mitigation

2.9.1. Genetically modified (GM) Bt corn

GM Bt corn has *Bacillus thuringiensis* (soil-borne bacteria) genes. It has insecticide activities due to the Cyt protein endotoxin which provides protection against *Lepidopteran coleopteran* pests that are found in cereal crops. Numerous Bt crops developed by inclusion of *B. thuringiensis* gene and inclusion of endotoxins that have a wide pesticide range have been developed. Some of the GM crops using this technique include: Maize, tobacco, wheat, peanuts, rice, tomato, cotton and walnuts. It should be noted that pest invasion has been closely linked with plant injury and fungi spore transmission leading to aflatoxin colonisation and buildup (Khan *et al*., 2021).

2.9.2. Biocontrol

The application of aggressive non-aflatoxigenic *A. parasiticus* and *A. flavus* strains in both before and after harvest as a strategy in aflatoxins control. *Aspergillus flavus* is predominantly associated with maize aflatoxin contamination. This species has various strains (morphotypes). Among these, the L strain is non-aflatoxigenic and S strain is aflatoxigenic (Probst *et al*., 2007; Probst *et al*., 2011).

Several field studies have reported significant aflatoxins production reduction in the ranges of 70% to 90%. Two products have been registered by the US Environmental Protection Agency (EPA). The two commodities possess non-aflatoxigenic strains that work as biological pesticides hence reducing mycotoxins present in peanut and cotton (Hell and Mutegi, 2011; Probst *et al*., 2011; Khan *et al*., 2021).

The two products are:

AF36 (*A. flavus* non-aflatoxigenic strains) for minimisation of aflatoxin contamination in corn and cottonseed.

A. flavus NRRL21882 (Afla-Guard®), that is efficient in reduction of aflatoxin production in both before and after-harvest stages (Hell and Mutegi, 2011; Khan *et al*., 2021).

In Africa, BN30 which has aflatoxigenic S-strain co-inoculated was shown to reduce aflatoxin contamination in corn significantly (Khan *et al*., 2021; Migwi *et al*., 2020).

In Kenya, 4 non-aflatoxigenic *A. flavus* strains have been identified. Aflasafe® KE01, biocontrol product; comprises of them as active ingredient. After successful safety and efficacy investigations Aflasafe® Ke01 was approved and registered for aflatoxin control in 2015 by the Pest Control Products Board (PCPB) (Migwi *et al*., 2020).

2.10. SPIRULINA PLATENSIS

2.10.1. Introduction

Globally, increased use of synthetic drugs has been associated with various adverse effects. Subsequently, there is increased global utilization of naturally occurring products that are considered to be economical, effective therapeutically, accessible and acceptable culturally among people living in resource limited countries (Abeer 2015; Farombi *et al.,* 2015). Furthermore, several pharmaceuticals in clinical use are obtained from plants (Sharma, 2006).

Several studies have been done on many herbal products and have confirmed they have some vital pharmacological effects. For instance, some beneficial effects in the

treatment of viral hepatitis have been confirmed in Glycyrrhizin, Silymarin, Curcumin and Schiszandra (Sharma, 2006; Yakoot *et al.,* 2012; Rethinasamy, 2017).

In addition to the above, naturally occurring food items and antioxidants including vitamins and other phytochemicals have attracted increased recognition. They act as chemo preventive agents against genotoxicity and oxidative damage. They are rich in several antioxidant substances, including: tocopherols, carotenoids, flavonoids, thiols and vitamins such as ascorbic acids (Mala *et al*., 2009; Abeer, 2015; El-beltagi *et al*., 2020).

Epidemiological and experimental data have revealed a number of natural and synthetic substances that have chemo preventive and chemoprotective effects against AF (Yakoot *et al*., 2012; Farombi *et al.,* 2015; Rethinasamy, 2017). Moreover, there is a need to discover and develop chemo preventive agents that remove AF present in food and feed without affecting their sensory and nutritional properties (Iqbal *et al*., 2019).

In conclusion, demand for herbal products and medicine has increased globally. Herbal drugs have gained popularity because of cultural beliefs, cost, their safety profile, efficacy and effectiveness (Mala *et al*., 2009; Tanuja *et al*., 2016; Makhuvele *et al*., 2020). Moreover, increased global demand in the utilization of phytochemicals and plant products has been documented (Makhuvele *et al*., 2020). Lastly, phytochemical analysis of plant derivatives and secondary phytochemicals has exponentially increased as a new drug source (Tanuja *et al*., 2016). Consequently, among these naturally occurring substances is the *Arthrospira platensis* species that was investigated in this study.

2.10.2. Historical use

During the 16th century invasion of Mexico by the Spanish invaders; they came across the Aztecs who lived in the Mexican valley, Tenochtitlan capital. It was then noted that the Aztecs collected some of their food from a nearby lake. Early Spanish writers described them as fishermen who had fine nets for collection of blue coloured substance “techuitlatl” that was found in the lake’s lagoons. A blue-greenish cake was then prepared from the substance (Habib, 2008; Ravi *et al.,* 2010).

It is documented that the Kanembu people who inhabited Lake Chad’s shores used clay pots to collect wet algae. The water was then drained out using cloth bags. It was then sun dried by spreading it in the sandy shores of the lake. Semi-dried algae were then chopped into small squares and sold in the villages. Sun drying process was done using mats (Habib, 2008; Ravi *et al.,* 2010).

Dihé (algae cake) was then broken into small pieces and mixed with slices of tomatoes and peppers. It was then consumed along with beans, millet or meat. This consists of approximately 70% of Kanembu meals (Habib, 2008). Pregnant women often ate the dihé cakes directly. Its dark colour was associated with the protective effect of their unborn baby against sorcerers (Habib, 2008). Lastly, Spirulina was also applied as an external poultice as a remedy of some diseases (Habib, 2008).

2.10.3. Renewed interest on Spirulina

A report was published in 1940 by Dangeard, a French phycologist; regarding algae cake consumption by Chad’s Kanembu sub-tribe (Habib, 2008). It was later found to be present in Kenya's Rift Valley lakes namely: Elementaita and Nakuru. It was also discovered in Ethiopia's lakes Kilotes and Aranguadi (Habib, 2008).

Spirulina thrives naturally in tropical and sub-tropical lakes rich in carbonate, bicarbonate and high PH. Huge quantities of spirulina can be found in Great Rift Valley lakes of East Africa, Lake Chad in Central Africa and Mexico's Lake Texcoco. *Arthrospira platensis* is present in South America, Asia and Africa. *Arthrospira maxima* is present only in Central America (Maddaly *et al*., 2010).

Jean Leonard a Belgian, in 1964 - 65, documented edible green cakes found in local markets N'Djamena in Chad for sale. The cakes were obtained from shores of Lake Chad. He associated algal blooms and the dried edible cakes that were available for sale in the local markets (Habib, 2008; Ravi *et al.,* 2010).

The International Association of Applied Microbiology recommended Spirulina as a potential "a future food source". Phytochemical analysis of the nutritional characteristics of spirulina revealed it contains 60 to 70% protein as its dry weight. It contains several essential amino acids. (Habib, 2008; Ravi *et al.,* 2010).

This preliminary investigation was adequate to enable the initiation of many industrial research projects in the 1970s. Due to its micro-organisms containing chlorella, yeast and some bacteria; this was then thought to be the easiest source of inexpensive proteins (Habib, 2008). Interestingly, US Space Program NASA has also investigated spirulina as a potential space travel food source (Capelli *et al*., 2010; Ravi *et al.,* 2010; DiNicolantonio *et al*., 2020). Furthermore, due to its nutritional properties, UN has identified it as one of the future foods (Abu-Elala *et al*., 2016).

2.10.4. Spirulina species

Various spirulina species have been identified: *Spirulina platensis, Spirulina pacifica* (also known as *Arthrospira platensis, Arthrospira maxima*). The most common is

Arthrospira platensis species. It has been grown on a large scale worldwide. However, *Arthrospira maxima* can be found in Central and South America (Habib, 2008; Ravi *et al.,* 2010; Farag, 2016).

Spirulina belongs to Phylum: *Cyanobacteria*, Class: *Cyanobacteriaceae*, Order: *Nostocales*, Family: *Oscillatoriaceae*, Genus: *Spirulina* (Ravi *et al.,* 2010; Arpita *et al*., 2014; Farag, 2016).

2.10.5. Health benefits of spirulina

Globally, Spirulina has been recognized as "a super food" owing to its nutritional composition and diversity (Ali *et al.,* 2012; Qinghua, 2016; Moneera *et al*., 2021). It has been confirmed to be an excellent food supplement by FAO of the UN (Ali *et al.,* 2012; Moneera *et al*., 2021). It was identified as one of the rich natural whole food sources. It has various macronutrients and micronutrients like iron, gamma-linolenic acid, vitamins, proteins and minerals (Sharma 2006; Hetta *et al*., 2014; Gutierrez *et al.,* 2015; El-beltagi *et al*., 2020). Consequently, its marketed as a food supplement (Capelli *et al.,* 2010; Ali and Mohammed, 2012; Qinghua, 2016).

In addition to these, Spirulina has been reported to enhance reproductive performance. It has also been used in the treatment therapy of anaemia, hypertension, arthritis, diabetes and cardiac disorders (Ali and Mohammed, 2012; Farag, 2016; Qinghua, 2016). Furthermore, anti-inflammatory, antimicrobial, antitumor, radio-protective and antiviral properties have been reported. Spirulina is known to have linoleic acid, phycocyanin, tocopherols and phenolic compounds. It has good antioxidant and oxygen scavenging properties (Asieh *et al*., 2016; Fatma, 2018; Ogechi e*t al*., 2021).

It has also been used in the preparations of nutritional drinks, frozen desserts, beverages, fruit juices, popcorn and food bars (Moneera *et al*., 2021). Moreover, current data has reported supplementation of food substances with various concentrations of spirulina. This augments nutritional quality and sensory characteristics of the food substances. Furthermore, it has also been used in the formulation of special foods for the elderly (Moneera *et al*., 2021; Ramírez-Rodrigues *et al*., 2021). Lastly it has been used in the cosmetic, natural dye in both pharmaceutical and food products industries (Asieh *et al*., 2016; Ramirez-Rodrigues *et al*., 2021).

2.10.6. Probable mechanism of action of *Spirulina platensis*

When the rate of Reactive Oxygen Species (ROS) formation rises or regenerative mechanism reduces; as well as when the number of scavengers for ROS reduces; oxidative stress will occur (Sharma 2006; Omar, 2013). ROS attack and destroy many tissues and cells thus causing many diseases. ROS include: peroxyl radical, superoxide anion, hydroxyl radical and hydrogen peroxide. They can cause protein oxidation, lipid peroxidation and death of cells. Subsequently, malondialdehyde (MDA), 4-hydroxynonenal and lipid peroxides (LPOs) have been pinpointed as the principal markers of oxidative stress.

Oxidative stress has been associated with increased protein oxidation. Experimental animal models have demonstrated this. One of the ways applied in the determination of protein oxidation is by protein carbonyls levels determination. Protein carbonyls are produced during protein oxidative cleavage. Amino acids like glutamic acid, threonine, proline, lysine, histidine and arginine undergo direct oxidation (Sharma, 2006; Omar, 2013).

Determination of protein carbonyl levels as the principal oxidative stress biomarkers gives some advantages when compared with determination of other products of oxidation. First, they are formed relatively earlier. Secondly, carbonylated proteins are more stable when compared to other oxidation products.

Derivatization 2, 4-dinitrophenylhydrazine (DNPH) in the assay determinations for detection of protein carbonyl groups in most of the assays. A dinitrophenyl (DNP) hydrazone stable product is formed. This product can then be detected and measured by different methods including: electrophoresis followed by Western blot immunoassay, enzyme-linked immunosorbent assay (ELISA) and spectrophotometric assay. It is important to note that, derivatization with DNPH and then assay of protein carbonyl groups is the most commonly used method in the determination of protein oxidation (Sharma, 2006; Omar, 2013).

TBARS (TBA-MDA) (Thiobarbituric Acid Reactive Substances) is the most commonly used method in lipid peroxidation determination. This is due to its simplicity and cheapness. The principle of this method consists in the reaction of Thiobarbituric acid reacts with MDA in acidic conditions and at an elevated temperature leading to formation of a pink MDA-(TBA) complex, which can be measured spectrophotometrically at 532 nm. TBARS method measures MDA equivalents formed during lipid peroxidation. However, other aldehydes produced during lipid peroxidation may react with TBA. The results of the assay are expressed in µmol of MDA equivalents (Sochor *et al*., 2012).

Oxidative stress can also be determined by measurement of malondialdehyde (MDA), 4-hydroxynonenal and lipid peroxides (LPOs). However, these are not commonly used (Isabella *et al.,* 2003).

Among the body's protective mechanisms against ROS is the antioxidation system. This comprises of enzymes like; catalase, glutathione peroxidase, superoxide dismutase (SOD), glutathione reductase. It also comprises antioxidants like uric acid, glutathione, vitamin E and vitamin C.

Moreover, some indigenous foods and antioxidants derived from food such as phenolic phytochemicals and vitamins have attracted increased attention in the recent past. They have been identified as potential chemo preventive agents that can reverse genotoxicity and oxidative damage. They contain numerous antioxidant substances including thiols, carotenoids, flavonoids, tocopherols, phenolics and vitamins such as ascorbic acid (Yakoot *et al*., 2012; Abeer, 2015; Farombi *et al.,* 2015). Subsequently, antioxidant, anti-inflammation and immunomodulation characteristics of *Spirulina platensis* may have a paramount role in chemoprotective effect against AF induced toxicities in this study.

Lastly, other proposed mechanisms of chemoprevention against aflatoxicosis include the induction of phase 2 metabolic enzymes. Rodent model experiments for chemoprevention of AFB_1 induced hepatocarcinogenesis have reported enzyme induction to be associated with the protective effects of naturally substances and synthetic substances like ethoxyquine, oltipraz, BHA and coumarin and (Farombi *et al*., 2015).

2.10.7. Spirulina platensis safety

Many toxicological investigational studies have confirmed its safety. The US FDA has listed and classified it in the class "Generally Recognized as Safe" (GRAS) (Yakoot *et al.,* 2012). No acute or chronic toxicities have been reported thus making it safe and fit for human consumption (Table 2.3); (Ali and Mohammed, 2012; Gutierrez *et al.,* 2015; Qinghua, 2016; Asieh *et al*., 2016).

Interestingly, renewed interest in *Spirulina platensis* has been based on the fact that it is believed to be easily absorbed, non-toxic and its protective effects against various chemical agents and drug induced toxicities (Ibrahim *et al*., 2015). However, some side effects have been documented (Table 2.3).

Table 2. 3: Toxicity of Spirulina

(Adapted from Gutierrez *et al.,* 2015)

Toxicity test	**Dose**	**Results**
Acute	oral single (800mg/Kg) in rats	No weight changes, no allergic reactions and no mortality
Sub - chronic	Feeding mice / rats (30% of feed level) for 13 weeks	No adverse effects on weight, blood, kidneys or laboratory parameters
	60% of feed level	Increased weight of kidney, heart and lungs, nephrocalcinosis syndrome
Chronic	48% of feed level	No adverse reactions
Reproductive (Fertility, teratogenic, multigenerational studies, peri & post-natal studies)	30, 20 and 10% of feed level	Adverse effects were not reported
Genotoxic (short & long term)	10, 20 and 30% of feed level	No germinal mutations

2.10.8. Commercialization of Spirulina

During the 1970s, a large-scale production by Sosa Texcoco was started. Its commercial activities involved three steps: planting, harvesting and processing (Maddaly *et al*., 2010). Currently, Spirulina is produced and used in many different nations including: Chile, Germany, Brazil, France, Spain, Canada, Egypt, Belgium, United States, Argentina, Philippines, Ireland and India.

In some countries it is certified as a food additive. It is also the main ingredient in some processed foods and drinks (Qinghua, 2016). Among the leading Spirulina producing companies globally include: Hainan in China, Cyanotech in USA, Genix in Cuba and Marugappa Chettir in India. Spirulina is formulated as tablets, capsules and powders. The US FDA has approved Spirulina extract as a colouring agent in various foods including: candy, ice cream, dessert coatings, yoghourts, chewing gum, among others (Qinghua, 2016).

2.11. Aflatoxins studies using experimental animal models

A comparative study on AFB_1 metabolism using albino mice and rats revealed that albino mice have similar susceptibility levels to the acute oral effects of AFB_1 (Juma *et al*., 2015). However, rats were reported to be less resistant to the AFB_1 carcinogenic effects compared to mice. Secondly, mice had a higher AFB_1 rate of metabolism compared to rats. This has been largely associated with their cytochrome P450 activity which is higher in comparison to rats. Hence, this enhances the resistance of mice to the carcinogenic effects of AFB_1.

Experimental animal models that use mice have been performed by several investigators (Table 2.4) in the investigation of aflatoxin toxicity among other

toxicants (Verma, 2001; Brahmi *et al.,* 2011; Miao, 2016). It has previously been documented that, acute effects of aflatoxins can be evaluated using mice while its chronic effects can be evaluated using rats (Juma *et al*., 2015).

Development of mice occurs rapidly. Sexual maturity is attained at the age of 40 to 60 days. It has been approximated that each month of a mouse to be equivalent to two and half human years (Wilson *et al*., 2016). Subsequently, 8 weeks old male Swiss albino mice were used to carry out the experimental work. In the present study, only male mice were included because of their higher susceptibility to aflatoxins compared to the female mice (Gupta and Sharma 2011; Mishra *et al*., 2016).

Mice are more resistant to hepatotoxicity of AFB_1 because of their higher of GSTA metabolic activity against AFB_1. This is in contrast to rats which are highly susceptible to carcinogenicity and hepatotoxicity caused by AFB_1 (Ishida *et al*., 2020). Subsequently, such interspecies variations in their sensitivity to hepatotoxicity induced by AFB_1 might limit the extrapolation of experimental animal study findings to humans (Ishida *et al*., 2020).

Furthermore, the severity of the response intraspecies is determined by sex, age, diet, infectious agents' exposure, weight and presence of other contaminants (Bennett and Klich, 2003).

Interestingly, most of the studies done using animals such as mice, rats and chicken are preliminary and largely exploratory in nature. Secondly, further clinical investigations are usually recommended by using other animals like monkeys. These

usually require further assessment regarding their effectiveness in aflatoxin poisoning management before they can be applied for veterinary and clinical use.

Furthermore, there is limited data regarding the efficacy of herbal products administered through either oral or other routes (Juma *et al*., 2015). Overall, study findings extrapolation of animal models experiments to humans does not consider the various daily changes that may occur in human's aflatoxin exposure (Kensler *et al*., 2011).

Table 2. 4: Summary of studies conducted using chemoprotective substances against aflatoxicosis using different species of mice and rats

	Author (Year)	Animal species (No. of animals)	Experimental duration	AFB_1 levels used in the study	Substance evaluated	Effect evaluated
1.	Verma, (2001)	Male albino rats (70)	45 days	750 and 1500 g/kg body weight	Vitamin E	AF induced lipid peroxidation
2.	Farombi *et al*., (2005)	Wilstar albino rats (40)	6 weeks	2 mg/kg as single dose	Kolaviron, using Garcinia kola seeds	AFB_1 induced hepatic damage and genotoxicity
3.	Brahmi *et al.,* (2011)	balbC male mice (54)	5 weeks	250 µg/Kg body wt 3 days / week for 4 weeks	cactus cladode extract	Oxidative stress & genotoxicity of AFB_1
4.	Yassein and Zghair,	Swiss male albino mice (25)	35 days	25 µg/ 0.2 ml olive oil/ animal/ day	None	Toxicity and pathogenicity of AFB_1 and

	(2012)					AFG_1
5.	Abeer, (2015)	Male albino rats (40)	4 weeks	250 µg/kg body weight / day for 5 days a week for 28 days	Curcumin	AFB_1 induced on renal cortex histology & immune-histochemical
6.	Miao, (2016)	Male Kunming mice (50)	3 weeks	100 µg/kg body weight	Grape Seed Proanthocyanidin Extract	Sub-chronic Immune Injury Induced by AFB_1
7.	Yaman *et al*., (2016)	Sprague–Dawley rats (18)	90 days	25 µg of AF/rat/day for 90 days	Honey	Histopathological and biochemistry
8.	Mishra *et al* (2016)	Swiss male albino mice	30 days	2 µg/30 g bw	Bougainvillea spectabilis leaves	Protection against AF induced hepatotoxicity
9.	Seham *et al*., 2018	male albino mice (28)	28 days	5 µg/KG	Dietary Turmeric	Immunomodulatory Effect of Dietary Turmeric against AF in Mice

CHAPTER THREE

MATERIALS AND METHODS

3.1. Introduction

This chapter presents study materials and methods. It covers: materials and equipment, experimental design, animal husbandry and experimental protocol. It has evaluation of protective effect of *Spirulina platensis* extract against biochemical, histopathological and immune changes induced by AFB_1. Also, it presents evaluation of mechanism of action of *Spirulina platensis* extract against AFB_1. Lastly, it highlights ethical considerations, data collection and analysis.

3.2. Study site

The study was done at the Centre of Traditional Medicine and Drug Research (CTMDR) in Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. KEMRI is a State Corporation established in 1979 through the Science and Technology (Repealed) Act, Cap 250 of the Laws of Kenya. The institution currently operates under the Legal Notice No. 35 of March 2021. The GPS coordinates of the institution are 1.3140700^0 S, 36.8034200^0 E. The vision of the institution is to be a leading centre of excellence in human health research. KEMRI is mandated to carry research activities in human health in Kenya.

3.3. Materials and Equipment

3.3.1. Chemicals

All the required materials were obtained from well recognized and reputable suppliers to guarantee the quality of the materials. Importation of AFB_1 (AF031) was done from Fermentek® Ltd, Jerusalem, Israel. The AFB_1 received was of high analytical grade and it was used as per the study protocol without any further purification.

3.3.2. Preparation of AFB_1 stock solution

Ten (10) mg of AFB_1 was dissolved in seven (7) % Dimethyl sulfoxide (DMSO). Seventy (70) ml of DMSO was added to nine hundred and thirty (930) ml of distilled water in the preparation of 7% DMSO solution. Preparation of AFB_1 stock solution was done in the approved, calibrated fume hood (CTMDR Hood no: 3, Model FH1500(E) serial No: FH15E111807005 manufactured by Jinan Biobase co. Ltd, China). Aflatoxins undergoes light decomposition. Therefore, preparation was kept in amber coloured vials in a dark place (Lijuan *et al*., 2016; Hua-Lian *et al*., 2016; Colla *et al*., 2017).

3.3.3. Extraction of *Spirulina platensis* powder

Spirulina platensis powder (MMUSTMUG SPIRULINA®) was bought from MMUST botanical shop. A calibrated weighing balance (OHAUS® Pioneer PA 224 serial number B710800864) was used to weigh 210 g of *Spirulina platensis* powder. Weighed *Spirulina platensis* powder was then put into a 1-liter conical flask. Five hundred (500) ml of distilled water was subsequently added into the conical flask.

It was then covered with cotton wool and set in a water bath (Memmer® water bath, equipment Model No: W350, Serial No: 811009, KEMRI No: JJK 006). The water bath was heated and the temperature was maintained at 60^0C for 30 minutes. Filtration was done using Whatman® qualitative filter paper grade 1 (WHA 1001125). The filtrate obtained was kept in a 250 ml round bottom flask. It was subsequently covered with acetone and dry ice.

A freeze drier (Edwards® freeze dryer modulyo, KEMRI No: JJK0035) was then maintained at a pressure of 10 mbar and -30^0 C of temperature for 72 hours. 14.7 g of freeze-dried *Spirulina platensis* powder was obtained. Freeze-drying is a method that has been documented to give higher quality of products that are dehydrated. Subsequently, it protects the sample material from thermal degradation (Papalia *et al*., 2019). It was then kept in an airtight container at (2 to 8^{0}C) in a fridge. The extracted *Spirulina platensis* extract powder was then used to perform the experiments as laid out in the study protocol.

3.3.3.1. Preparation of *Spirulina platensis* aqueous suspension

A calibrated weighing balance (OHAUS® Pioneer PA 224 serial number B710800864) was used to measure 925 mg of *Spirulina Platensis* freeze dried powder. Then 98 ml of distilled water was added (Jinlu *et al*., 2019). Amber coloured container was used to store the aqueous suspension of *Spirulina platensis* at 0 to 8^0 C (Hua-Lian *et al*., 2016; Colla *et al*., 2017). It was then used to perform the experiments as laid out in the protocol.

3.4. Sampling design

Simple random sampling method was employed in the selection of 25 healthy male Swiss albino mice inbred at KEMRI animal house II.

3.5. Sample size calculation

Sample size determination was done using resource equation approach; Group comparison one-way ANOVA (Arifin *et al*., 2017; Charan and Kantharia, 2013).

The following formula was used:

$Df = N – k = kn – k = k (n – 1)$

Where:

N = total number of mice

k = number of groups

n = number of mice per group

Df = degrees of freedom

Reorganisation of the formula,

n = Df/k + 1

The Df were set at minimum (10) and maximum (20).

Therefore, based on these Df; minimum and maximum numbers of mice per group were determined as shown below.

Minimum n = 10/5 + 1 = 3

Maximum n = 20/5 + 1= 5

Hence, the total minimum and maximum numbers required are 15 and 25 mice respectively as shown below.

3 mice per group * 5 groups = 15 mice

5 mice per group * 5 groups = 25 mice

Subsequently, from the above sample size determination; 5 mice per group was adapted with the total number of groups being 5 in this study.

3.6. Animal husbandry

A request for inbred male Swiss albino mice aged 8 weeks was done by filling the experimental animal request form (appendix XII). Twenty-five male Swiss albino mice weighing 30 to 35 g were weighed using a calibrated animal scale (Shinano® animal scale, serial number 28099). They were randomly chosen and housed in labelled clean polypropylene cages (29 cm x 22 cm x 14 cm). They were then moved from the breeding room of KEMRI animal house II (Breeding room – Swiss albino

mice room I -13) to the experimental room of KEMRI animal house II (Experimental room I -3).

Animal experimental room temperatures ranged between 20^0 to 25^0 C during the study. Humidity was at 70%. The temperatures and humidity were taken twice a day at 9 am and at 3 pm. The room was well-ventilated and well built. It provided ideal experimental conditions; it is located away from excess noise, high temperatures and pollution. This reduced possible stress factors to animals before and during the experiment.

Free water access *ad labium* and standard pellet diet purchased from Unga® Feeds Limited, Kenya were allowed prior to the commencement of the experiment. Two weeks were allowed for acclimatisation of mice before the start of the experiments. 12-hour dark and light cycle was maintained. Weighing of the mice was done on days 0, 7, 14 and 28 (using weight monitoring log attached in appendix No. 1).

Standard animal handling procedures were followed. The National and Institutional guidelines for animal welfare protection during experiments were followed (National research Council of National academies, 2011). The requirements of The Prevention of Cruelty to Animal Act, chapter 360, Kenya; were adhered to. In addition to these, KEMRI Standard Operating procedures for CTMDR-Biological science laboratory for *in vivo* safety assays were strictly followed. Lastly, KEMRI Laboratory safety rules (KEMRI /CTMDR/LABS/DOC/LS/01 issue No: 1) were read and adhered to throughout the experimental procedures.

3.7. Aflatoxin analysis of experimental diet

Using a sample of experimental diet aflatoxin content was analysed. Rapid test kits were used to analyse aflatoxin content. The procedure was as per the instructions of

the manufacturer of enzyme immunoassay for detection of AFB_1 (Celer® AFLA B1) (Cat.nr.HU0040004) by Eurofins technologies®, Italy.

3.8. Experimental design

The study was done using *in vivo* inbred male Swiss albino mice as an experimental model. Experimental animal models that use mice have been performed by several investigators in the investigation of aflatoxins toxicity among other toxicants (Verma, 2001; Farombi *et al*., 2005; Brahmi *et al.,* 2011; Yassein and Zghair, 2012; Abeer, 2015; Miao, 2016; Mishra *et al*., 2016; Yaman *et al*., 2016).

Randomly twenty-five inbred male Swiss albino mice were randomly selected. One control group and 4 treatment groups were used in the study. Mice were randomly put into each of the 5 groups. They were housed in labelled polypropylene cages having a metallic top in the experimental room of the animal house II. Individual mice were identified using a tag having a study number.

Group I (Control): The mice were given a normal diet for 4 weeks.

Group II (*S. Platensis* 100 mg/kg/day): The mice were treated with *S. Platensis* extract 100 mg/kg/day orally by gastric tube for 4 weeks. In addition to the normal diet availed to the control group. Group II was used to monitor the effect of *S. Platensis* in the absence of AFB_1.

Group III (AFB_1 200 µg/kg/day): mice were treated with 200 µg/kg/day bw. of AFB_1, for 4 weeks administered orally by gavage. In addition to the normal diet availed to the control group.

Group IV (*S. Platensis* 100 mg/kg/day + AFB_1 200 µg/kg/day): mice in this group were pre-treated with *S. Platensis* 100 mg/kg/day, 1 hour before 200 µg/kg/day bw of

AFB_1 was given, for 4 weeks administered orally by gastric tube. In addition to the normal diet availed to the control group.

Group V (*S. Platensis* 200 mg/kg/day + AFB_1 200 µg/kg/day): mice in this group were pre-treated with *S. Platensis* 200 mg/kg/day, 1 hour before 200 µg/kg bw. of AFB_1 was given, for 4 weeks administered orally by gastric tube. In addition to the normal diet availed to the control group.

The experiment was done for 4 weeks. Documentation on mice health status was done using health status monitoring log (appendix II).

After 28 days, the mice were then euthanized by inhalation of carbon dioxide in an enclosed chamber. Their liver and kidney organs were dissected and washed using normal saline (0.9% NaCl). Dissected pieces were then put into neutralised 10% formalin for preservation before commencement of histopathological studies. They were well labelled for identification. They were stored at 0^0C - 8^0C until commencement of histopathological analysis procedures. All non-harvested mice tissues were disposed off as biohazard waste by incineration in accordance with biohazard waste disposal guidelines of KEMRI. This was done by filling the KEMRI Waste Tracking document (Form NEMA / WM/ 3) (Appendix XIV).

3.9. Mice feeding behaviour

The feeding behaviour of each of the groups was monitored. The mice ate most of their feed during the dark hours. Minimal feed was taken during daytime. Water intake was more during feed intake, minimal water intake took place in restricted mice.

3.10. Preparation of serum

Heart puncture technique after euthanization was done followed by aseptic blood collection into heparinized glass tubes. Centrifugation was then done for 10 minutes at 3000 g using a centrifuge. All collected samples were clearly labelled indicating the group and study number of the mice they were harvested from. Estimation of serum enzymes was then done using the clear supernatant liquid (Farombi *et al.,* 2005).

The following essential information was documented in a legible manner on the sample containers at the time of sample collection:

i. Mice study identification number
ii. Date and time of sample collection
iii. Initials of the investigator assistant collecting and labelling the sample.
iv. Test required to be performed

3.11. Evaluation of protective effect of *Spirulina platensis* extract on biochemical changes induced on liver by AFB_1.

The serum samples processed above were then subjected to biochemical analysis of globulin, plasma total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Laboratory analysis was done using an autoanalyzer at Pathologists Lancet Kenya Limited Laboratories, Nairobi, Kenya (Tamta *et al*., 2009).

3.12. Evaluation of protective effect of *Spirulina platensis* extract on histopathological changes induced by AFB_1.

Histopathological studies were done on the samples prepared as indicated above at the department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi. Tissue samples of liver and kidney were

embedded in paraffin wax and after that, staining with hematoxylin and eosin was done. Paraffin sections were cut serially at 6 mm thickness using a rotary microtome.

Microscopic examination was then done using Olympus®, CX21 microscope, Tokyo, Japan. Eyepiece lens magnification of X10, objective lens magnification of 3 types (X4, X10 and X40) were used to clearly visualise the tissues.

Total microscope magnification used

= magnification of objective lens x magnification of eye piece lens

Therefore, photomicrographs had X40, X100 and X400 magnifications.

During microscopic examination the mice tissues were inspected for the following histopathological changes.

i. Inflammation: Distension of vein, venule and capillary lumen with red blood cells.
ii. Necrosis: Premature death of cells in living tissues caused by cell injury.
iii. Haemorrhage: Red blood cells outside the lumen of a blood vessel(s)
iv. Fatty changes: Tissue deterioration of fats and their structure.

The histopathological changes were assessed by three independent assessors. For each animal tissue three reading fields were read. The grading was done semi-quantitatively using ordinal four step scale system (Meyerholz and Beck, 2019, Landmann *et al*, 2021) as shown below.

i. Normal: no distinguishable change (0 %)
ii. Mild: up to 30% changes noticed
iii. Moderate: changes of 31 -60%

iv. Severe: changes of 61 – 100 %

3.13. Investigation of protective effect of *Spirulina platensis* extract against AFB_1 induced immune depression.

Blood samples collection was done aseptically in serum separator tube (SST). Clotting was allowed for 2 hours at room temperature. Fifteen minutes of centrifugation was then done at 4000 Xg. 0.3 ml of serum was then measured from the centrifuged samples using a calibrated micropipette. The serum collected was then stored at -20^{0}C. During analysis the samples were defrosted at room temperature (25^{0}C ± 2^{0}C). They were subsequently used to perform the assay procedures as illustrated (Figures 3.1 and 3.2).

ELISA Kits for quantitative determination of mouse IgM, IgG and IgA were imported from Beijing Solabio Science & Technology co. Limited; China (Cat#SEKM-0098; Lot. No. 0604K9121, Lot No. 20191212 and Lot No. 0604K9122 respectively). The concentration IgM, IgG and IgA in serum were estimated using ELISA kits. Analysis procedures were followed in line with the manufacturer's manual directions of the ELISA kits used in the quantitative determination of immunoglobulin biomarkers in serum. A 96 microplate well (12 strips of 8 wells) template was used to tabulate the results (appendix XIII).

3.13.1. Assay procedure for quantitative determination of IgG

Preparation of reagents and test standards was done as recommended in the manual. The plate was washed 3 times before the assay

↓

100 µl of standard or sample was added to each well. Incubation for 2 hours at room temperature (25± 2^0C) was done

Aspirate and wash for 4

↓

100 µl of working solution of Biotin-conjugate anti-mouse IgG antibody was added to each well. Incubation for 1 hour at room temperature (25±

Aspiration of the contents

↓

100 µl of working solution of Streptavidin-HRP was added to each well. Allow it to stand for 30 minutes at room temperature (25± 2^0C)

Aspiration of the contents

↓

100 µl of substrate solution was added to each well. Allowed to stand for 15 minutes at room temperature (25± 2^0C). It was protected from light.

↓

50 µl of stop solution was added to each well. Then reading was done at 450 nm within 5 minutes

Figure 3. 1: Flow diagram showing procedure for quantitative determination of IgG

3.13.2 Assay procedure for quantitative determination of IgM & IgA

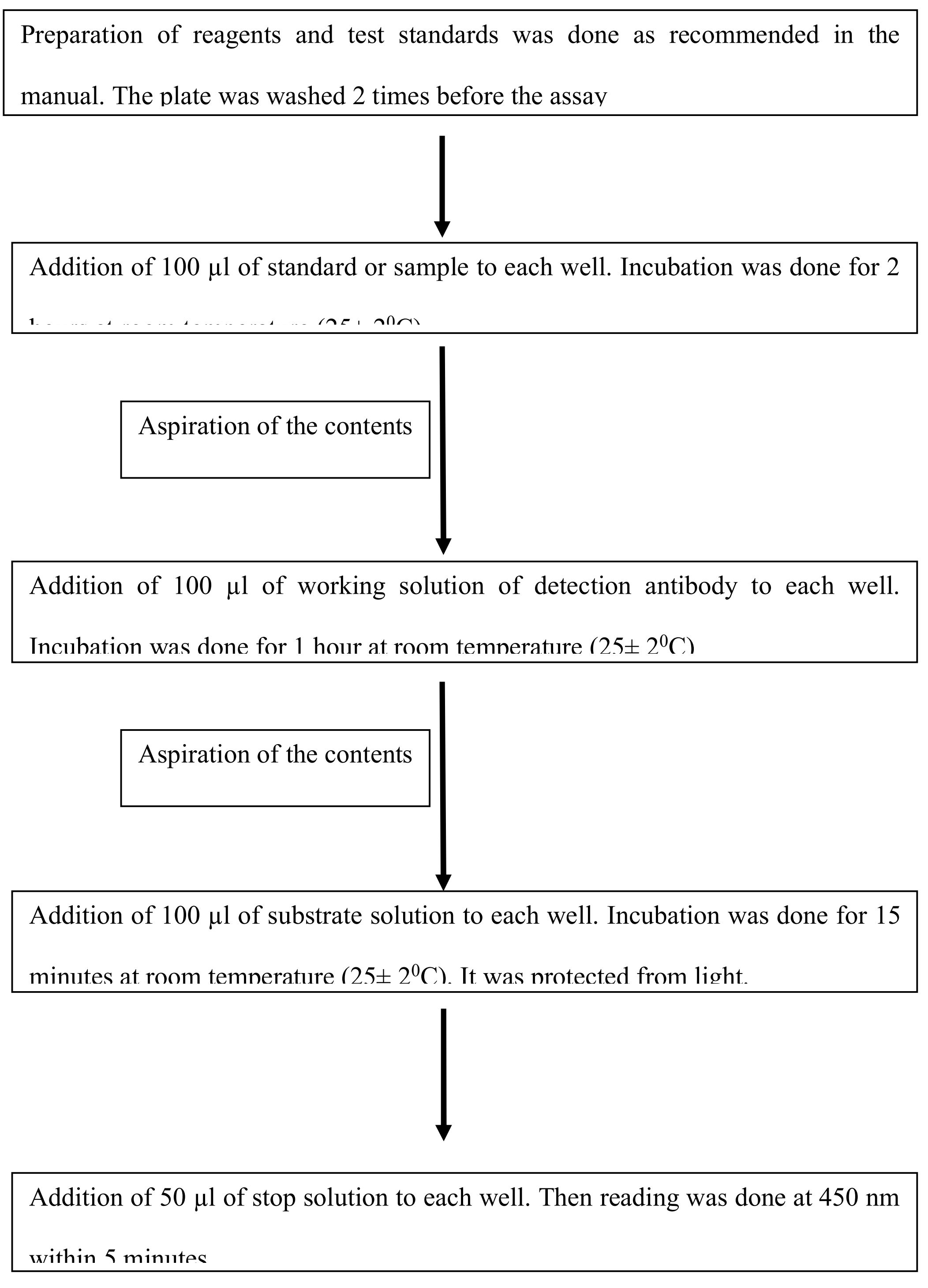


Figure 3. 2: Flow diagram showing procedure for quantitative determination of IgM and IgA.

3.14. Investigation of the probable mechanism of action of the protective effect *Spirulina platensis* extract against aflatoxin B_1 induced toxicity.

Malondialdehyde (MDA) and lipid hydroperoxide (LHP) formation in mice were used to assess lipid peroxidation. LHP was investigated by adapting the thiocyanate method. Determination of MDA was done using Farombi *et al.,* 2005 method. LHP was investigated by adapting thiocyanate method by Cavallini *et al*., (1983). The thiobarbituric acid reactive substances (TBARS) assay was measured using Farombi *et al.,* 2005 method. Determination of MDA is regarded as an excellent tool in lipid oxidation assessment. MDA is a final lipid oxidation product. It is regarded as oxidative stress and cell injury biomarker (Brahmi *et al.*, 2011).

3.14.1. Preparation of 0.15M KCL

Determination of the number of moles required in the given volume was done using the following formula.

Moles KCl = (0.100L) (0.15M) = 0.015 moles

Conversion of the moles to grams was then done as shown below.

Mass of KCl= (0.015 mole) (74.55 g/mol) =1.1 g KCl

1.1 g of potassium chloride was then weighed in a 100 ml volumetric flask. Dilution was then done using distilled water that was topped up to 100 mL. This solution was then used during the experiment as per the protocol in the next page.

3.14.2. Preparation of 50% Trichloroacetic acid (TCA)

Addition of 400 ml of distilled water was done into a half a litre volumetric flask. Trichloroacetic acid (TCA) 550.16 g was then added. It was then topped up to the 500 ml mark using distilled water. This solution was then used during the experiment as per the study protocol.

3.14.3. Assay procedure for thiobarbituric acid reactive substances (TBARS)

Fifty (50) mg of the kidney and 50 mg of the liver harvested, were mashed separately. The following procedures were then followed for each of the mashed samples of liver and kidney. Addition of ten (10) ml of distilled water was done. Then 1 ml of 0.15M KCl was added. Then centrifugation was done at 1000 Xg for 30 min. Then to 0.5 ml of supernatant solution, 0.5 ml 50% TCA was added. Centrifugation was then done at 5000 Xg for 5 min.

Aluminium foil was used to cover it, to protect it from light. It was then kept for sixty minutes (60) in a water bath set at 90^{0}C. Absorbance reading of the resulting solution was then done at 540 nm at room temperature (26.2^{0}C). A 96 microplate well template was used to tabulate the results (appendix XIII).

Absorbance was read using Multiscan Go® machine (Serial No: 1510 – 04920). Software used was Skanit® 4.1 for microplate readers version 4.1.0.43. All samples were read simultaneously. The results obtained were then exported to MS Excel® sheet for data analysis. A standard calibration curve was plotted using concentrations of the blank and thiobarbituric acid reactive substances.

3.15. Laboratory Safety and Precautions

Throughout the study KEMRI standard operating procedures were strictly adhered to. Secondly, KEMRI standard operating procedure for CTMDR - Biological Science Laboratory; in-vivo assays were adhered to. In addition, KEMRI Laboratory Safety rules reference number: KEMRI/CTMDR/LABS/DOC/LS/01 issue 1 were strictly followed.

Adequate safety precautions were observed while handling of AFB_1 as it is hepatotoxic, carcinogenic and mutagenic (Sung-Eun *et al*., 2001; Knipstein *et al*., 2015). Fermentek® Limited AFB_1 Safety Data Sheet precautions were adhered to throughout the experimental period. AFB_1 involving procedures were done in the approved, calibrated fume hood (CTMDR Hood no: 3, Model FH1500(E) serial No: FH15E111807005, Manufactured by Jinan Biobase co. Limited, China).

Personal protective equipment: face mask, eye protection, dust coat and gloves were used throughout the experimental period. Work areas were sanitised using 70% alcohol before and after the experiments.

Control animals and AFB_1 exposed animals were kept in separate well labelled cages. The cages did not house any other animals during the study. During the experimental period, proper documentation of the study procedures was strictly adhered to.

Cleaning of the animal cages was done twice a week. Seventy (70) % alcohol was used in the saturation of animal feed remains, soiled beddings and any other disposable waste prior to disposal.

Mice were transferred to animal feed and AFB_1 free cages after 28 days. They were then taken to the experimental room where blood and organs were harvested as per

the study protocols. Based on KEMRI waste disposal guidelines, the remaining mice tissues were disposed of as biohazardous waste.

Soaking of glassware equipment used during the experiments in 5% aqueous sodium hypochlorite was done to destroy residual aflatoxins. They were then cleaned before their re-use (Lijuan *et al*., 2016).

All chemicals and reagents used in this protocol were procured from reputable suppliers who supply KEMRI stores. All chemicals and reagents procured were stored as per the individual recommendations of the manufacturer so as to guarantee their safe handling and stability throughout the experiment period.

3.16. Handling and transportation of biological specimen

All biological specimens generated during the study were considered and treated as potentially hazardous. The principal investigator ensured safe packaging, labelling and transfer of any biological specimens from the experimental room in the animal house II to the analytical laboratories of KEMRI, Pathologists Lancet Limited and the department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, UON. Samples were packaged in well-sealed, labelled containers to avoid spillage during their handling and transportation. Face mask, eye goggles, dust coat and gloves were put on during the handling of biological specimens.

3.17. Handling and disposal of waste

The waste generated by the study was sorted into 2 colour coded plastic bags (black and red). They were used for non-infectious (black) and infectious (red) waste. Bags

were well labelled. The polythene bags containing the infectious material were leak proof. All sharps used in the study were kept in the sharp's safety box regardless of whether or not they were contaminated. Waste disposal polythene bags were placed in all points where particular categories of waste were generated.

The waste collected was disposed of as frequently as required. All remaining animal tissues at the end of the experiment were disposed of as biohazardous waste as per KEMRI waste disposal guidelines. This was done by filling the KEMRI waste tracking document (FORM NEMA/WM/3) (appendix XIV). The waste was then taken to the KEMRI incinerator for combustion.

3.18. Data and information collection

The following data capture tools were used to capture the general data during the experiment: Weight monitoring tool (appendix I) and health status monitoring tool (appendix II).

For the objectives number 3 and 4, a 96- well template (appendix XIII) was filled. Reading of absorbance of the samples was done using Multiscan Go® machine of Serial No: 1510 – 04920. Its software was Software Skanit® 4.1 for microplate readers version 4.1.0.43. The results obtained were then exported to MS Excel® 2013 sheet for data processing and analysis.

3.19. Data analysis and presentation

Data entry, its management, its preliminary summaries like means, their percentages and other descriptive statistics were done using Microsoft Excel® 2013 software. Python® 3.0 with statistical libraries, a data analytical software was used to perform data analysis. Expression of data was as the mean ± standard deviation (SD). One-

way ANOVA statistical test was performed in the comparison of group means. Data was statistically significant if (P <0.05). If statistically significant differences were found (P<0.05), post-hoc comparisons between multiple groups were carried out using Tukey's Honestly Significantly Differenced (HSD).

Non-parametric analysis using Mann-Whitney U test was done for two group comparisons of the semi-quantitative ordinal data of histopathology photomicrographs. Dunnet's post-hoc-tests were then done for resulting pairwise comparisons.

All data was presented in tabular format. Photomicrographs were used in presentation of histopathology findings.

3.20. Ethical Considerations

Study approval from the Directorate of Postgraduate studies MMUST was obtained, (appendix V). Secondly, study approval was acquired from Institutional Ethics Review Committee (IERC), MMUST (appendix VI). Thirdly, approval was sought from National Commission for Science, Technology and Innovation (NACOSTI) was obtained, (appendix VII). NACOSTI study licence number: *A20847* (appendix VIII), was received. Lastly, KEMRI Scientific and Ethics Review Unit (SERU); Animal Use and Care committee granted study approval to carry out the study in KEMRI laboratories (appendix XI).

CHAPTER FOUR

RESULTS

4.1. Introduction

Presentation of study results are clustered as per specific study objectives in this chapter.

4.2. Weight changes

There were some weight changes in mice observed (Table 4.1). On day 1, group I (control) had the highest mean weight (34.6 g), the lowest mean weight was 31.8 in group IV (*S. platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day.

Table 4. 1: The mean weight in grams of male Swiss albino mice during the experiment period

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Control (Group I)	34.6	36.2	37	37	36.4
SP 100 mg/Kg/day (Group II)	32.2	32.2	33	32.8	33
AFB_1 200 µg/Kg/day (Group III)	32.2	31.6	31.8	31.6	31.4
SP 100 mg/Kg/day + AFB_1 200 µg/Kg/day (Group IV)	31.8	31.6	31.4	31.4	31.4
SP 200 mg/Kg/day + AFB_1 200 µg/Kg/day (Group V)	32.6	32.4	33	32.8	33.2

On day 28, group I (control) had the highest mean weight (36.4 g), the lowest mean weights were found in group III (AFB_1 200 µg/Kg/day) and group IV (*S. platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day.

A drop in average weight in group III (AFB_1 group) mice was noted. A gradual weight increase in mice in group I (control) was observed. A median weight growth for mice in groups 2 (*S. Platensis* 100 mg/Kg/day), 4 (*S. Platensis* 100 mg/Kg/day + AFB1 200 µg/Kg/day) and 5 (*S. Platensis* 100 mg/Kg/day + AFB1 200 µg/Kg/day) was noted, (Table 4.1).

One-way ANOVA analysis showed that among the 5 groups, the mean weight differences were statistically significant at $P < 0.05$, F (66.61). Some group-to-group differences were observed in the mean weight changes using Tukey's Honestly Significantly Differenced (HSD) for do post-hoc comparison (Table 4.2).

Table 4. 2: Post-hoc comparison of mean weight using Tukey's Honestly Significantly Differenced (HSD)

	Group I	Group II	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	3.60	2.612897	4.587103	15.434873	0.0010*
1	Group I	Group III	4.52	3.532897	5.507103	19.379340	0.0010*
2	Group I	Group IV	4.72	3.732897	5.707103	20.236833	0.0010*
3	Group I	Group V	3.44	2.452897	4.427103	14.748878	0.0010*
4	Group II	Group III	0.92	-0.067103	1.907103	3.944467	0.0755
5	Group II	Group IV	1.12	0.132897	2.107103	4.801960	0.0214*
6	Group II	Group V	0.16	-0.827103	1.147103	0.685994	0.9000
7	Group III	Group IV	0.20	-0.787103	1.187103	0.857493	0.9000
8	Group III	Group V	1.08	0.092897	2.067103	4.630462	0.0278*
9	Group IV	Group V	1.28	0.292897	2.267103	5.487955	0.0074*

Note: * Statistically significant

Statistically significant differences were noted in all group-to-group comparisons except for Group II (100 mg/kg/day of *S. platensis* extract) when compared with group V (*S. Platensis* 200 mg/kg/day + 200 µg/kg/day of AFB1) and group III (AFB1 200 µg/Kg/day) when compared with group IV (*S. platensis* 100 mg/kg/day + 200 µg/kg/day of AFB1); which both had a P value of 0.900.

4.3. Evaluation of the protective effect of *Spirulina platensis* extract on biochemical changes induced by AFB_1.

There were some biochemical changes in serum observed. The highest AST levels were observed in group III (AFB_1 200 µg/Kg/day) with a mean value of (256±115.99 IU/L). Intermediate mean values of (230.5±96.03 IU/L) and (229.80±95.01 IU/L) were found in the mice in group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** respectively. The lowest AST mean levels were seen in mice that received *S. Platensis* 100 mg/Kg/day (group II) and in group I (control) with a mean level of (131.60±19.32 IU/L) and (176.75±44.34) respectively, (Table 4.3).

The highest ALT levels were observed in group III (AFB_1 200 µg/Kg/day**)** with a mean value of (51.50±8.19 IU/L). Intermediate mean values of (47.25±10.81 IU/L) and (49.80±7.92 IU/L) were found in the mice in group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** respectively. The lowest ALT mean levels were seen in mice group II (*S. Platensis* 100 mg/Kg/day) and in group I (control) with AST mean levels of (43.20±12.38 IU/L) and (44.00±6.83 IU/L) respectively.

The highest ALP levels were observed in group I (control) with a mean value of (59.40±6.91 IU/L). Intermediate mean values of (49.75±4.11 IU/L) and (51.75±11.89 IU/L) were found in the mice in group III (AFB_1 200 µg/Kg/day) and group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day) respectively. Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) had the lowest ALP mean levels of (26.50±13.48 IU/L).

Table 4. 3: Biochemical changes in the serum of mice after 28 days exposure period.

	Normal reference (Serfilippi *et al*., 2003, Otto *et al*., 2016)	**Control (Group I)**	**SP 100 mg/Kg (Group II)**	**AFB_1 200 µg/Kg(Group III)**	**SP 100 mg/Kg + AFB_1 200 µg/Kg (Group IV)**	**SP 200 mg/Kg + AFB_1 200 µg/Kg (Group V)**
AST (IU/L)	48 (40-200)	176.75±44.34	131.60±19.32	256.0±115.99	230.5±96.03	229.80±95.01*
ALT (IU/L)	30 (24-40)	44.00±6.83	43.20±12.38	51.50±8.19	47.25±10.81	49.80±7.92*
Bilirubin (total) (µmol/L)	2.3 (2.1 – 2.4)	2.40±0.55	2.20±0.45	2.25±0.50	2.0±0.01	2.0±0.01
ALP (IU/L)	90 (80-100)	59.40 ±6.91	41.80±20.57	49.75±4.11	51.75±11.89	26.50±13.48*
Total protein (g/L)	50.0 (48-53.3)	66.00±3.39	71.40±10.06	69.25±4.79	76.25±11.38	77.25±12.12
Albumin (g/L)	27 (24.2-31.0)	34.2±16.4	34.2±1.30	34.0±0.82	34.25±1.89	34.00±1.41
Globulin (g/L)	24.5 (17-32)	31.8±3.35	37.20±9.20	35.25±4.99	42±9.83	43.25±11.17

Mean Values ±SD *statistically significant ($p \leq 0.05$)

With regard to bilirubin (total) levels the mean values ranged between 2.0 µmol/L and 2.4 µmol/L for all the 5 groups. The results were statistically insignificant with a p value of 0.3202.

The highest total protein levels were observed in group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** and group IV (*S. platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** with mean values of (77.25±12.12 g/L) and (76.25±11.38 g/L) respectively. Intermediate mean values of (71.40±10.06 g/L) and (69.25±4.79 g/L) were found mice in group II (*S. platensis* 100 mg/Kg/day) and group III (AFB_1 200 µg/Kg/day respectively.

With regard to albumin levels the mean values ranged between 34.0 g/L and 34.2 g/L across the 5 groups. The results were statistically insignificant with a p value of 0.9596.

The highest globulin levels were observed in group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** with a mean value of (43.25±11.17 g/L). Intermediate mean values of (35.25±4.99 g/L and (37.2±9.20 g/L) were found in the mice in group II (*S. platensis* 100 mg/Kg/day**)** and group III (AFB_1 200 µg/Kg/day**)** respectively. The lowest globulin mean levels was observed in the control group mice with a mean level of (31.8±3.35 g/L). The results were statistically insignificant with a p-value of 0.3202.

Overall, one-way ANOVA analysis revealed that ALT, AST and ALP changes among the 5 groups were statistically significant (P= 0.0491, 0.0519 and 0.004) respectively.

Some group-to-group differences were observed after post-hoc comparison of ALT levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.4).

Table 4. 4: Post-hoc comparison of ALT using Tukey's Honestly Significantly Differenced (HSD)

	Group	Group	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	6.60	-9.6600	22.8600	1.7179	0.7205
1	Group I	Group III	0.95	-15.3100	17.2100	0.2473	0.0540*
2	Group I	Group IV	1.70	-14.5600	17.9600	0.4425	0.9000
3	Group I	Group V	2.55	-13.7100	18.8100	0.6637	0.9000
4	Group II	Group III	7.55	-8.7100	23.8100	1.9651	0.0526*
5	Group II	Group IV	8.30	-7.9600	24.5600	2.1603	0.5518
6	Group II	Group V	4.05	-12.2100	20.3100	1.0541	0.0490*
7	Group III	Group IV	0.75	-15.5100	17.0100	0.1952	0.9000

8	Group III	Group V	3.50	-12.7600	19.7600	0.9110	0.0390*
9	Group IV	Group V	4.25	-12.0100	20.5100	1.1062	0.9000

Note: * Statistically significant (p≤0.05)

It was observed that comparison of group I (control) and group III (AFB_1 200 µg/Kg/day); group II (*S. platensis* 100 mg/Kg/day) and group III (AFB_1 200 µg/Kg/day); group III (AFB_1 200 µg/Kg/day) and group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day); had P values of 0.054, 0.0526 and 0.03900 respectively. All the other group to group comparisons had P values of more than 0.05.

Some group-to-group differences were observed after post-hoc comparison of AST levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.5).

Table 4. 5: Post-hoc comparison of AST using Tukey's Honestly Significantly Differenced (HSD)

	Group	Group	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	58.2	-66.857592	183.257592	1.969591	0.6245
1	Group I	Group III	94.9	-30.157592	219.957592	3.211584	0.0195*
2	Group I	Group IV	124.4	-0.657592	249.457592	4.209916	0.0517*
3	Group I	Group V	48.9	-76.157592	173.957592	1.654863	0.7445
4	Group II	Group III	36.7	-88.357592	161.757592	1.241993	0.9000
	Group II	Group IV	66.2	-58.857592	191.257592	2.240325	0.5213
6	Group II	Group V	9.3	-115.757592	134.357592	0.314728	0.9000

7	Group III	Group IV	29.5	-95.557592	154.557592	0.99833 2	0.9000
8	Group III	Group V	46.0	-79.057592	171.057592	1.55672 1	0.0482*
9	Group IV	Group V	75.5	-49.557592	200.557592	2.55505 4	0.3986

Note: * Statistically significant ($p \leq 0.05$)

It was observed that comparison of group I (control) and group III (AFB_1 200 μg/Kg/day); group I (control) and group IV (*S. platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day); group III (AFB_1 200 μg/Kg/day) and group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day); had P values of 0.01954, 0.0517 and 0.04819 respectively. All the other group to group comparisons had P values of more than 0.05.

Some group-to-group differences were observed after post-hoc comparison of ALP levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.6).

Table 4. 6: Post-hoc comparison of Alkaline phosphatase using Tukey's Honestly Significantly Differenced (HSD)

	Group I	Group II	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	17.60	-5.208611	40.40861	3.265705	0.1830
1	Group I	Group III	9.65	-13.158611	32.45861	1.790571	0.0493*
2	Group I	Group IV	7.65	-15.158611	30.45861	1.419468	0.8340
3	Group I	Group V	32.90	10.091389	55.708611	6.104642	0.0028*
4	Group II	Group III	7.95	-14.858611	30.75861	1.475134	0.8130
5	Group II	Group IV	9.95	-12.858611	32.75861	1.846237	0.6715
6	Group II	Group V	15.30	-7.508611	38.10861	2.838937	0.2984
7	Group III	Group IV	2.00	-20.808611	24.80861	0.371103	0.9000
8	Group III	Group V	23.25	0.441389	46.05861	4.314071	0.0444*
9	Group IV	Group V	25.25	2.441389	48.05861	4.685174	0.0256*

Note: * Statistically significant ($p \leq 0.05$)

The results showed that comparison of group I (control) when compared with group III (AFB_1 200 µg/Kg/day); group I (control) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day); group III (AFB_1 200 µg/Kg/day) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day); as well as, group IV (*S. platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) had P values of 0.04928, 0.0028, 0.0444 and 0.0256 respectively. All the other group to group comparisons had P values of more than 0.05.

4.4. Assessment of the protective effect of *Spirulina platensis* extract on histopathological changes induced by AFB_1.

Histopathological examination was carried out to ascertain *S. platensis* extract effect on AFB1 induced kidney and liver toxicity.

4.4.1. Histopathological changes induced by AFB_1 on the liver

In group I (control), it was observed that the mice tissues were normal. There was no localised haemorrhage, no hepatocellular necrosis and no fatty changes observed (Plate 4.1 A).

Group II (100 mg/kg/day *S. platensis),* revealed the mice tissues were predominantly normal. There was no localised haemorrhage, no hepatocellular necrosis and no fatty changes observed (Plate 4.1 B).

Group III (200 µg/kg/day of AFB_1), showed that the mice tissues had severe tissue injury. Congestion of the central and portal veins was noted. In addition, diffuse hepatocellular necrosis and localised haemorrhage were seen (Plate 4.1 C).

In group IV (100 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1), severe tissue injury was observed. Congestion and hepatocellular necrosis with a few viable hepatocytes were observed in all the mice in the group, (Plate 4.1 D).

Group V (200 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1, showed moderate tissue injury. Generalised congestion and moderate hepatocellular necrosis were seen. Haemorrhage and fatty changes were seen in two of the mice in the group, (Plate 4.1 E).

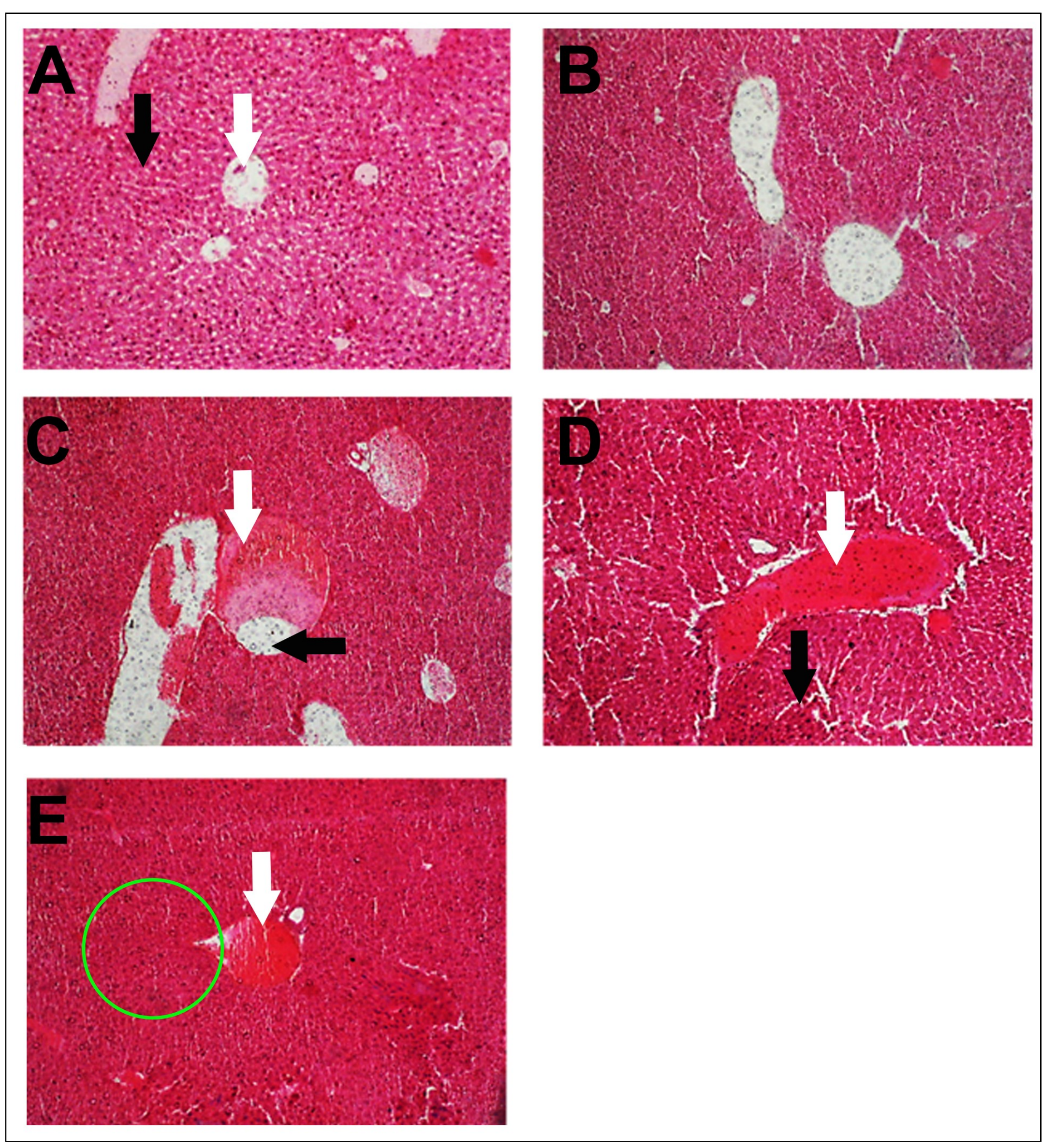


Plate 4. 1: Photomicrographs of the liver tissues (X40; Hematoxylin and Eosin staining)

(A) Group I (control) showing normal architecture of central veins (black arrow) and normal sheets of hepatocytes (white arrow)
(B) Group II (*S. Platensis* 100 mg/Kg/day) showing normal sheets of hepatocytes and normal central veins
(C) Group III (AFB_1 200 µg/kg/day) showing haemorrhagic zone (white arrow)
(D) Group IV (100 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1) showing diffuse hepatocellular necrosis
(E) Group V (200 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1) sheets of hepatocytes undergoing restoration from hepatocellular necrosis (circled) indicated by mild vacuolation and minimal inflammation

4.4.2. Semi-quantitative grading of liver tissues

A total of 75 liver tissue fields were read under the microscope; 3 tissue fields per mice. Group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had the highest number of liver tissue fields graded as normal using semi-quantitative grading, 23 and 22 respectively. All the other groups (group III, group IV and group V) none of their liver tissue fields were graded as normal, (Table 4.7).

Table 4. 7: Semi-quantitative grading of liver tissues

	Normal	Mild	Moderate	Severe
Control (Group I)	23	2	0	0
***S. Platensis* 100 mg/Kg/day (Group II)**	22	3	0	0
AFB_1 200 µg/Kg/day (Group III)	0	0	1	24
***S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day (Group IV)**	0	0	15	9
***S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day (Group V)**	0	5	18	2

Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) had the highest number of liver tissue fields (5) graded as mild using semi-quantitative grading. Group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had 2 and 3 of liver tissue fields graded as mild using semi-quantitative grading respectively. None of the liver tissue fields were graded as mild using semi-quantitative grading in group III (AFB_1 200 μg/Kg/day) and group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day).

Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) and group IV (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) had the highest number of liver tissue fields graded as moderate using semi-quantitative grading, 18 and 9 respectively. In group I (control) and group II (*S. Platensis* 100 mg/Kg/day) none of their liver tissue fields were graded as moderate.

Group III (AFB_1 200 μg/Kg/day) and group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day) had the highest number of liver tissue fields graded as severe using semi-quantitative grading, 24 and 15 respectively. Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) had 2 liver tissue fields graded as severe. None of the liver tissue fields were graded as severe in group I (Control) and group II (*S. Platensis* 200 mg/Kg/day).

Overall, non-parametric analysis using Mann-Whitney U test for five group comparisons of the semi quantitative ordinal data of liver histopathology photomicrographs revealed the results were statistically significant with a p-value of 0.04894.

The results showed that comparison of group I (control) when compared with group III (AFB_1 200 µg/Kg/day); group I (control) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) as well as group III (AFB_1 200 µg/Kg/day) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day), they had P values of 0.043270, 0.04880 and 0.045511 respectively. All the other group to group comparisons had P values of more than 0.05, (Table 4.8).

Table 4. 8: Post-hoc comparison of semi-quantitative data of liver tissues using Dunnet's test

	Group I	Group II	Group III	Group IV	Group V
Group I	1.0000	1.00000	0.043270*	1.00000	0.04880*
Group II	1.00000	1.00000	1.00000	1.00000	0.99999
Group III	1.00000	1.00000	1.00000	1.00000	0.04551*
Group IV	1.00000	1.00000	1.00000	1.00000	0.99999
Group V	0.99998	0.99999	0.99996	0.99999	1.0000

Note: Statistically significant ($P \leq 0.05$)

4.4.3. Histopathological changes induced by AFB_1 on the kidney

Group I (control) revealed that the mice tissues were normal. There was no localised haemorrhage, no necrosis and no fatty changes observed, (Plate 4.2 A).

Inspection of group II (100 mg/kg/day *S. platensis),* showed that the mice tissues were predominantly normal. There was no localised haemorrhage, no necrosis and no fatty changes were observed (Plate 4.2 B).

In group III (200 µg/kg/day of AFB_1), the mice tissues had severe tissue injury with extensive tissue damage (degeneration) of the renal tubules and localised haemorrhage were noted. Thickened glomerular basement membrane was seen. In addition to these, inflammation of renal tubules was observed, (Plate 4.2 C).

The mice tissues of group IV (100 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1), had severe tissue injury. Inflammation of renal tubules was observed in all the mice in the group. Extensive haemorrhage and extensive fatty changes were also noted (Plate 4.2 D).

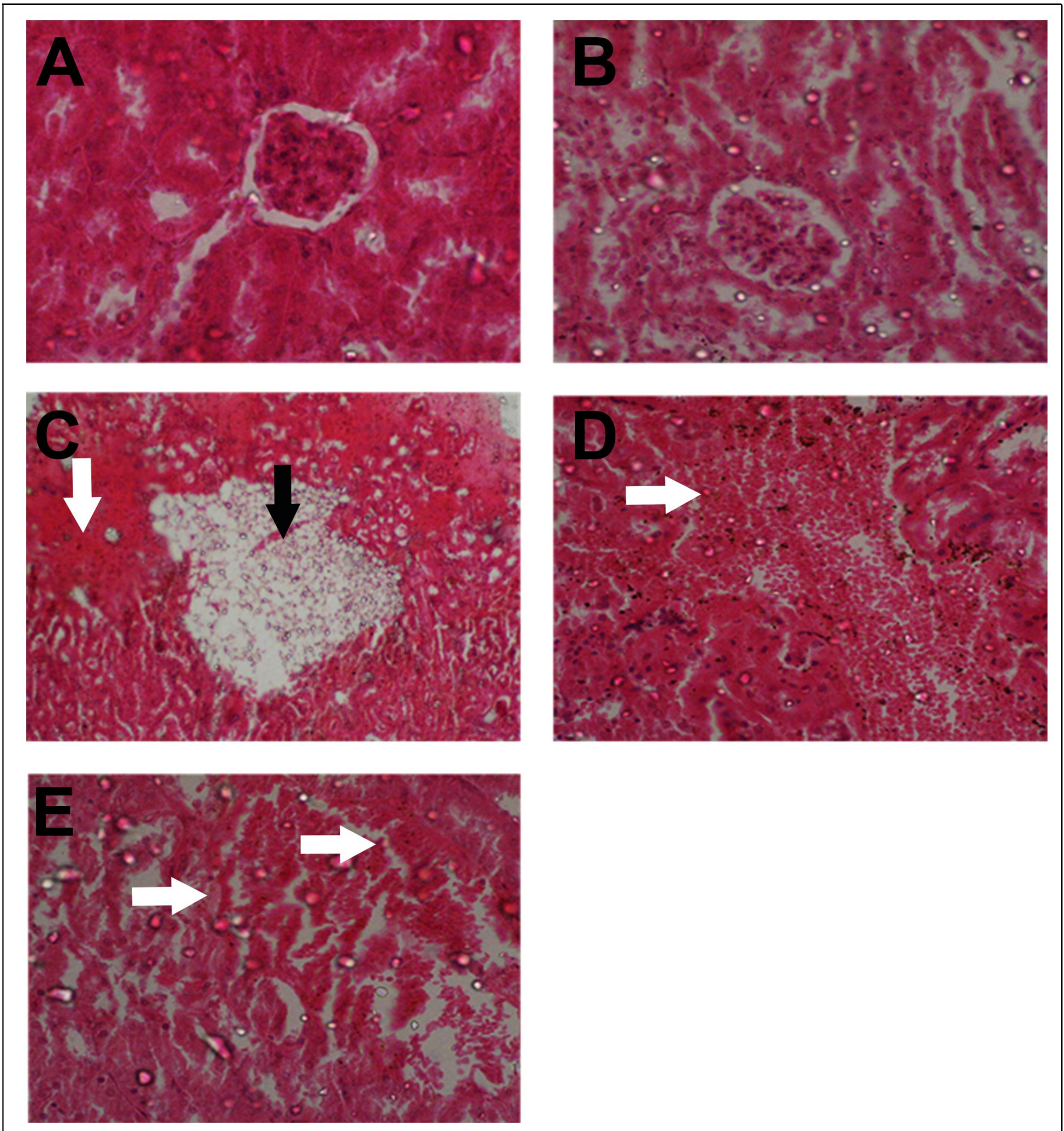


Plate 4. 2: Photomicrographs of kidney tissues (X400; Hematoxylin and Eosin staining)

(A) Group I (control) showing normal renal tubules
(B) Group II (*S. Platensis* 100 mg/Kg/day) showing normal renal tubules

(C) Group III (AFB_1 200 µg/kg/day) showing necrosis (degeneration) of the renal tubules and fatty changes (white and black arrows)
(D) Group IV (*S. platensis* 100 mg/kg/day + 200 µg/kg/day of AFB_1) showing severe tissue necrosis
(E) Group V (*S. platensis* 200 mg/kg/day + 200 µg/kg/day of AFB_1) showing moderate renal tubules (white arrows)

Examination of group V (200 mg/kg/day *S. platensis* + 200 µg/kg/day of AFB_1), revealed that the mice tissues had moderate tissue injury. Generalised inflammation and fatty changes were seen. Destruction of glomerular tuft, haemorrhage and fatty changes were seen in two of the mice in the group, (Plate 4.2 E).

4.4.4. Semi-quantitative grading of kidney tissues using ordinal four step scale system

A total of 75 kidney tissue fields were read under the microscope; 3 tissue fields per mice. Group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had the highest number of kidney tissue fields graded as normal using semi-quantitative grading, 24 and 23 respectively. All the other groups (group III, group IV and group V) none of their kidney tissue fields were graded as normal, (Table 4.9).

Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) had the highest number of kidney tissue fields (3) graded as mild using semi-quantitative grading. Group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had 1 and 3 of kidney tissue fields graded as mild using semi-quantitative grading respectively. None of the kidney tissue fields were graded as mild using semi-quantitative grading in group III (AFB_1 200 µg/Kg/day) and group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day).

Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) and group IV (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) had the highest number of kidney

tissue fields graded as moderate using semi-quantitative grading, 17 and 7 respectively. In group III (AFB_1 200 μg/Kg/day) 4 kidney tissue fields were graded as moderate. None of the kidney tissue fields were graded as severe in group I (Control) and group II (*S. Platensis* 200 mg/Kg/day).

Table 4. 9: Semi-quantitative grading of kidney tissues

	Normal	Mild	Moderate	Severe
Control (Group I)	24	1	0	0
***S. Platensis* 100 mg/Kg/day (Group II)**	23	2	0	0
AFB_1 200 μg/Kg/day (Group III)	0	0	4	21
***S. Platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day (Group IV)**	0	0	15	10
***S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day (Group V)**	0	3	17	5

Group III (AFB_1 200 μg/Kg/day) and group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day) had the highest number of kidney tissue fields graded as severe using semi-quantitative grading, 21 and 18 respectively. Group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) had 5 kidney tissue fields graded as severe. None of the kidney tissue fields were graded as severe in group I (Control) and Group II (*S. Platensis* 200 mg/Kg/day).

Overall, non-parametric analysis using Mann-Whitney U test for five group comparisons of the semi quantitative ordinal data of kidney histopathology photomicrographs revealed the results were statistically significant with a p-value of 0.04981.

Table 4. 10: Post-hoc comparison of semi-quantitative data of kidney tissues using Dunnet's test

	Group I	Group II	Group III	Group IV	Group V
Group I	1.000000	1.00000	0.04710*	1.0	0.048810*
Group II	1.000000	1.00000	0.04992*	1.0	0.999990
Group III	1.000000	1.00000	1.000000	1.0	0.04502*
Group IV	1.000000	1.00000	1.000000	1.0	1.000000
Group V	0.999995	0.99999	0.999998	1.0	1.000000

Note: Statistically significant ($P \leq 0.05$)

The results showed that comparison of group I (control) when compared with group III (AFB_1 200 μg/Kg/day); group II (*S. platensis* 200 mg/Kg/day) when compared with group III (AFB_1 200 μg/Kg/day); group I (control) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) as well as group III (AFB_1 200 μg/Kg/day) when compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day), they had P values of 0.04710, 0.04992, 0.04881 and 0.04502 respectively. All the other group to group comparisons had P values of more than 0.05, (Table 4.10).

4.5. Evaluation of protective effect of *Spirulina platensis* extract against AFB_1 induced immune dysfunction.

There were immune changes in IgG, IgM and IgA serum levels observed. For IgG, group III (AFB_1 200 μg/Kg/day) had a higher mean level of 0.2808 pg/ml when compared to the other 4 groups whose average values were between 0.1739 to 0.1749 pg/ml, as shown in Table 4.11.

Table 4. 11: Mean values for IgG, IgM and IgA in mice

	IgG (pg/ml)	IgM (ng/ml)	IgA (ng/ml)
Control (Group I)	0.1746 ± 0.001	0.0916 ± 0.003	0.7147 ± 0.001
SP 100 mg/Kg/day (Group II)	0.1739 ± 0.002	0.0975 ± 0.003	0.7133 ± 0.004
AFB_1 200 µg/Kg/day (Group III)	0.2808 ± 0.243	0.0866 ± 0.019	0.7075 ± 0.010
SP 100 mg/Kg/day + AFB_1 200µg/Kg (Group IV)	0.1746 ± 0.001	0.0899 ± 0.003	0.7123 ± 0.0102
SP 200 mg/Kg/day + AFB_1 200 µg/Kg/day (Group V)	0.1749 ± 0.001	0.1005 ± 0.004	0.7124 ± 0.005

Mean values ± SD

In contrast to IgG, with regard to IgM, group III (AFB_1 200 µg/Kg/day) had a reduced mean of 0.0866 ng/ml when compared to the other 4 groups whose mean values ranged from 0.0899 to 0.1005 ng/ml. Likewise, for IgA, group III (AFB_1 200 µg/Kg/day) had a drop in mean value of 0.7075 ng/ml when compared to the other 4 groups whose mean values were between 0.7123 to 0.7147 ng/ml. The p values were 0.01448, 0.04512 and 0.0541 for IgM, IgG and IgA respectively.

Some group-to-group differences were observed after post-hoc comparison of IgG levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.12).

Table 4. 12: Post-hoc comparison of IgG mean values using Tukey's Honestly Significantly Differenced (HSD)

	Group	Group	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	0.0059	-0.0114	0.023088	1.4396	0.8266
1	Group I	Group III	0.0050	-0.0122	0.022208	1.2234	0.0090*
2	Group I	Group IV	0.0017	-0.0155	0.018968	0.4274	0.9000
3	Group I	Group V	0.0087	-0.0084	0.026088	2.1766	0.5458
4	Group II	Group III	0.0108	-0.0064	0.028068	2.6630	0.0136*
5	Group II	Group IV	0.0076	-0.0096	0.024828	1.8670	0.6636
6	Group II	Group V	0.0030	-0.0142	0.020228	0.7370	0.9000
7	Group III	Group IV	0.0032	-0.0140	0.020468	0.7960	0.0671
8	Group III	Group V	0.0138	-0.0034	0.031068	3.3999	0.0155*
9	Group IV	Group V	0.0106	-0.0066	0.027828	2.60470	0.3800

Note: * Statistically significant ($p \leq 0.05$)

It was observed that comparison of group III (AFB_1 200 μg/Kg/day) compared with group I (control); group III (AFB_1 200 μg/Kg/day) compared with group II (*S. platensis* 100mg/Kg/day), as well as; group III (AFB_1 200 μg/Kg/day) compared with group V (*S. platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day) had P values of 0.009, 0.0136 and 0.0155 respectively. All the other group to group comparisons had P values of more than 0.05.

Some group-to-group differences were observed after post-hoc comparison of IgM levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.13).

Table 4. 13: Post-hoc comparison of IgM mean values using Tukey's Honestly Significantly Differenced (HSD)

	Group	Group	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	0.0014	-0.0121	0.0149	0.4387	0.9000
1	Group I	Group III	0.0072	-0.0063	0.0208	2.2686	0.0511
2	Group I	Group IV	0.0025	-0.0111	0.0160	0.7708	0.9000
3	Group I	Group V	0.0023	-0.0112	0.0158	0.7207	0.0523
4	Group II	Group III	0.0058	-0.0077	0.0194	1.8299	0.6778
5	Group II	Group IV	0.0011	-0.0124	0.0146	0.3321	0.9000
6	Group II	Group V	0.0009	-0.0126	0.0144	0.2820	0.9000
7	Group III	Group IV	0.0048	-0.0087	0.0183	1.4978	0.8044
8	Group III	Group V	0.0049	-0.0086	0.0185	1.5479	0.0178*
9	Group IV	Group V	0.0002	-0.0133	0.0137	0.0501	0.0190*

Note: * Statistically significant ($p \leq 0.05$)

It was observed that comparison of group III (AFB_1 200 μg/Kg/day) and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day**)**; group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 μg/Kg/day**)** and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 μg/Kg/day**)** had P values of 0.0178 and 0.0190 respectively. All the other group to group comparisons had P values of more than 0.05.

Some group-to-group differences were observed after post-hoc comparison of IgA levels, using Tukey's Honestly Significantly Differenced (HSD), (Table 4.14).

Table 4. 14: Post-hoc comparison of IgA mean values using Tukey's Honestly Significantly Differenced (HSD)

	Group	Group	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	0.0007	-0.2047	0.2060	0.014015	0.9000
1	Group I	Group III	0.1062	-0.0992	0.3115	2.188400	0.5411
2	Group I	Group IV	0.0000	-0.20530	0.2054	0.000824	0.9000
3	Group I	Group V	0.0003	-0.20500	0.2057	0.007007	0.0190 *
4	Group II	Group III	0.1069	-0.09848	0.3122	2.202415	0.5357
5	Group II	Group IV	0.0007	-0.20462	0.2061	0.014839	0.9000
6	Group II	Group V	0.0010	-0.20432	0.2064	0.021022	0.9000
7	Group III	Group IV	0.1061	-0.0992	0.3115	2.187575	0.5414
8	Group III	Group V	0.1058	-0.0995	0.3112	2.181392	0.0544
9	Group IV	Group V	0.0003	-0.2050	0.2056	0.006183	0.0560

Note: * Statistically significant ($p \leq 0.05$)

It was observed that comparison of group I (control) and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** had P values of 0.019. All other group to group comparisons had P values of more than 0.05.

4.6. Investigation on the probable mechanism of action of protective effect *Spirulina platensis* extract against AFB_1 induced toxicity.

There were some changes on the mean concentration of MDA equivalents (µmol) in the liver and kidney serum levels during the study period. It was observed that liver samples of group III (AFB_1 200 µg/Kg/day) had elevated mean concentration when compared to group I (control); (0.963±0.2100 vs. 0.2654±0.0741).

It was also observed that liver samples of group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** had lower mean concentrations of MDA equivalent when compared to group III (AFB_1 200 µg/Kg/day), (0.8505±0.5046 and 0.6044±0.4044 vs. 0.963±0.2100); as shown in Table 4.15.

Table 4. 15: Mean concentration of MDA equivalents (µmol) in the liver and kidney samples

	Control (Group I)	**Spirulina 100 mg/Kg/day (Group II)**	**AFB_1 200 µg/Kg/day (Group III)**	**Spirulina 100 mg/Kg + AFB_1 200 µg/Kg/day (Group IV)**	**Spirulina 200 mg/Kg + AFB_1 200 µg/Kg/ day (Group V)**
Kidney	0.109±0.0420	0.1512±0.1458	0.7445±0.1194	0.175±0.1574	0.2158±0.661*
Liver	0.2654±0.0741	0.2772±0.3115	0.963±0.2100	0.8505±0.5046	0.6044±0.4044

Note: * Statistically significant ($p \leq 0.05$)

Similarly, it was observed that kidney samples of group III (AFB_1 200 µg/Kg/day) had elevated mean concentration when compared to group I (control); (0.109±0.0420 vs. 0.7445±0.1194). It was also observed that kidney samples of group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** and group V (*S. Platensis* 200

mg/Kg/day + AFB_1 200 µg/Kg/day) had lower mean concentrations of MDA equivalent when compared to group III (AFB_1 200 µg/Kg/day); (0.175±0.1574 and 0.2158±0.661 vs. 0.7445±0.1194).

One-way ANOVA analysis revealed that the mean concentration of MDA equivalents (µmol) in the kidney samples among the 5 groups were statistically significant at P< 0.0011, F (7.026). In contrast to the kidney samples; One-way ANOVA analysis revealed that the mean concentration of MDA equivalents (µmol) in the liver samples among the 5 groups were statistically insignificant at P< 0.089, F (2.35). Subsequently, post-hoc comparison using Tukey's Honestly Significantly Differenced (HSD) was done on the mean concentration of MDA equivalents (µmol) in the kidney samples. It was found that there were some group-to-group differences after post-hoc comparison using Tukey's Honestly Significantly Differenced (HSD) in the liver samples as shown in Table 4.16.

Table 4. 16: Post-hoc comparison using Tukey's Honestly Significantly Differenced (HSD) of the mean concentration of MDA equivalents (µmol) in the kidney samples

	Group I	Group II	Diff	Lower	Upper	q-value	p-value
0	Group I	Group II	0.0422	-0.249851	0.33425	0.61153	0.9000
1	Group I	Group III	0.3005	0.008449	0.59255	4.35460	0.0418*
2	Group I	Group IV	0.0660	-0.226051	0.35805	0.95642	0.9000
3	Group I	Group V	0.4167	0.124699	0.70880	6.03921	0.0051
4	Group II	Group III	0.2583	-0.033751	0.55035	3.74308	0.0992
5	Group II	Group IV	0.0238	-0.268251	0.31585	0.34489	0.9000
6	Group II	Group V	0.3745	0.082499	0.6666	5.42768	0.0810
7	Group III	Group IV	0.2345	-0.057551	0.52655	3.39816	0.0155*
8	Group III	Group V	0.1163	-0.175801	0.408301	1.684601	0.0473*
9	Group IV	Group V	0.3508	0.05870	0.64280	5.08279	0.0139*

Note: * Statistically significant ($p \leq 0.05$)

It was observed that comparison of group I (control) with group III (AFB_1 200 µg/Kg/day); group IV (AFB_1 200 µg/Kg/day) compared with group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)**; group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day**)** compared with group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day); group III (AFB_1 200 µg/Kg/day) compared with group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day**)** had P values of 0.0418, 0.0155, 0.01389 and 0.04733 respectively. All the other group to group comparisons had P values of more than 0.05.

CHAPTER FIVE

DISCUSSION

5.1. Introduction

Discussion of study findings is presented in this chapter. It covers discussion on body weight changes, the protective effect of *S. Platensis* extract on biochemical changes and histopathological changes on liver and kidney and immune changes induced by $AFB_{1.}$ It also covers discussion on the probable mechanism of action of protective effect of *S. Platensis* extract against AFB_1 induced toxicity.

5.2. Body weight changes

Administration of a dose of 200 µg/Kg/day of AFB_1 (group III) led to decreased mean weight of mice in comparison to group I (control), (Table 4.1). This was in agreement with an earlier study findings (Tomkova *et al*., 2001). The reduction in body weight has been attributed to poor feeding, reduced growth, and inefficient feed utilisation (Faridha *et al*., 2006). Body weight change has been regarded as a very responsive indicator of poisonous effects of toxicants (Khattab *et al*., 2016). Body weight loss is usually among the preliminary indicators of onset of toxic effects. A toxic dose is usually associated with 10% or more body weight reduction (Tanuja *et al*., 2016).

A rise in mean weight was gradually noted in mice in group II (*S. Platensis* 100 mg/Kg/day) as well as group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day). One way ANOVA analysis showed that the changes in body weight among the 5 groups were statistically significant, $P< 0.05$, F (66.61). Post-hoc pairwise comparison using Tukey's Honestly Significantly Differenced (HSD) showed that there were significant statistical differences in all the groups ($P< 0.05$). However, group II (*S. platensis* extract 100 mg/Kg/day) when compared with group V (S. *Platensis* extract

200 mg/Kg/day + AFB_1 200 µg/Kg/day) and Group III (AFB_1 200 µg/Kg/day) in comparison with group IV (*S. platensis* extract 100 mg/kg + 200 µg/kg/day of AFB_1) that had $P>0.05$.

Therefore, study findings demonstrated that supplementation of 200 mg/kg/day of *S. platensis* extract followed by administration AFB_1 200 µg/Kg/day inhibits weight loss in male Swiss albino mice caused by AFB_1 intoxication ($P<0.001$).

5.3. Evaluation of the protective effect of Spirulina platensis extract on biochemical changes induced by AFB1.

Administration of AFB_1 at a dose of 200 µg/Kg/day (group III) led to a rise in ALT mean levels when compared with group I (control), (44.0±6.83 IU/L vs. 61.0±8.19 IU/L; p=0.054). This was in line with earlier investigators, who have all reported elevated ALT mean levels in aflatoxins exposed experimental animals, (Farombi *et al*., 2005; Nili-Ahmadabadi *et al*., 2011; Tanuja *et al*., 2016).

There was no significant variation in the mean ALT levels of control (group I) and *S. platensis* extract when administered at a dosing level of 100 mg/kg/day (group II); p (0.6245). This demonstrated that *S. platensis* does not cause hepatotoxicity. This was in agreement with previous investigators, who have all reported that *S. Platensis* is safe and good for human consumption, (2012; Gutierrez *et al.,* 2015; Qinghua, 2016; Asieh *et al*., 2016).

Administration of *S. Platensis* extract at a dose of 200 mg/Kg/day followed by AFB_1 at a dose of 200 µg/Kg/day (group V) lowered mean ALT level (49.8±7.9 IU/L vs. 51.5±8.19 IU/L; p=0.039). This revealed that administration of *S. Platensis* at higher dose of 200 mg/Kg/day had hepatoprotective effect against AFB1 induced toxicity.

Aflatoxin B_1 given at 200 μg/Kg/day (group III) increased the mean levels of AST compared with the AFB_1 untreated group (control); (176.75±44.34 IU/L vs. 256±115.99 IU/L, respectively; p=0.0195). This has also been demonstrated with previous investigators (Farombi *et al*., 2005; Ghada *et al*., 2012; Tanuja *et al*., 2016). In addition to this, AFB_1 hepatotoxic effect has been confirmed in vivo on mice (Pickova *et al*., 2021). However, this contradicted with a previous study that reported no changes in mean AST levels (Angelica *et al*., 2017). This variation in study findings may be due to sampling time variation and dosing frequency. Their study evaluated the activity of AFB_1 single oral acute dose in C57B1/6 mice while in our study, repeated doses of AFB_1 were administered.

Administration of *S. Platensis* extract at a dose of 200 mg/Kg/day followed by AFB_1 200 μg/Kg/day (group V); reversed the elevation of AST levels (229.8±95 IU/L vs. 256±115.99 IU/L, respectively; p=0.04819). This was reported in a previous study in rats using honey, a natural antioxidant, (Yaman *et al*, 2016).

In this study, AFB_1 induced hepatotoxicity was assessed by elevation of ALT and AST mean serum levels. The significant reduction in the mean serum levels of these two enzymes by *S. platensis* extract revealed its hepatoprotective effect on AFB_1 induced hepatotoxicity as previous reported, (Farombi *et al*., 2005). They demonstrated significant reduction in ALT and AST enzyme levels after their elevation by AFB_1 exposure using kolaviron; a natural antioxidant commonly found in Nigeria.

Administration of *S. Platensis* extract at a dose of 100 mg/Kg/day followed by AFB_1 at a dose of 200 μg/Kg/day (group IV) and also administration of *S. Platensis* extract at a dose of 200 mg/Kg/day followed by AFB_1 at a dose of 200 μg/Kg/day (group V),

led to statistically significant reversal of ALP mean levels; P (0.0444). This was in line with an earlier study (Tanuja *et al*., 2016). In their study conducted using *Phyllanthus niruri (Euphorbiaceae*); reduced ALP serum levels were reported.

Consequently, our study findings revealed that supplementation of *S. Platensis* extract at doses ranging from 100 mg/Kg/day to 200 mg/Kg/day reverses elevation of ALT and AST levels.

In the present study, the mean levels of total bilirubin in male Swiss albino mice remained similar for the 5 groups ranging from 2.0 µmol/L to 2.4 µmol/L; (p= 0.3202). This was in line with a previous study; that found the levels of bilirubin to be within their normal range. In addition to these, total bilirubin is a prognostic marker of chronic liver damage, (Tanuja *et al*., 2016). Consequently, the current study being a sub-acute study, the changes observed in total bilirubin levels were minimal.

There were insignificant changes in the mean serum levels of albumin across the 5 groups, (p= 0.960). They ranged from 34.0 g/L to 34.2 g/L. The results revealed that *S. Platensis* extract administered at a dose of 100 mg/Kg/day (group II); administration of *S. Platensis* at a dose of 100 mg/Kg/day followed by administration of AFB_1 at a dose of 200 µg/Kg/day (group IV) and also administration of *S. Platensis* at a dose of 200 mg/Kg/day followed by AFB_1 at a dose of 200 µg/Kg/day (group V) did not cause any significant changes to albumin levels when compared to the group I (control). This may suggest that *S. Platensis* extract does not play any role in the synthesis, metabolism and elimination of albumin (Tanuja *et al*., 2016).

In this study, there were statistically insignificant changes in the mean levels of globulin and total protein across the 5 groups (p= 0.30 and 0.3202) respectively. This was contrary to an earlier study (Tanuja *et al*., 2016) that reported elevated levels of

albumin and total proteins. The elevated albumin and total proteins mean levels in Tanuja *et al*., 2016 study, were attributed to the presence of these nutrients in *Phyllanthus niruri* leaf extract that was being investigated.

Supplementation of *S. Platensis* extract administered at doses ranging from 100 mg/Kg/day to 200 mg/Kg/day reverses elevation of ALT, AST and ALP mean levels. There were statistically insignificant changes in the mean levels of albumin, globulin, total protein and bilirubin across the 5 groups.

5.4. Evaluation of the protective effect of Spirulina platensis extract on histopathological changes induced by AFB1.

5.4.1. Histopathological changes in liver

Histopathological examination was done to further confirm the activity of S. *Platensis* on AFB_1 induced liver toxicity. Histopathological examination of liver tissues of group I (control) and *S. Platensis* extract administered at a dose of 100 mg/Kg/day (group II) revealed that the liver mice tissues were normal. There was no localised haemorrhage, no hepatocellular necrosis and no fatty changes noted. In addition to these, semi-quantitative grading using ordinal four step scale system revealed that group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had majority of their liver tissues graded as normal. This was in line with previous investigators (Devendran and Balasubramanian, 2011; Ali and Mohammed 2012; Gutierrez *et al.,* 2015; Asieh *et al*., 2016). All these have reported normal liver histopathological findings in liver tissues of aflatoxins untreated groups.

Mice given a dose of 200 µg/Kg/day of AFB1 (group III) had severe tissue injury. Congestion of the central and portal veins was also noted. In addition to these, localised haemorrhage and congestion were seen. These findings revealed that AFB_1

administered at a dose of 200 µg/Kg/day causes hepatic tissue injury and extensive tissue damage. Semi-quantitative grading using ordinal four step scale system revealed that group III mice had majority of their liver tissues graded as severe. This was in agreement with previous investigators (Ghada *et al*., 2012; Bbosa, 2013; Yaman *et al*, 2016). All these have reported hepatic tissue injury and damage after AFB_1 exposure. Furthermore, these hepatotoxic effects of AFB_1 have been well reported in various animal species (Mishra and Chitrangada, 2003; Yaman *et al*, 2016).

Administration of S. *Platensis* extract at a lower dose of 100 mg/Kg/day followed by administration of AFB_1 at a dose of 200 µg/Kg/day (group IV); microscopic examination of mice tissues revealed moderate tissue injury with moderate tissue damage. This demonstrated that co-administration of *S. Platensis* extract at a lower dose of 100 mg/kg/day followed by AFB_1 at a dose of 200 µg/Kg/day led to moderate hepatic tissue damage when compared to tissues obtained from mice in group III (AFB_1 200 µg/Kg/day) that had severe tissue damage. Semi-quantitative grading using ordinal four step scale system revealed that group IV mice had majority of their liver tissues graded as moderate. Similar results have been reported in rats in experimental aflatoxicosis treated with honey, (Yaman *et al*., 2016).

Administration of *S. Platensis* extract at a higher dose of 200 mg/Kg/day followed by AFB_1 at a dose of 200 µg/Kg/day (group V); revealed that there was moderate hepatic tissue damage. This revealed that supplementation of S. *Platensis* extract administered at a dose of 200 mg/kg/day led to moderate hepatic tissue damage when compared to tissues obtained from mice in group III (AFB_1 200 µg/Kg/day) that had severe tissue damage. Semi-quantitative grading using ordinal four step scale system revealed that group V mice had majority of their liver tissues graded as moderate. This was in line

with previous studies (Ghada *et al*., 2012; Yaman *et al*., 2016). These studies were conducted using other natural substances that have antioxidant properties.

Comparison of hepatic tissues photomicrographs of group IV (*S. Platensis* 100 mg/Kg + AFB_1 200 µg/Kg/day) and group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) suggests that hepatoprotective effect of *S. Platensis* against AFB_1 induced toxicity may be dose related. However, this should be interpreted with caution until further studies are performed.

Overall, these study findings demonstrated that supplementation of *Spirulina platensis* extract at doses ranging from 100 mg/Kg/day to 200 mg/Kg/day has hepatoprotective effect against AFB_1 (200 µg/Kg/day) induced toxicity in male Swiss albino mice as evidenced by histopathology photomicrographs.

5.4.2. Histopathological changes in the kidney

Histopathological examination of kidney tissues of control group (group I) and mice treated with *S. Platensis* extract at a dose of 100 mg/Kg/day (group II) revealed that the kidney tissues were predominantly normal. There was no localised haemorrhage, no necrosis and no fatty changes observed. In addition to these, semi-quantitative grading using ordinal four step scale system revealed that group I (control) and group II (*S. Platensis* 100 mg/Kg/day) had majority of their kidney tissues graded as normal. This was in agreement with previous investigators (Devendran and Balasubramanian, 2011; Gutierrez *et al.,* 2015; Qinghua, 2016). These investigators have all reported no toxic effects on mice and rats that are unexposed to aflatoxins.

In contrast to group I (control), administration of AFB_1 at a dose of 200 µg/Kg/day (group III) revealed severe tissue injury. Degeneration of the renal tubules and localised haemorrhage were also noted. Semi-quantitative grading using ordinal four

step scale system revealed that group III mice had majority of their kidney tissues graded as severe. This was in agreement with previous investigators (Mishra and Chitrangada, 2003; Abeer, 2015; Yaman *et al*, 2016). All these investigators have documented chronic renal tissue damage after aflatoxins exposure.

Co-administration of *S. Platensis* extract administered at a lower dose of 100 mg/Kg/day followed by administration of AFB_1 at a dose of 200 μg/Kg/day (group IV); revealed renal tissues that had moderate tissue damage. Congestion and moderate necrosis of renal tubules were observed in all the mice in the group. Moderate haemorrhage and fatty changes were also noted. Semi-quantitative grading using ordinal four step scale system revealed that group IV mice had majority of their kidney tissues graded as moderate. This revealed that co-administration of 100 mg/kg/day of *S. Platensis* followed by AFB_1 administered at a dose of 200 μg/Kg/day led to moderate renal tissue damage when compared to renal tissues obtained from mice in group III (AFB_1 200 μg/Kg/day) that had severe tissue damage. Similar results have been reported in rats in experimental aflatoxicosis using honey; a natural substance that has significant antioxidant properties (Yaman *et al*., 2016).

Co-administration of *S. Platensis* extract administered at a dose of 200 mg/Kg/day followed by administration of AFB_1 at a dose of 200 μg/Kg/day (group V) resulted in moderate tissue injury with minimal tissue damage. Generalised congestion and mild necrosis were also seen. Semi-quantitative grading using ordinal four step scale system revealed that group V mice had majority of their kidney tissues graded as moderate. This demonstrated that supplementation of *S. Platensis* at a dose of 200 mg/kg/day led to minimal renal tissue damage when compared to renal tissues obtained from mice in group III (AFB_1 200 μg/Kg/day) that had severe renal tissue damage.

Comparison of renal tissues photomicrographs of group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day) and group V (*S. Platensis* 200 mg/Kg + AFB_1 200 µg/Kg/day) suggests that renoprotective effect of *S. Platensis* against AFB_1 induced toxicity in male Swiss albino mice may be dose related. However, this should be interpreted with caution until further investigations are carried out.

Overall, these study findings demonstrated that co-administration of *S. platensis* extract at doses ranging from 100 mg/Kg/day to 200 mg/Kg/day has reno-protective effects against AFB_1 (200 µg/Kg/day) induced toxicity in male Swiss albino mice as evidenced by beneficial changes observed on histology.

5.5. Evaluation of the protective effect of *Spirulina platensis* extract on Immune dysfunction induced by AFB_1

Immunotoxicity caused by naturally occurring compounds present in food and environmental contaminants has remained a subject of increasing interest for several years (Genevieve *et al*., 2000). Several investigators have reported that chronic ingestion of aflatoxin contaminated feeds and food causes immune depression in both animals and human beings respectively (Jalila et al., 2016; Rushing *et al*., 2019; Oladeji *et al*., 2020). AFB_1 affects immune system either in a synergistic or additive manner (Cardwell, 2000). Estimations of the mean levels of serum immunoglobulins like IgM, IgA and IgG are the commonly applied methods in the determination of the effect of a drug or toxin on the immune system (Bbosa *et al*., 2013, Rania *et al*., 2015; Jalila *et al*., 2016).

Administration of a dose of 200 µg/Kg/day of AFB_1 (group III) led to decreased IgA mean levels when compared with group I (control); (0.7075 ± 0.010 vs. 0.7147 ± 0.001). This finding was in concurrence with earlier studies that reported reduced levels of IgA (Bbosa *et al*., 2013; Williams *et al*., 2004). Pre-treatment using a dose of

100 mg/Kg/day of *S. Platensis* (group II) gave a mean value for IgA that was slightly lower than the mean for group I (control); (0.7133 ± 0.004 vs. 0.7147 ± 0.001). Pre-treatment of *S. Platensis* extract using a dose level of 100 mg/Kg/day and 200 mg/Kg/day as observed in group IV and group V respectively; led to a reduction of IgA levels to the levels below the control (group I) but higher than group III (AFB_1 200 µg/Kg/day) values; (0.7123 ± 0.0102 and 0.7124 ± 0.005 vs. 0.7147 ± 0.001). One-way ANOVA analysis showed that the p value was 0.01448 for comparison of IgA mean values. Subsequently, post-hoc comparison of IgA mean values using Tukey's Honestly Significantly Differenced (HSD) done showed that group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day) compared with group I (control), (P= 0.0190) as well as group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) compared with group III (AFB_1 200 µg/Kg/day), (P= 0.05); were statistically significant. This study result was in agreement with an earlier study on immunomodulatory effect of *S. platensis* (Ravi *et al*., 2010). Therefore, this study results showed that pre-treatment of *S. platensis* at dose level of between 100 to 200 mg/Kg/day inhibited depression of IgA levels caused by 200 µg/Kg/day of AFB_1.

With regard to IgM, AFB_1 administered at a dose of 200 µg/Kg/day (group III) reduced the average levels of IgM when compared with control (group I); (0.0866 ± 0.019 vs. 0.0916 ± 0.003). This result was in agreement with previous findings which have reported a drop in average levels of IgM (Bbosa *et al*., 2013; Williams et al., 2004). *Spirulina platensis* given at 100 mg/Kg/day (group II) gave a mean value for IgM that was higher than the mean for the group I (control); (0.0975 ± 0.003 vs. 0.0916 ± 0.003). This revealed that *S. Platensis* pre-treatment in the absence of aflatoxins, may enhance the mean levels of IgM. This incidental finding suggests that *Spirulina platensis* has potential immune system boosting properties.

Spirulina Platensis given at dose of 100 mg/Kg/day followed by administration of 200 µg/Kg/day of AFB_1 (group IV) elevated IgM levels to levels similar to those in group I (control); (0.0899 ± 0.003 vs. 0.0916 ± 0.003). Likewise, *S. Platensis* extract given at a higher dose level of 200 mg/Kg/day followed by AFB_1 given at a dose of 200 µg/Kg/day (group V) elevated IgM levels to a level higher than control (group I); (0.1005 ± 0.004 vs. 0.0916 ± 0.003).

One-way ANOVA analysis showed that the p value was 0.01448 in the comparison of IgM mean values. Post-hoc comparison of IgM mean values using Tukey's Honestly Significantly Differenced (HSD) revealed that group III (AFB_1 200 µg/Kg/day) compared with group I (control), (P= 0.05105); group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) compared with group III (AFB_1 200 µg/Kg/day), (P= 0.0178); group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) compared with group I (control), (P=0.0523); as well as group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) compared with group IV (AFB_1 100 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) were statistically significant. Consequently, this study results showed that pre-treatment of *S. platensis* at dose level of between 100 to 200 mg/Kg/day inhibited depression of IgM levels caused by 200 µg/Kg/day of AFB_1.

With regard to IgG, a dose of 200 µg/Kg/day of AFB_1 increased average level of IgG when compared with control (group I); (0.2808 ± 0.243 vs 0.1746 ± 0.001). This result was in harmony with previous findings by Samir *et al*., 2010; that reported increased levels of immunoglobulins. However, reduced IgG serum levels have been reported in experimental animal model (Bbosa *et al*., 2013). Subsequently, variation in study findings may be ascribed to immune system effects of AFB_1 being

determined by; duration of exposure, dose used and species under investigation (Bbosa *et al*., 2013; Miao *et al*., 2016).

Co-administration of *S. Platensis* at a lower dosing level of 100 mg/Kg/day and a higher dose of 200 mg/Kg/day as seen in groups 4 and 5 respectively; followed by AFB_1 200 µg/Kg/day administration, led to a drop in IgG levels to levels similar to group I (control); (0.1746±0.001 and 0.1749±0.001 vs 0.2808±0.243). One-way ANOVA analysis result showed statistically significant differences ($P=0.04512$), for comparison of IgG mean values. Post-hoc comparison of IgG mean values using Tukey's Honestly Significantly Differenced (HSD) demonstrated that group III (AFB_1 200 µg/Kg/day) compared to group I (control), ($P=0.009$); group III (AFB_1 200 µg/Kg/day) compared with group II (*S. Platensis* 200 mg/Kg/day), ($P=0.0136$); group V (AFB_1 200 µg/Kg/day + *S. Platensis* 200 mg/Kg/day) compared with group III (AFB_1 200 µg/Kg/day), ($P=0.0155$); were statistically significant. Therefore, this study results showed that pre-treatment of *S. platensis* at dose level of between 100 to 200 mg/Kg/day inhibited increment of IgG levels caused by 200 µg/Kg/day of AFB_1.

An experimental animal model using birds has documented that aflatoxins reduce the levels of immunoglobulins IgA, IgG and IgM. Also, aflatoxins have been documented to lower the complement activity in chicken (Bbosa *et al*., 2013). Significantly, animal studies performed using rats, poultry and pigs have shown that ingestion aflatoxin contaminated food causes immune depression (Williams *et al*., 2004). However, this should be interpreted with caution as some studies have documented an elevation in the levels of IgM and IgA. Immune depression may occur depending on the dose used in the study (Samir *et al*., 2010). However, some investigators have reported no changes in the immune system. The variation in these findings could be

ascribed to AFB_1 effects on the immune system being dependent on the dose, exposure duration and the species being investigated (Miao *et al*., 2016).

Overall, there are conflicting reports concerning immune depression caused by aflatoxins. This may partially be explained by variations in experimental designs, the specific immune response investigated and other secondary effects of the administered aflatoxins dose.

5.6. Investigation on the probable mechanism of action of protective effect of *Spirulina platensis* extract against AFB_1 induced toxicity.

In the current investigation, malondialdehyde (MDA) and lipid hydroperoxide (LHP) formation in mice were used to assess lipid peroxidation. This study (Table 4.11), shows the mean concentration of MDA equivalents (µmol) in the kidney samples were predominantly higher for mice that received 200 µg/Kg/day of AFB_1 (group III) in comparison to group I (control). This result was in agreement with earlier studies (Nili-Ahmadabadi *et al*., 2011; Vaziriyan *et al*., 2018; Shahid *et al*., 2019). All these investigators have previously documented increased mean concentration of MDA while investigating other naturally occurring antioxidant substances.

In addition to these, previous investigators have documented that AFB_1 increases free radicals' formation, lipid oxidation and promotes oxidative damage. Subsequently, this has been reported to cause severe cell damage and cell death in both human beings and experimental animals (Cheng-Feng *et al*., 2000, Stefano *et al*., 2007; Vaziriyan *et al*., 2018).

It should be noted that oxidative damage will occur when the formation of free radicals (hydrogen peroxide, hydroxyl radical and superoxide radical) exceeds

antioxidant capacity in the tissue. The antioxidants involved include: non-enzymes vitamin C and glutathione. Enzymes having antioxidant activity include catalase (CAT), glutathione peroxidase and superoxide dismutase (SOD). Both these enzymes and non-enzyme antioxidants are the principal determinants of the cell defence mechanisms. Consequently, stress caused by oxidation has been identified as a key factor in the mechanism of AFB_1 toxicity (Nili-Ahmadabadi *et al*., 2011; Vaziriyan *et al*., 2018; Shahid *et al*., 2019).

Spirulina Platensis given at a dosing level of 100 mg/Kg/day followed by 200 µg/Kg/day of AFB_1 (group IV) reduced the mean concentration of MDA equivalents (µmol) in the liver and kidney compared to group III (AFB_1 200 µg/Kg/day). Similarly, *Spirulina Platensis* given at a dosing level of 200 mg/Kg/day followed by 200 µg/Kg/day of AFB_1 (group V) further reduced the mean concentration of MDA equivalents (µmol) in the liver and kidney compared to group III (AFB_1 200 µg/Kg/day). These results were in agreement with an earlier investigation conducted using *cactus Opuntia ficus indica* plant that also has significant antioxidant properties. It grows in arid and semi-arid regions; in the Mediterranean basin and Africa (Brahmi *et al*, 2011).

One-way ANOVA analysis showed that there were statistically significant differences ($P< 0.0011$, F (7.026) in the mean concentration of MDA equivalents (µmol) in the kidney samples among the 5 groups. Hence the evidence that the mean concentration of MDA equivalents (µmol) of the kidney samples across the groups were statistically different. Subsequently, Post-hoc comparison using Tukey's Honestly Significantly Differenced (HSD) was done on the mean concentration of MDA equivalents (µmol) in the kidney samples. The result revealed that, apart from group III (AFB_1 200 µg/Kg/day when compared with group I (control); group I (control) when compared

with group V (*S. Platensis* 200 mg/Kg/day + AFB_1 200 µg/Kg/day); group II (*S. Platensis* 100 mg/Kg/day) when compared with group V (*S. Platensis 2*00 mg/Kg/day + AFB_1 200 µg/Kg/day) as well as group IV (*S. Platensis* 100 mg/Kg/day + AFB_1 200 µg/Kg/day) when compared with group V (*S. Platensis* 200 mg/Kg/day + AFB1 200 µg/Kg/day); all which had statistically significant differences ($P<0.05$).

One-way ANOVA analysis showed statistically insignificant differences in the mean concentration of MDA equivalents (µmol) in liver samples among the 5 groups (P< 0.089, F (2.35). Hence, the evidence that the mean concentration of MDA equivalents (µmol) of the liver samples across the groups were not different.

One possible explanation of the above findings may be, that, there is significant variation observed in rodents with regard to resistance to AFB_1 toxicity. Mice have been found to be more resistant to AFB_1 hepatotoxicity. This has been attributed to their high GSTA toward AFB_1 metabolic activity which is high. This is in contrast to rats that are regarded to be among the highly sensitive animals to carcinogenicity and AFB_1 induced hepatotoxicity (Ishida *et al*., 2020).

AFB_1 can induce gene mutation, damage to DNA and chromosomal anomalies in both human beings and animals. Aflatoxin B_1 has been reported to be a pro-toxic chemical (Nili-Ahmadabadi *et al*., 2011; Qian *et al*., 2013; Ishida *et al*., 2020). Furthermore, AFB_1 metabolite known as 8, 9-epoxide is a readily reactive substance that can bind to DNA, protein and other living cells thereby forming 'adducts' (Vincent *et al.,* 2000; Stefano *et al*., 2007; Ghada *et al.,* 2012). Qian *et al*., 2013 reported that conversion of AFB_1 8, 9-epoxide to AFB_1-8, 9-diol which binds to lysine present in albumin resulting in formation of AFB_1-lysine adducts. Consequently, AFB_1-lysine adducts have been determined and confirmed as a viable human aflatoxin exposure biomarker.

Subsequently, any adducts produced during these activities have been associated with disruption of the normal cell function. Furthermore, the adduct formed above and biomarkers of AFB_1 like AFB1-N7-guanine adduct are valuable tools in the assessment of chemo preventive agents in both human beings and animals (Qian *et al*., 2013).

Overall, these study findings suggest that *Spirulina platensis* may reduce the mean concentration of MDA equivalents (µmol) in the kidney samples. Subsequently, it may reduce the concentration of MDA and lipid peroxidation.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1. Introduction

This chapter presents summary of study findings, conclusions drawn and recommendations as per study findings and suggestions for further research.

6.2. Conclusions

1. On protective effect of *Spirulina platensis* extract against biochemical changes induced by AFB_1. Study results showed that *Spirulina platensis* extract at doses of between 100 to 200 mg/Kg/day inhibits elevation of ALT and AST levels.

2. On evaluation of protective effect of *Spirulina platensis* extract against histopathological changes induced by AFB_1 on the kidney and liver; this study revealed that *Spirulina platensis* extract at doses ranging from 100 to 200 mg/Kg/day reverses histopathological changes in liver and kidney as shown by the histopathology photomicrographs.

3. On the investigation of the protective effect of *Spirulina platensis* extract against AFB_1 induced immune dysfunction, this study revealed that AFB_1 induced immune changes in IgG and IgM could be inhibited by pre-administration *Spirulina platensis* extract at doses ranging from 100 to 200 mg/Kg/day. The study findings were statistically insignificant with regard to IgA.

4. Lastly, on the evaluation of the probable mechanism of action of protective effect *Spirulina platensis* extract against AFB_1 induced toxicity, this study showed that *Spirulina platensis* extract reduces the mean concentration of malondialdehyde (MDA) equivalents (μmol). Subsequently, this reduces lipid peroxidation and

AFB_1 toxicity. The study findings were statistically insignificant with regard to mean concentration of MDA equivalents (µmol) in liver samples.

In conclusion, the use of *Spirulina platensis* as a food supplement has been extensively researched by other investigators and its safety confirmed. This study has further demonstrated that *Spirulina platensis* could effectively reverse the effects of AFB_1 that cause liver and kidney damage by reducing the mean concentration of malondialdehyde (MDA) equivalents and thereby reducing oxidative stress. Overall, this study has demonstrated that *Spirulina platensis* has the potential of being used as a feed and food additive to protect the liver and kidney organs from AFB_1 found in feeds and food. Subsequently, this may improve the quality of human life.

6.3. Recommendations

1. This study recommends clinical trials of *Spirulina platensis* to assess its protective effects against AFB_1 induced hepatotoxicity in humans and other animals.
2. This study recommends clinical trials of *Spirulina platensis* to assess its protective effects against AFB_1 induced histopathological changes in liver and kidneys in humans and other animals.
3. This study recommends clinical trials of *Spirulina platensis* to assess its protective effects against immune dysfunction induced by AFB_1. In addition to these, the study recommends clinical trials to assess the use of *Spirulina platensis* as an immune booster.
4. The investigator recommends clinical trials to assess the use of *Spirulina platensis* as an antioxidant.

6.4. Suggestions for further research

This study findings lay the groundwork for further investigation on *Spirulina platensis*. The following suggestions were identified for further studies.

1. This study recommends further phytochemistry studies to determine the specific *Spirulina Platensis* phytoconstituents that cause inhibition biochemical changes, inhibition of immune changes and reversal of histopathological changes.
2. This study recommends further studies to evaluate the highest dose of *Spirulina Platensis* that will give the best prophylaxis against AFB_1 induced hepatotoxicity.
3. This study recommends further studies to evaluate the required doses of *Spirulina Platensis* that inhibit histopathological changes induced by AFB_1 toxicity in primates and extrapolation to human-beings.

4. In addition to these, this study recommends further investigations to establish the highest doses of *Spirulina platensis* required when it is used as an immune booster in animals and human-beings. Also, the study recommends evaluation of the dose of *Spirulina Platensis* required to reverse AFB_1 induced toxicity in clinical trials.
5. Furthermore, investigations are recommended in other experimental animals and human-beings so as to evaluate further the mechanisms responsible for the protective effect of *Spirulina Platensis* with regard to biochemical changes, histopathological changes and immune system.
6. Moreover, more investigations are required to determine whether *Spirulina Platensis* reduces lipid peroxidation directly through reduced reactive oxygen species formation or by reduced in tissue sensitivity to peroxidation arising from suppressed antioxidant defence system.

REFERENCES

Abeer, M.E. (2015). Protective effect of curcumin against experimentally induced aflatoxicosis on the renal cortex of adult male albino rats: a histological and immunohistochemical study. *International Journal of Clinical Experimental Pathology*, *8*(6), 6019 – 6030.

Abu-Elala, N.M., Galal, M.K., Abd-Elsalam, R.M., Mohey-Elsaeed, O.R.N.M., (2016). Effects of Dietary Supplementation of *Spirulina platensis* and Garlic on the Growth Performance and Expression Levels of Immune-related Genes in Nile tilapia (*Oreochromis niloticus*). *Journal of Aquaculture Research and Development, 7*, 433. Doi:10.4172/2155-9546.1000433.

Adhiambo, S.O and Ohingo, M. (2004). Dietary aflatoxin exposure and impaired growth in young children from Kisumu district, Kenya: cross sectional study. *African Journal of Health Sciences*, *11*(1-2), 43-54.

Alakonya, A.E., Monda, E.O. and Ajanga, S. (2009). Short Communication - fumonisin B1 and aflatoxin B_1 levels in Kenyan maize. *Journal of plant pathology*, *91*(2), 459-464.

Ali, S.K. and Mohammed, A.S. (2012). Review article: Spirulina – An overview. *International Journal of Pharmacy and Pharmaceutical Sciences*, *4*(3), 9-15.

Alix, P., Imourana, A.K., Isabelle, P.O. (2016). Review article: Impact of mycotoxin on immune response and consequences for pig health. *Animal Nutrition* *2*(2), 63-68. http://dx.doi.org/10.1016/j.aninu.2016.03.001.

Alperen, G.E.Y and Kultigin, C. (2021). Combined toxic effects of aflatoxin B_2 and the protective role of resveratrol in Swiss albino mice. *Scientific Reports 11*:18081. https://doi.org/10.1038/s41598-021-95879-7.

An, Y., Shi, X., Tang, X., Wang, Y., Shen, F., *et al*., (2017). Aflatoxin B_1 induces reactive oxygen species-mediated autophagy and extracellular trap formation in macrophages. *Frontiers in Cellular and Infection Microbiology, 7*(53). Doi: 10.3389/fcimb.2017.00053.

Angelica, T.I., Elisa, Y.H., Paula, L.A., Ana, P.F.R., Karina K.M., *et.al*., (2017). Impact of a single oral acute dose of aflatoxin B_1 on liver function/cytokines and the lymphoproliferative response in C57Bl/6 Mice. *Toxins, 9*(11), 374; Doi:10.3390/toxins9110374.

Aniket, L., Roch-C., Cheng, C., Je-Ruei, L. and Kuan-Chen, C. (2018). Protective and detoxifying effects conferred by dietary selenium and curcumin against AFB_1-Mediated Toxicity in Livestock: A Review. *Toxins, 10(1*), doi:10.3390/toxins10010025.

Arifin, W.N., and Zahiruddin, W.M. (2017). Sample size calculation in animal studies using resource equation approach. *Malaysian Journal of Medical Sciences*, *24*(5),101–105. https://doi.org/10.21315/mjms2017.24.5.11.

Arpita, M., Misra, N., Srivastav, D., Umapathy, D., Kumar, S. (2014). Spirulina-The Nature's wonder: A review. *Scholars Journal of Applied Medical Sciences*, *2*(4C), 1334-1339.

Asieh, A., Mohammad, F., Ali, M.L, Hossain, S. and Ali, C. (2016). A Review on antioxidant properties of Spirulina. *Journal of Applied Biotechnology Reports*, *3*(1), 345-351.

Asiki, G., Seeley, J., Srey, C., Baisley, K., Lightfoot, T. (2014). A pilot study to evaluate aflatoxin exposure in a rural Ugandan population. *Tropical Medicine and International Health*, *19*(5), 592–599. Doi:10.1111/tmi.12283.

Azziz-B, Lindblade, K., Gieseker, K., Schurz, R., Stephanie, K., *et al*., (2005). Case–Control Study of an acute aflatoxicosis Outbreak, Kenya. *Environmental Health Perspectives*, *113*(12), 1779-1783. Doi: 10.1289/ehp.8384.

Bbosa, K.D., Lubega, A.O., Kyegombe,W.W.D., (2013). Review of the biological and health effects of aflatoxins on body organs and body systems. http://dx.doi.org/10.5772/51201.

Beijing Solarbio Science & Technology Co., Ltd; China; *Elisa Kit user Manual*; Catalog number SEKM-0098. www.solarbio.com.

Bennett, J.W. and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews,* *16*(3), 497–516. DOI: 10.1128/CMR.16.3.497–516.2003.

Brahmi, D., Bouaziz, C., Ayed, Y., Mansour, H.B., Zourgui, L. *et al*., (2011). Chemo preventive effect of *Cactus Opuntia ficus indica* on oxidative stress and genotoxicity of aflatoxin B_1. *Nutrition and Metabolism, 8(*73), 1-16. Doi:10.1186/1743-7075-8-73.

Capelli, B., and Cysewski, G.R. (2010). Potential health benefits of Spirulina microalgae: A review of the existing literature. *Nutra foods, 9*(2), 19-26.

Cardwell, K.F. (2000). Mycotoxin contamination of foods in Africa: Antinutritional factors. *Food and Nutrition Bulletin*, *21*(4), 488-492. Doi:10.1177/156482650002100427.

Carvajal, M.M. (2015). Metabolic changes of aflatoxin B_1 to become an active carcinogen and the control of this toxin. *Immunome Research, 11*(104). DOI:10.4172/1745-7580.10000104.

Charan, J., and Kantharia, N.D. (2013). How to calculate sample size in animal studies? *Journal of pharmacology and pharmacotherapeutics, 4*(4),303–306. DOI: 10.4103/0976-500X.119726.

Chebon, S., Wanyoike, W., Bii, C., Gathumbi, J. and Ogoyi, D. (2016). Incidence of aflatoxigenic fungi and aflatoxins in maize cultivated in highland and mid-altitude agro-ecological zones in Kenya. *Journal of Scientific Research and Reports, 9*(6), 1-12. Doi.org/10.9734/JSRR/2016/17836.

Cheng-Feng, Y., Jin, L., Han-Ming, S. and Choon-Nam, O. (2000). Protective effect of ebselen on aflatoxin B_1-induced cytotoxicity in primary rat hepatocytes. *Pharmacology and Toxicology*, *86*(4), 156–161. Doi: 10.1034/j.1600-0773. 2000.d01-29.x.

Christopher, P.W. and Yun, Y.G (2010). Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis, 31*(1),71–82. Doi:10.1093/carcin/bgp264.

Colla, L.M., Bertol, C.D., Ferreira, D.J., Bavaresco, J. and Costa, J.A.V. (2017). Thermal and photo-stability of the antioxidant potential of *Spirulina*

platensis powder. *Brazilian Journal of Biology, 77*(2), 332-339. http://dx.doi.org/10.1590/1519-6984.14315.

Costa, U.A., Speroni, E., Cervellati, R., Piva, G., *et al*., (2007). Carnosic acid from rosemary extracts: a potential chemoprotective agent against aflatoxin B_1. An in vitro study. *Journal of Applied Toxicology*; *27*(2), 152–159. Doi: 10.1002/jat.1186.

Craig, P.T. (2013). Review Article: The molecular epidemiology of chronic aflatoxin driven impaired child growth. *Hindawi Publishing Corporation Scientifica*, 2013(152879). http://dx.doi.org/10.1155/2013/152879.

Da Silva, J.V.B, Oliveira, C.A.F. and Ramalho, L.N.Z. (2022). An overview of mycotoxins, their pathogenic effects, foods where they are found and their diagnostic biomarkers. *Food Science and Technology, Campinas*, *42*. https://doi.org/10.1590/fst.48520.

Deabes, M.M., Darwish, H.R., Abdel-Aziz, K.B., Farag, I.M., Nada, S.A., *et al*., (2012). Protective Effects of *Lactobacillus rhamnosus GG* on aflatoxins induced toxicities in male albino mice. *Journal of Environment and Analytical Toxicology, 2*(3),132. Doi:10.4172/2161-0525.1000132.

Devendran, G. and Balasubramanian, U. (2011). Biochemical and histopathological analysis of aflatoxin induced toxicity in liver and kidney of rat. *Asian Journal of Plant Science and Research, 1*(4):61-69. ISSN: 2249 – 7412.

Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N. and Panneerselvam, A. (2011). Aflatoxins and aflatoxicosis in human and animals. *Dr. Ramon G. Guevara-Gonzalez (Ed.),* ISBN: 978-953-307-395-8, Doi: 10.5772/22717.

DiNicolantonio, J.J., Bhat, A.G. and Okeefe, J. (2020). Effects of S*pirulina* on weight loss and blood lipids: A review. *Open Heart*, *7*(1), e001003. Doi:10.1136/openhrt-2018-001003.

Dohnal, V., Wu, Q. and Kuc, K., (2014). Review Article: Metabolism of aflatoxins: Key enzymes and interindividual as well as interspecies differences. *Archives of Toxicology, 88*, 1635–1644. Doi: 10.1007/s00204-014-1312-9.

El-beltagi, H.S., Dhawi, F., Ashoush, I.S., Ramadan, K.M.A. (2020). Antioxidant, anti-cancer and ameliorative activities of *Spirulina platensis* and pomegranate juice against hepatic damage induced by CCl4. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 48*(4),1941-1956. Doi:10.15835/48412151.

Ellen, E.Y., Johnni, H.D, Lauren, S.L, Michael, E.R., Ekaterina, M.P., *et al*., (2013). Human aflatoxin exposure in Kenya, 2007: A cross-sectional study. *Food Additives and Contaminants: Part A*, *30*(7), 1322-1331. Doi:10.1080/19440049.2013.789558.

Eva, G.L., Ernesto, M. and Susana P.M. (2011). Aflatoxins and their impact on human and animal health: An emerging problem. Dr. Ramon G. Guevara-Gonzalez (Ed.), ISBN: 978-953-307-395-8. Doi: 10.5772/26196.

Farag, M., Alagawany, M., Ezzat, M. and Dhama, K., (2016). Review Article: Nutritional and Healthical Aspects of *Spirulina (Arthrospira)* for Poultry, Animals and Human. *International Journal of Pharmacology, 12*(1), 36-51. Doi: 10.3923/ijp.2016.36.51.

Faridha, A., Faisal, K. and Akbarsha, M.A. (2006). Duration-dependent histopathological and histometric changes in the testis of aflatoxin B_1-treated mice. *Journal of Endocrinology and Reproduction, 10*(2), 117-133.

Farombi, E.O., Adepoju1, B.F., Ola-Davies, O.E. and Emerole, G.O. (2005). Chemoprevention of aflatoxin B_1-induced genotoxicity and hepatic oxidative damage in rats by kolaviron, a natural biflavonoid of *Garcinia kola* seeds. *European Journal of Cancer Prevention*, *14*(3), 207-214. Doi: 10.1097/00008469-200506000-00003.

Fatma, M.E., Mohamed, A.A.S., Ahmed, M.E.W., Rehab-Allah, A.I. and Mohamed, A.A.S. (2018). Protective effect of Spirulina against cyclophosphamide-induced neurotoxicity in mice. *Egyptian Journal of Basic and Applied Sciences*, *5*(3), 191-196. Doi: 10.1016/j.ejbas.2018.06.001.

Feibo, X., Peiyan, W., Qiucheng, Y., Bing, S., Hongyan, Y., *et al*., (2019). Lycopene alleviates AFB_1-induced immunosuppression by inhibiting oxidative stress and apoptosis in the spleen of mice. *Food and Function, 10*, 3868–3879. Doi.org/10.1039/C8FO02300J.

Genevieve, S.B. and James J.P., (2000). Immunomodulation by fungal toxins. *Journal of Toxicology and Environmental Health, Part B*, *3*(2), 109 – 143. Doi: 10.1080/109374000281113.

Geraldo, R.G.G., (2011). Aflatoxins biochemistry and molecular biology. DOI:10.5772/896.

Gershim, A., Janet, S., Chou, S., Kathy, B., Tracy, L. *et al*., (2014). A pilot study to evaluate aflatoxin exposure in a rural Ugandan population. *Tropical*

Medicine and International Health, *19* (5), 592–599. DOI:10.1111/tmi.12283.

Ghada Z.A. Soliman, A.M.H., and Arafa, M. (2012). Protective effect of *curcuma longa* or *nigella sativa* on aflatoxin B_1-induced hepato-toxicity in rats in relation to food safety on public health. *Medical Journal of Cairo University*, *80*(2), 191 – 203.

Gong, Y., Hounsa, A., Egal, S., Turner, P.C., Sutcliffe, A.E. *et al*., (2004). Postweaning exposure to aflatoxin results in impaired child growth: A longitudinal study in Benin, West Africa. *Environmental Health Perspectives, 112(*13), 1334-1338. Doi: 10.1289/ehp.6954.

Gong, Y.Y., Shona, W., Mwatha, J., Routledge, M., Castelino, J. *et al*., (2012). Aflatoxin exposure may contribute to chronic hepatomegaly in Kenyan school children. *Environmental Health Perspectives, 120*(6), 893 -896. Doi: 10.1289/ehp.1104357.

Gong, Y.Y., Watsona, S. and Routledge, M.N. (2016). Aflatoxin exposure and associated human health effects: A review of epidemiological studies. *Food Safety, 4(*1), 14–27. Doi: 10.14252/foodsafetyfscj.2015026.

Gordon, S.S. (2008). Risk assessment of aflatoxins in food in Africa. *Food Additives and Contaminants, 25(*10), 1246-1256. Doi: 10.1080/02652030802036222.

Guan, Y., Chen, J., Nepovimova, E., Long, M., Wu, W., *et al*., (2021). Aflatoxin detoxification using microorganisms and enzymes. *Toxins,1*3(1), 46. Doi.org/10.3390/toxins13010046.

Gugliandolo, E., Peritore, A.F., D'Amico, R., Licata, P. and Crupi, R. (2020). Evaluation of neuroprotective effects of quercetin against aflatoxin B_1-intoxicated mice. *Animals*, *10*(5), 898. DOI:10.3390/ani10050898.

Gupta R. and Sharma V. (2011). Ameliorative effects of *Tinospora Cordifolia* root extract on histopathological and biochemical changes induced by aflatoxin-B_1 in mice kidney. *Toxicology International, 18*(2).

Gutierrez-Salmean, F.L. and Chamorro, G. (2015). Revision nutritional and toxicological aspects of *Spirulina (Arthrospira). Nutricion Hospitalaria, 32*(1), 34-40. DOI: 10.3305/nh.2015.32.1.9001.

Guyonnet D., Belloir C., Suschetet M., Siess M. and Le Bon A. (2002). Mechanisms of protection against Aflatoxin B_1 genotoxicity in rats by organosulphur compounds from garlic. *Carcinogenesis*, *23*(8), 1335 -1342. Doi: 10.1093/carcin/23.8.1335.

Habib, M.A.B, Parvin, M., Huntington, T.C, Hasan, M.R., (2008). A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. *FAO Fisheries and Aquaculture Circular. No. 1034*. Rome, Italy, Page 33. ISSN 2070-6065.

Hamid, A.S., Tesfamariam, I.G., Zhang, Y. and Zhang, Z.G., (2013). Aflatoxin B_1-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention (Review). *Oncology letters 5*(4), 1087-1092, Doi: 10.3892/ol.2013.1169.

Hell, K. and Mutegi, C. (2011). Aflatoxin control and prevention strategies in key crops of Sub-Saharan Africa. *African Journal of Microbiology Research, 5*(5), 459-466, Doi: 10.5897/AJMR10.009.

Hetta, M., Rehab M., Waled, E., Mohamed, I., Gamila, E., *et al*., (2014). Antiviral and antimicrobial activities of *Spirulina Platensis*. *World Journal of Pharmacy and Pharmaceutical sciences, 3(*6), 31-39. ISSN 2278 – 4357.

Hua-Lian, W., Guang-Hua, W., Wen-Zhou, X., Tao, L. and Hui, H. (2016). Stability and antioxidant activity of food-grade phycocyanin isolated from *Spirulina platensis*. *International Journal of Food Properties, 19(*10), 2349-2362. Doi: 10.1080/10942912.2015.1038564.

Ibrahim, A. and Abdel-Daim, M.M. (2015). Modulating effects of *Spirulina platensis* against tilmicosin-induced cardiotoxicity in mice. *Cell Journal*. *17*(1): 137-144. Doi: 10.22074/cellj.2015.520.

Iqbal, M., Abbas, M., Adil, M., Nazir, A. and Ahmad, I. (2019). Aflatoxins biosynthesis, toxicity and intervention strategies: A review: *Chemistry International 5*(3), 168-191. Doi.org/10.5281/zenodo.1570747.

Isabella, D., Ranieri, R., Daniela, G., Aldo, M. and Roberto, C. (2003). Review: Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta, 329*(1-2), 23–38. Doi: 10.1016/s0009-8981(03)00003-2.

Ishida, Y., Yamasaki, C., Iwanari, H., Yamashita, H., Ogawa, Y. *et al*., (2020). Detection of acute toxicity of aflatoxin B_1 to human hepatocytes in vitro and in vivo using chimeric mice with humanized livers. *Plos One, 15*(9): e0239540. Doi.org/10.1371/journal., pone.0239540.

Jalila, B. S, Samir, A., Rania, J., Zohra, H. and Ridha, O. (2015). Potential preventive role of lactic acid bacteria against Aflatoxin M_1 immunotoxicity and genotoxicity in mice. *Journal of Immunotoxicology, 12(*2), 107-114, Doi: 10.3109/1547691X.2014.904025.

Jalila, B.S., Rania, J., Hakimeh, S., Kambiz, A.N., Ridha, O., *et al*., (2016). Immuno-physiological alterations from AFB_1 in rats counteracted by treatments with *Lactobacillus paracasei* BEJ01 and montmorillonite clay mixture. *Journal of Immunotoxicology, 13(*5), 628-637, Doi: 10.3109/1547691X.2016.1145157.

Jinlu, H., Yaguang, L., Sepideh, P., Sufang, W., Zhenhong, P., *et al.,* (2019). Dose effects of orally administered spirulina suspension on Colonic Microbiota in healthy mice. *Frontiers in cellular and infection microbiology; 9*, 1-13.

John, L.R. (2008). Discovery of aflatoxins and significant historical features. *Toxin Reviews, 27(*3-4), 171-201, Doi: 10.1080/15569540802462040.

Juma, K.K., Fulakeza, R.M.J., Ngeranwa, J.N., Ngugi, M.P., Mburu, N.D. (2015). Evidence based phytopharmacological potential of herbal extracts in post-ingestion management of mycotoxins in animal models. *Journal of Clinical Toxicology, 5*(4), 1 -8. Doi:10.4172/2161-0495.1000260.

Kaiming, T. (2020). Aflatoxin and its tragic tragedies in Kenya. *Journal of Young Investigators*, *38*(2). Doi:10.22186/jyi.38.2.10-12.

Kang'ethe, E.K., and Langat, K.A. (2009). Aflatoxin B_1 and M_1 contamination of animal feeds and milk from urban centres in Kenya. *African Health Sciences, 9(*4), 218 -26.

Kang'ethe, E.K., Gatwiri, M., Sirma, A.J., Ouko, E.O., Mburugu-Musoti, C.K., *et al*., (2017). Review: Exposure of Kenyan population to aflatoxins in foods with special reference to Nandi and Makueni counties. *Food Quality and Safety, 1*(2), 131–137. Doi:10.1093/fqs/fyx011.

Kensler, T.W., Roebuck, B.D., Wogan, G.N. and Groopman, J.D. (2011). Aflatoxin: A 50-Year Odyssey of mechanistic and translational toxicology. *Toxicological sciences, 120* (Supplementary 1), S28–S48. Doi:10.1093/toxsci/kfq283.

Kew, M. (2013). Aflatoxin as a cause of hepatocellular carcinoma. *Journal of Gastrointestinal Liver Disease*, *22*(3), 305-310.

Khalil, A.A., Gabal, A.A., Abdellatef, A.A., Zahran, N.M, and Deraz, S.F. (2019). A natural approach to control induced fumonisin B_1-histopathological nephrotoxic and hepatotoxic changes in rats. *Journal of Applied Pharmaceutical Science, 9*(S1), 017-029. Doi: 10.7324/JAPS. 2019.S103.

Khan, R., Ghazali, F.M., Mahyudin, N.A., Samsudin, N.I.P., (2021). Aflatoxin biosynthesis, genetic regulation, toxicity, and control strategies: A Review. *Journal of Fungi, 7*(8). Doi.org/10.3390/jof7080606.

Khattab, H. AH, Wazzan, M.A.M. and Al-Ahdab, M.A., (2016). Nephroprotective potential of artichoke leaves extract against gentamicin in rats: Antioxidant mechanisms. *Pakistan Journal of Pharmaceutical Sciences, 29*(5), 1775-1782.

Kiama, T.N., Lindah, J.F., Sirma, A.J., Senerwa, D.M., Waithanji, E.M., *et al*., (2016). Kenya dairy farmer perception of moulds and mycotoxins and

implications for exposure to aflatoxins: A gendered analysis. *African Journal of Food, Agriculture, Nutrition and Development*, *16*(3), 11106-11125. Doi: 10.18697/ajfand.75. ILRI10.

Kiarie, G.M., Dominguez-Salas, P., Kang'ethe, S.K., Grace, D. and Lindah, J. (2016). Aflatoxin exposure among young children in urban low-income areas of Nairobi and association with child growth. *African Journal of Food, Agriculture, Nutrition and Development, 16(*3), 10968 – 10990. Doi: 10.18697/ajfand.75. ILRI02.

Kimanya, M.E., Meulenaer, B.D., Roberfroid, D., Lachat, C. and Kolsteren, P. (2010). Fumonisin exposure through maize in complementary foods is inversely associated with linear growth of infants in Tanzania*. Molecular Nutrition and Food Research, 54*(11), 1659–1667 Doi 10.1002/mnfr.200900483.

Knipstein, B., Jiansheng, H., Barr, E., Sossenheimer, P., Dietzen, D., *et al*., (2015). Dietary aflatoxin-induced stunting in a novel rat model: evidence for toxin-induced liver injury and hepatic growth hormone resistance. *Pediatric Research, 78*(2): 120–127.

Kobra, S., Bamdad, R.Z., Mahmoud, M., Amir, H.J., Faezeh, V.H., *et al*., (2018). Immunotoxicity of aflatoxin M_1: As a potent suppressor of innate and acquired immune systems in a sub-acute study. *Journal of the Science of Food and Agriculture, 98*(15), 5884–5892. Doi: 10.1002/jsfa.9240.

Kovesi, B., Kulcsar, S., Cserhati, M., Erdelyi, M., Ancsin, Z., *et al*., (2021). Modification of the effects of aflatoxin B_1 on the glutathione system and its

regulatory genes by zeolite. *Acta Veterinaria Hungarica, 69(*1), 23–30. Doi: 10.1556/004.2021.00002.

Kumar, P., Mahato, P., Madhu, K., Tapan, K.M. and Sang, G.K., (2017). Aflatoxins: A global concern for food safety, human health and their management. *Frontiers in microbiology, 7*,2170. Doi:10.3389/fmicb.2016.02170.

Lalah, J.O., Omwoma, S., Dora, A.O. and Orony, D.A.O., (2019). Aflatoxin B_1: Chemistry, environmental and diet sources and potential exposure in human in Kenya. intechopen.88773. Doi: http://dx.doi.org/10.5772/intechopen.88773.

Landmann M., Scheibner D., Graaf A., Gischke M., Koethe S., *et al.,* (2021). A semi-quantitative scoring system for histopathological and immunohistochemical assessment of leisons and tissue tropism in avian influenza. *Viruses, 13*, 868. Doi: https://doi.org/10.3390/v13050868.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., *et.al.*, (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. *Environmental Health Perspectives, 113*(12), 1763-1767. Doi: 10.1289/ehp.7998.

Lijuan, X., Min, C. and Yibin, Y. (2016). Development of methods for determination of aflatoxins. *Critical Reviews in Food Science and Nutrition, 56(*16), 2642-2664. Doi: 10.1080/10408398.2014.907234.

Maddaly, R., Sai, L.D, Syed, A. and Solomon, F.D.P. (2010). Review: The beneficial effects of *Spirulina* focusing on its immunomodulatory and antioxidant properties. *Nutrition and Dietary supplements. 90(8)*:1817-40.

Makhuvele, R., Naidu, K., Gbashi, S., Thipe, V., Adebo, O., *et al*., (2020). The use of plant extracts and their phytochemicals for control of toxigenic fungi and mycotoxins. *Heliyon*, *6*(10). Doi.org/10.1016/j.heliyon. 2020.e05291.

Makun, H.A., Dutton, M.F., Njobeh, P.B., *et al*., (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa. *Trends in Vital Food and Control Engineering*, Prof. Ayman Amer Eissa (Ed.), ISBN: 978-953-51-0449-0, InTech. Pg 188 – 234.

Mala, R., Sarojini, M., Saravanababu, S. and Umadevi, G. (2009). Screening for antimicrobial activity of crude extracts of *Spirulina platensis*. *Journal of Cell and Tissue Research, 9*(3) 1951-1955.

Maryam, J., (2015). A Review on Aflatoxins reduction in food. *Iranian Journal of Health, Safety & Environment*, *3*(1), 445-459.

Meyerholz D.K. and Beck A.P., (2019). Fundamental concepts for semi-quantitative tissue scoring in translational research. *Institute for Laboratory Animal Research Journal*, *59*(1), 13-17.

Miao, L., Zhang, Y., Peng, L., Yang, S., Zhang, W., *et al*., (2016). Intervention of grape seed proanthocyanidin extract on the sub-chronic immune injury in rats induced by aflatoxin B_1. *International Journal of Molecular Science, 17*(4):516. Doi: 10.3390/ijms17040516.

Migwi, B., Mutegi, C., Mburu, J., Wagacha, J., Cotty, P. *et al*., (2020). Assessment of willingness-to-pay for Aflasafe KE01, a native biological control product for aflatoxin management in Kenya. *Food Additives and Contaminants: Part A, 37(*11), 1951-1962. DOI: 10.1080/19440049.2020.1817571.

Milad, M. (2011). Evaluation of different mycotoxin binders on aflatoxin B_1 (*Aspergillus parasiticus*) produced on Rice (*Oriza sativa*) on Fertility, Hatchability, Embryonic Mortality, Residues in Egg and Semen Quality. *Advances in Environmental Biology, 5*(13), 3818-3825.

Mishra, H.N. and Chitrangada, D., (2003). A Review on biological control and metabolism of aflatoxin. *Critical Reviews in Food Science and Nutrition, 43*(3), 245–264.

Mishra, N., Tandonn, V.L., Dhama, K., Khandia, R., Munjal, A., (2016). Does *Bougainvillea spectabilis* protect swiss albino mice from aflatoxin-induced hepatotoxicity? *Advances in Animal and Veterinary Sciences, 4*(5), 250-257, Doi | http://dx.doi.org/10.14737/journal.,aavs/2016/4.5.250.257.

Mogilnaya, O.A., Puzyr, A.P., Baron, A.V., and Bondar, V.S., (2010). Hematological parameters and the state of liver cells of rats after oral administration of aflatoxin B_1 alone and together with nanodiamonds. *Nanoscale Research Letters 5*:908–912. DOI 10.1007/s11671-010-9571-8.

Mona, N.M.S. (2015). Protective effect of Spirulina against paracetamol induced hepatic injury in rat. *Journal of Experimental Biology and Agricultural Sciences, 3* (1), 45 -53.

Moneera, O.A., Najla, A.A., Amal, N.A., and Nora, M.A., (2021). Physicochemical properties, nutritional value, and sensory attributes of a nectar developed using date palm puree and spirulina. *International Journal of Food Properties, 24*(1), 845-858, Doi: 10.1080/10942912.2021.1938604.

Morbidity and Mortality Weekly Report, (MMWR) (2004). Outbreak of Aflatoxin poisoning — Eastern and Central Provinces, Kenya, January – July, 2004. *MMWR September: 54* (34) 790 – 793.

Mutegi, C.K., Cotty, P.J. and Bandyopadhyay, R. (2018). Prevalence and mitigation of aflatoxins in Kenya (1960-to date). *World Mycotoxin Journal, 11*(3), 341-357. Doi: 10.3920/WMJ2018.2362 341.

Muthomi, J.W., Njenga, L.N., Gathumbi, J.K., Chemining'wa, G.N., (2009). The occurrence of aflatoxins in maize and distribution of mycotoxin producing fungi in Eastern Kenya. *Plant Pathology Journal, 8*(3) 113-119. Doi: 10.3923/ppj.2009.113.119.

Mutiga, S.K., Hoffmann, V., Harvey, J.W., Milgroom, M.G. and Nelson, R.J., (2015). Assessment of Aflatoxin and Fumonisin contamination of maize in Western Kenya. *Phytopathology, 105*(9), 1250-61. Doi: 10.1094/PHYTO-10-14-0269-R.

Mwihia, J.T., Straetmans, M., Ibrahim, A., Njau, J., Muhenje, O. et al (2008). Aflatoxin levels in locally grown maize from Makueni District, Kenya. *East African Medical Journal, 85(*7).

Nathan, M., Gijs, K., Monique, D., Marie-luise, R. and Ria, D. *et al*., (2021). The aflatoxin situation in Africa: Systematic literature review. *Comprehensive Reviews in Food Science and Food Safety*. Doi: 10.1111/1541-4337.12731.

National Research Council of National Academies (2011). Guide on care and use of laboratory animals. *The National Academies Press*, Washington DC; 8th edition.

Nduati, N.N., Njeru, P.N., Mwaniki, M. and Reid, G., (2017). Aflatoxin variations in maize flour and grains collected from various regions of Kenya. *African Journal of Food Agriculture Nutrition and Development, 17*(1), 11743-11756 Doi: 10.18697/ajfand.77.16875.

Ndung'u, J.W., Makokha, A.O., Onyango, C.A., Mutegi, C.K., Wagacha, J.M., *et al*., (2013). Prevalence and potential for aflatoxin contamination in groundnuts and peanut butter from farmers and traders in Nairobi and Nyanza provinces of Kenya. *Journal of Applied Biosciences, 65*, 4922 – 4934. Doi: 10.4314/jab. v65i0.89579.

Negash, D. (2018). A Review of aflatoxins: Occurrence, prevention, and gaps in both food and feed safety. *Journal of Nutritional Health and Food Engineering, 8*(2), 190 – 197. Doi: 10.15406/jnhfe.2018.08.00268.

Nili-Ahmadabadi, A., Tavakoli, F., Hasanzadeh, G.R.., Rahimi, H.R., Sabzevari, O., (2011). Protective effect of pre-treatment with thymoquinone against Aflatoxin B_1 induced liver toxicity in mice. *Journal of Pharmaceutical Sciences*, *19*(4), 282-7.

Noreddine B, (2020). Review: Aflatoxins: Producing-moulds, structure, health issues and incidence in Southeast Asian and Sub-Saharan African Countries. *International Journal of Environmental Research and Public Health, 17(4),*12-15; Doi: 10.3390/ijerph17041215.

Noreddine, B., (2019). Retrospective and prospective look at aflatoxin research and development from a practical standpoint. *International Journal of*

Environmental Research and Public Health, 16(19), 3633; Doi:10.3390/ijerph16193633.

Noreddine, B., (2020). Review: Chronic and acute toxicities of aflatoxins: mechanisms of action. *Journal of Environmental Research and Public Health, 17(2)*, 423; Doi:10.3390/ijerph17020423.

Nour, H., Chadi, S., Denis, J.M. and Abdulsamie, H., (2020). Involvement of hepatic lipid droplets and their associated proteins in the detoxification of aflatoxin B_1 in aflatoxin-resistance BALB/C mouse. *Toxicology Reports, 7*, 795–804. Doi: 10.1016/j.toxrep.2020.06.005.

Obade, M., Andang'o, P., Obonyo, C. and Lusweti, F., (2015). Exposure of children 4 to 6 months of age to aflatoxin in Kisumu County, Kenya. *African Journal of Food Agriculture and Development, 15*(2). Doi: 10.18697/ajfand.69.14020.

Obura, A., (2013). Aflatoxicosis: Evidence from Kenya. *International Food Policy Research Institute*, USA, Focus 20 Brief 2.

Ogechi, S.E., Soelaiman, I., Chin, K., (2021). Health beneficial properties of Spirulina in preventing non-communicable diseases - The green metabolic regulator from the sea. *Sains Malaysiana, 50*(3), 803-819. http://dx.doi.org/10.17576/jsm-2021-5003-21.

Oladeji, E.A., Gondwe, T., Akello, J., Maziya-Dixon, B. and Mukanga, M., (2020). Relationship between serum aflatoxin concentrations and the nutritional status of children aged 6–24 months from Zambia. *International Journal of*

Food Sciences and Nutrition, 71(5), 593-603. Doi: 10.1080/09637486.2019.1689547.

Omar, H., (2013). Mycotoxin induced oxidative stress and disease. *Mycotoxin and Food Safety in Developing Countries*. Chapter 3, 64-94. http://dx.doi.org/10.5772/51806.

Omara, T., Kiprop, A., Wangila, P., Wacoo, A., Kagoya, S. *et al.,* (2021). Review Article: The scourge of aflatoxins in Kenya: A 60-Year Review (1960 to 2020). *Hindawi Journal of Food Quality*, *2021*, https://doi.org/10.1155/2021/8899839.

Oskoueian, E., Abdullah, N., Zulkifli, I., Ebrahimi, M., Karimi, E. *et al*., (2015). Cytoprotective effect of palm kernel cake phenolics against aflatoxin B_1 induced cell damage and its underlying mechanism of action. *BMC Complementary and alternative medicine, 15*(392), 1-12.

Otto P.G., Rathkolb B., Oestereicher M.A., Lengger C.J., Moerth C. *et al.,* (2016). Clinical Chemistry reference intervals for C57BL/6J, C57BL/6N, and C3HeB/FeJ mice (*Mus Musculus*). *Journal of American Association for laboratory animal science*, *55*(4), 375 – 386.

Papalia, T., Sidari, R., and Panuccio, M.R., (2019). Impact of different storage methods on bioactive compounds in *Arthrospira platensis* biomass. *Molecules 24*(15), 2810. Doi:10.3390/molecules24152810.

Patrick, J., Jennifer, A., Stacy, L. and Mark, R. *et al*., (2006). Evaluation of acute immunotoxicity of aerosolized Aflatoxin B_1 in Female C57BL/6N Mice.

Journal of Immunotoxicology, 3, 11–20. DOI: 10.1080/15476910500468635.

Pickova, D., Ostry, V. and Malir, F., (2021). A Recent overview of producers and important dietary sources of Aflatoxins. *Toxins,13*(3*)*, 186. https://doi.org/10.3390/toxins13030186.

Pickova, D., Ostry, V., Toman, J. and Malir, F., (2021). Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation. *Toxins, 13,* 399. https://doi.org/10.3390.

Probst, C., Bandyopadhyay, R., Price, L.E. and Cotty, P.J., (2011). Identification of Atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. *Plant Disease*, *95*(2). Doi:10.1094/PDIS-06-10-0438.

Probst, C., Njapau, H. and Cotty, P.J., (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. *Applied and Environmental Microbiology,* *73*(8), 2762–2764. Doi:10.1128/AEM.02370-06.

Qian, G., Wang, F, Tang, L., Massey, M.E., Mitchell, N.J. *et al*., (2013). Integrative toxicopathological evaluation of aflatoxin B_1 exposure in F344 rats. *Toxicologic Pathology, 41*, 1093-1105. Doi: 10.1177/0192623313477256.

Qinghua, W., Lian L., Anca, M, Blanka, K., Dan, W, Kamil, K., (2016). Review articles: The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview. *Archives of Toxicology*. DOI 10.1007/s00204-016-1744-5.

Ramírez-Rodrigues, M.M., Estrada-Beristain, C., Metri-Ojeda, J., Pérez-Alva, A. and Baigts-Allende, D.K. (2021). *Spirulina platensis* protein as sustainable ingredient for nutritional food products development. *Sustainability*, *13*, 6849. https://doi.org/10.3390/su13126849.

Rania J, Samir A, Jalila B.S, Ridha B.Y, Zohra H et al., (2015). Ability of Lactobacillus plantarum MON03 to mitigate aflatoxins (B_1 and M_1) immunotoxicities in mice. *Journal of Immunotoxicology, 12*(3): 290–299.

Ravi M., Lata S., Azharuddin S., and Paul S.F., (2010). Review: The beneficial effects of Spirulina focusing on its immunomodulatory and antioxidant properties. *Nutrition and Dietary Supplements, 2*(73–83).

Rethinasamy, V., (2017). Review: Bioprospecting of medicinal plants for detoxification of aflatoxins. *International Journal of Nutrition, pharmacology, neurological Disease*. *7*(60-3).

Rushing, B.R. and Selim, M.I. (2019). Review: Aflatoxin B_1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food and Chemical Toxicology, 124*, 81–100.

Sahar, T.A., Abeer, S.H., Manal, A.H., and Mogda, K.M., (2011). Influence of Rosemary extract on immune responses and oxidative stress in mice intoxicated by aflatoxins. *Nature and Science, 9*(10), 54-63. (ISSN: 1545-0740).

Samir, A., Jalila B.S., Mosaad, A.A., and Ridha, O., (2010). Immunotoxicological and biochemical effects of Aflatoxins in rats prevented by Tunisian

Montmorillonite with reference to HSCAS. *Immunopharmacology and Immunotoxicology; 32*(3): 514–522.

Samir, A., Jalila, B-S-A., Rania, J., Ridha B., Younes et al., (2016). Interaction of aflatoxin B_1 and fumonisin B_1 in mice causes immunotoxicity and oxidative stress: Possible protective role using lactic acid bacteria. *Journal of Immunotoxicology*, *13*(1), 46-54. DOI: 10.3109/1547691X.2014.997905.

Seham, A.H., Dalia, A.E., Heba, M.A.A., and Rewan, H.A., (2018). Immunomodulatory effect of dietary turmeric against aflatoxins in mice: histological and immunohistochemical study. *Egyptian Academic Journal of Biological Sciences D. Histology & Histochemistry; 10*(1): 97 – 79. ISSN: 2090 – 0775.

Serfilippi L.M., Stackhouse D.R., Rusell B., and Spainhour C.B., (2003). Serum clinical chemistry and haematology reference values in outbred stocks of albino mice from three commonly used venders and two inbred strains of albino mice. *Contemporary topics*; *42*(3).

Shahid, A.R., Lvhui S., Ni-Ya Z., Mahmoud, M. K., Zhao, L. *et al*., (2019). Grape Seed Proanthocyanidin extract alleviates Aflatoxin B_1-Induced immunotoxicity and oxidative stress via modulation of NF-κB and Nrf2 signaling pathways in broilers. *Toxins, 11(*23). DOI:10.3390/toxins11010023.

Shan, Y. (2019). The toxic effects of Aflatoxin B_1: An Update. DOI: http://dx.doi.org/10.5772/intechopen.88775.

Sharma, A.C., Proshad, R., Kormoker, T., Islam, M.S., and Chandra, K., (2018). A review on aflatoxins in stored grain food, their sources, mechanisms and possible health hazard. *Archives of Agriculture and Environmental Science, 3*(4), 416-423, https://dx.doi.org/10.26832/24566632.2018.0304015.

Sharma, K. D. (2006). Bioprospecting for drug research and functional foods for prevention of diseases – Role of flavonoids in drug development. *Journal of scientific & industrial research*, *65*, 391- 401.

Shivender, S.S. and Anupreet, K. (2012). Review: Aflatoxin B_1: Toxicity, characteristics and analysis. *Global Advanced Research Journal of Chemistry and Material Science*, *1*(4), 063-070, Available online: http://garj.org/garjest/index.htm.

Silvia, M., Andrea, P., Andrea, A., Salvatore, V., Susan, C., *et al*., (2018). Review: Aflatoxin B_1 and M_1: Biological properties and their involvement in cancer development. *Toxins, 10*, 214; DOI:10.3390/toxins10060214.

Sirma, A.J., Senerwa, D.M., Grace, D., Makita, K., Mtimet, N., *et al.,* (2016). Aflatoxin B_1 occurrence in millet, sorghum and maize from four agro-ecological zones in Kenya. *African Journal of Food, Agriculture, Nutrition and Development, 16*(3), 10992- 11003.

Sochor, J., Ruttkay-Nedecky, B., Babula, P., Adam, V., Hubalek, J. *et al*., (2012). Automation of methods for determination of lipid peroxidation. Available at: http://dx.doi.org/10.5772/45945.

Sriwattanapong, K., Slocum, S.L., Chawanthayatham, S., Fedeles, B.I., Egner, P.A., *et al.,* (2017). Pregnancy alters Aflatoxin B_1 metabolism and increases

DNA damage in mouse liver. *Toxicological Sciences,160*(1). DOI: 10.1093/toxsci/kfx171.

Stefano, C., Anelì, U., Ester, S., Rinaldo, C., Gianfranco, P., *et al*., (2007). Carnosic acid from rosemary extracts: a potential chemoprotective agent against aflatoxin B_1. An in vitro study. *Journal of Applied Toxicology, 27,* 152–159. https://doi.org/10.1002/jat.1186.

Sung-Eun, L., Bruce, C.C, Russell, J.M., Shin, H., and Hoi-Seon, L., (2001). Inhibitory effects of naturally occurring compounds on aflatoxin B_1 biotransformation. *Journal of Agricultural and Food Chemistry, 49*, 5171−5177

Tamta, A., Manu, C. and Rajesh, S., (2009). A 28-days sub-acute toxicity study in Swiss albino mice to evaluate toxicity profile of Neurotol Plus (Mannitol and Glycerol Combination). *International Journal of Biomedical Science, 5(*4), 428 – 433.

Tanuja, S., Ruchi, R.K., Vimal, K., Anjali, S., (2016). Evaluation of biochemical and histological effects on liver of Swiss albino mice due to acute oral toxicity of aqueous leaf extract of *Phyllanthus niruri. International Journal of Pharmacognosy and Phytochemical Research; 8*(1); 85-90.

Tomkova, I., Sev, Z. Kova, C., Levkut. M., Revajov, V., Conkov, E., et al., (2001). Effect of aflatoxin B_1 on CD3 T cells and alkaline phosphatase in the intestine of mice. *Mycopathologia, 154,* 15–19.

Vaziriyan, M., Banaee, M., Nemadoost, H.B. and Mohiseni, M., (2018). Effects of dietary exposure to aflatoxins on some plasma biochemical indices of

common carp (*Cyprinus carpio*). *Iranian Journal of Fisheries Sciences 17*(3), 487-502. DOI: 10.22092/IJFS.2018.116552.

Verma, R.J. and Anita, N., (2001). Ameliorative effect of vitamin E on aflatoxin induced lipid peroxidation in the testis of rats. *Asian Journal of Andrology, 3*(3), 217-221.

Vincent, P.K., Elizabeth, M.E., Margaret, M.M., Simon, A.C., Graeme, J.M., *et al*., (2000). Chemoprevention of Aflatoxin B_1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B_1-Aldehyde reductase, the Glutathione *S*-Transferase A_5 and P_1 Subunits, and NAD(P) H: Quinone Oxidoreductase in Rat Liver. *Cancer Research, 60*, 957–969.

WHO (2018). Aflatoxins. *Food safety digest*. REF. No.: WHO/NHM/FOS/RAM/18.1.

WHO and the U.S. Centers for Disease Control and Prevention, (2005). Public Health strategies for preventing aflatoxin exposure. Workgroup report for the international mycotoxin workshop, Geneva.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., *et al*., (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American Journal of Clinical Nutrition, 80*(5),1106-22. Doi: 10.1093/ajcn/80.5.1106.

Wilson S., Cunha de O., Hastreiter B.S., Soares de O.B. *et al*., (2016). Hematological and biochemical reference values for C57BL/6, Swiss Webster and

BALB/c mice. *Brazilian Journal of Veterinary Research and Animal Science, 53*(2), 138-145. Doi: 10.11606/issn.1678-4456.v53i2p138-145.

Xing-Jiu, H., Yang-Kyu, C., Hyung-Soon, I., Oktay, Y., Euisik, Y., *et al*., (2006). Review aspartate aminotransferase (AST/SGOT) and alanine aminotransferase (ALT/GPT) detection techniques. *Sensors 6*, 756-782. http://www.mdpi.com/journal/sensors.

Yakoot, M. and Salem, A. (2012). *Spirulina platensis* versus silymarin in the treatment of chronic hepatitis C virus infection - A pilot randomized, comparative clinical trial, *BMC Gastroenterology, 12*(32).

Yaman, T., Yener, Z. and Celik, I., (2016). Histopathological and biochemical investigations of protective role of honey in rats with experimental aflatoxicosis. *BMC Complementary and Alternative Medicine*, *16*(232), 1-11. DOI 10.1186/s12906-016-1217-7.

Yassein and Zghair (2012). Protective role of honey in rats with experimental aflatoxicosis, BMC Complementary and alternative study of toxicity and pathogenicity of aflatoxin B_1 and G_1 in Rats. *Al-anbar Journal of Veterinary Science*, *5*(1), 23-31.

Yassein, S.N. and Zghair, Z.R. (2012). Study of toxicity and pathogenicity of aflatoxin B_1 and G_1 in mice. *Al-Anbar Journal of Veterinary Sciences, 5*(1), ISSN: 1999-6527.

Yi Jiang, P.E., Peter, P., Jia-Sheng, W., William, O., *et al*. (2008). Aflatoxin-related immune dysfunction in health and in Human Immunodeficiency Virus

Disease. *Clinical and Developmental Immunology*, 1-12; Doi:10.1155/2008/790309.

Zain, M.E., (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society 15*, 129–144. Doi: 10.1016/j.jscs.2010.06.006.

Zamir-Nasta, T., Ahmadi, A., Yazdkhasti, M., Pazhouhi, M., Jalili, C. *et al.,* (2021). Effects of exposure to Aflatoxin G_1 on the plasma biochemical factors and histopathological properties of renal tissue in mice. *Iranian Journal of Toxicology*, *15*(2):127-134. http://dx.doi.org/10.32598/ijt.15.2.787.1.

APPENDICES

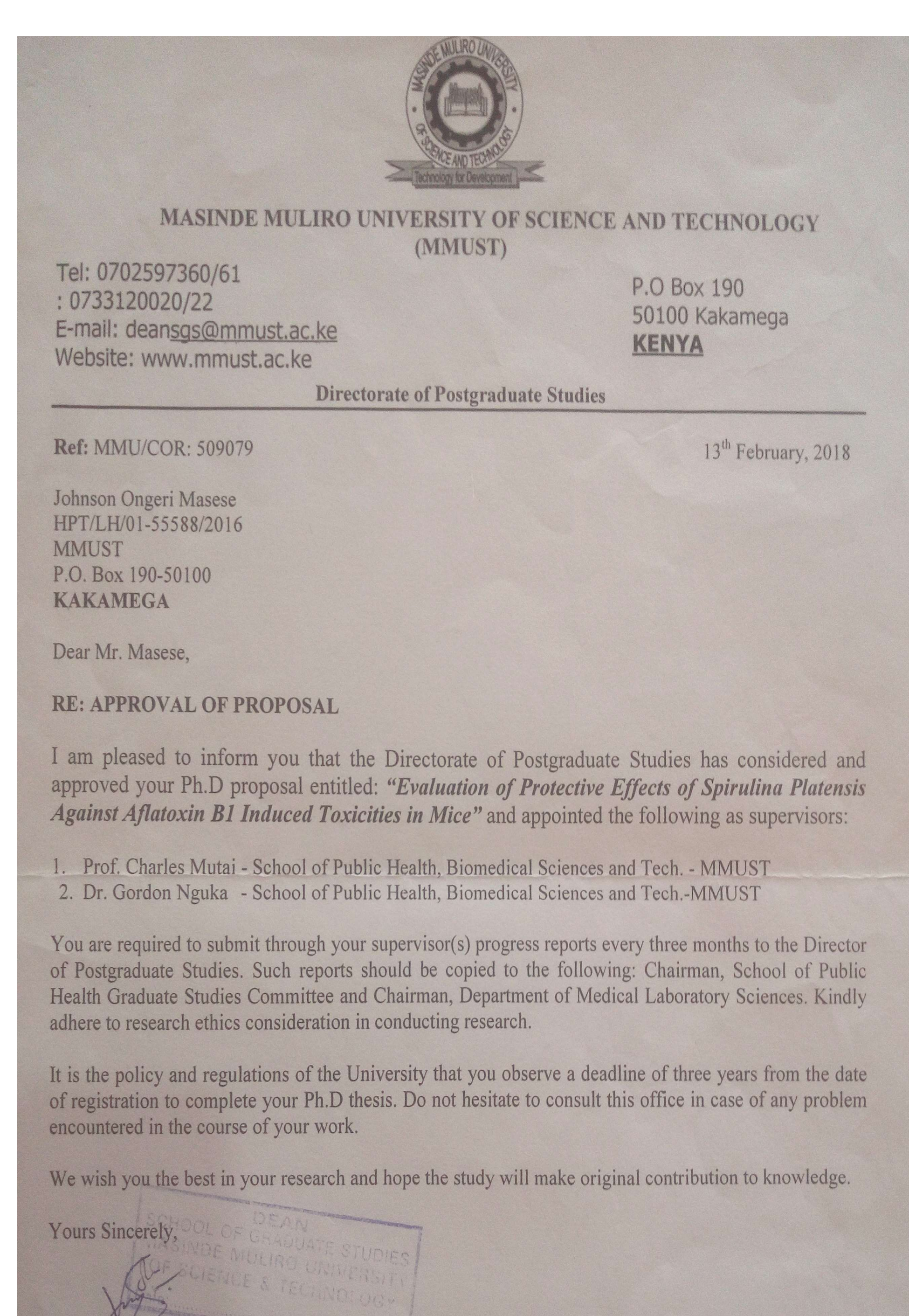
Appendix I: Weight Monitoring Tool

Mice study number	Weight (grams) Day of study			
	0	7	14	28
GROUP I				
1				
2				
3				
4				
5				
GROUP II				
6				
7				
8				
9				
10				
GROUP III				
11				
12				
13				
14				
15				
GROUP IV				
16				
17				
18				
19				
20				
GROUP V				
21				
22				
23				
24				
25				

Appendix II: Tool For Monitoring Health Status of Mice during the Study

	DATE	MICE STUDY No.	GROUP No.	HEALTH STATUS
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

Appendix III: Study approval by Directorate of Postgraduate Studies - MMUST



MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY
(MMUST)

Tel: 0702597360/61
: 0733120020/22
E-mail: deansgs@mmust.ac.ke
Website: www.mmust.ac.ke

P.O Box 190
50100 Kakamega
KENYA

Directorate of Postgraduate Studies

Ref: MMU/COR: 509079

13th February, 2018

Johnson Ongeri Masese
HPT/LH/01-55588/2016
MMUST
P.O. Box 190-50100
KAKAMEGA

Dear Mr. Masese,

RE: APPROVAL OF PROPOSAL

I am pleased to inform you that the Directorate of Postgraduate Studies has considered and approved your Ph.D proposal entitled: ***"Evaluation of Protective Effects of Spirulina Platensis Against Aflatoxin B1 Induced Toxicities in Mice"*** and appointed the following as supervisors:

1. Prof. Charles Mutai - School of Public Health, Biomedical Sciences and Tech. - MMUST
2. Dr. Gordon Nguka - School of Public Health, Biomedical Sciences and Tech.-MMUST

You are required to submit through your supervisor(s) progress reports every three months to the Director of Postgraduate Studies. Such reports should be copied to the following: Chairman, School of Public Health Graduate Studies Committee and Chairman, Department of Medical Laboratory Sciences. Kindly adhere to research ethics consideration in conducting research.

It is the policy and regulations of the University that you observe a deadline of three years from the date of registration to complete your Ph.D thesis. Do not hesitate to consult this office in case of any problem encountered in the course of your work.

We wish you the best in your research and hope the study will make original contribution to knowledge.

Yours Sincerely,

DEAN
SCHOOL OF GRADUATE STUDIES
MASINDE MULIRO UNIVERSITY
OF SCIENCE & TECHNOLOGY

Prof. John Obiri
DIRECTOR DIRECTORATE OF POSTGRADUATE STUDIES

Appendix IV: Study approval by Institutional Ethics Review Committee (IERC)

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY

Tel: 056-31375 P. O. Box 190-50100
Fax: 056-30153 Kakamega, Kenya
E-mail: rel@mmust.ac.ke
Website: www.mmust.ac.ke

Institutional Ethics Review Committee (IERC)

MMU/COR: 403012 vol2 (15) Date: 17/4/2018

Johnson Ongeri
Masinde Muliro University of science and technology
P.O. Box 190-50100
Kakamega

Dear Mr. Ongeri,

RE: Evaluation of protective effects of Spirulina plentensis against aflatoxin B1 induced toxicities in mice.

Thank you for submitting your proposal entitled as above for initial/continuation review. This is to inform you that during the 10th IERC meeting held on the 12th April, 2018, the committee conducted the initial review and approved (with minor revisions) the above Referenced application for one year.

This approval is valid from 17th April, 2018 through to 17th April, 2019. Please note that authorization to conduct this study will automatically expire on 18th April, 2019. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the MMUST IERC by 17th march, 2019.

Approval for continuation of the study will be subject to submission and review of an annual report that must reach the MMUST IERC secretariat by 17th march, 2019. You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to MMUST IERC prior to implementation.

Please note that any unanticipated problems or adverse effects/events resulting from the conduct of this study must be reported to MMUST IERC. Also note that you are required to seek for research permit from NACOSTI prior to the initiation of the study.

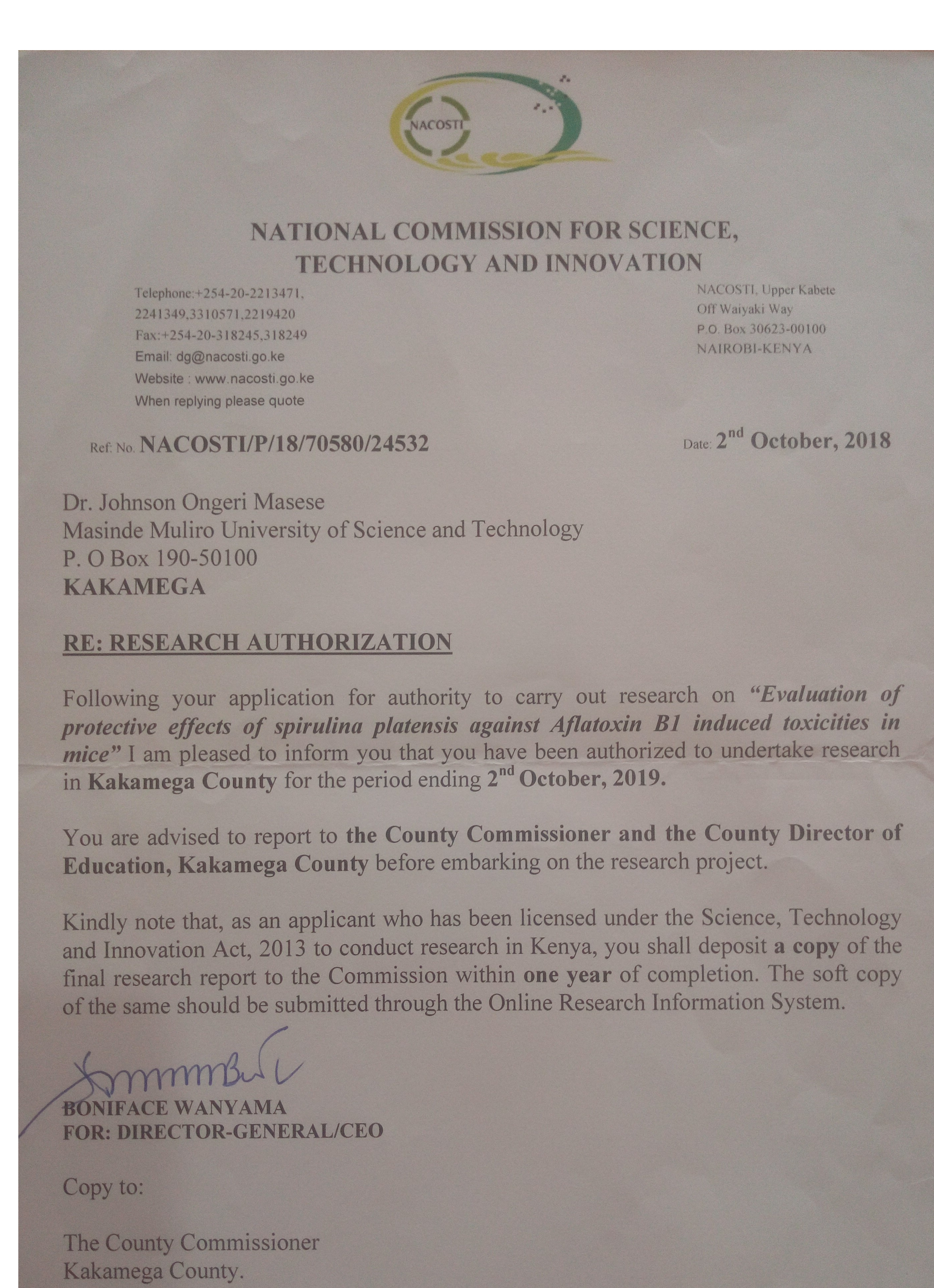
Yours faithfully

Dr Gordon Nguka (PhD)
Chairman, Institutional Ethics Review Committee

Copy to:

- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)
- DVC (A & F)
- DVC (A&SA)

Appendix V: Research authorization by NACOSTI



NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471, 2241349,3310571,2219420
Fax:+254-20-318245,318249
Email: dg@nacosti.go.ke
Website : www.nacosti.go.ke
When replying please quote

NACOSTI, Upper Kabete
Off Waiyaki Way
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/18/70580/24532**

Date: **2nd October, 2018**

Dr. Johnson Ongeri Masese
Masinde Muliro University of Science and Technology
P. O Box 190-50100
KAKAMEGA

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Evaluation of protective effects of spirulina platensis against Aflatoxin B1 induced toxicities in mice"*** I am pleased to inform you that you have been authorized to undertake research in **Kakamega County** for the period ending **2nd October, 2019.**

You are advised to report to **the County Commissioner and the County Director of Education, Kakamega County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

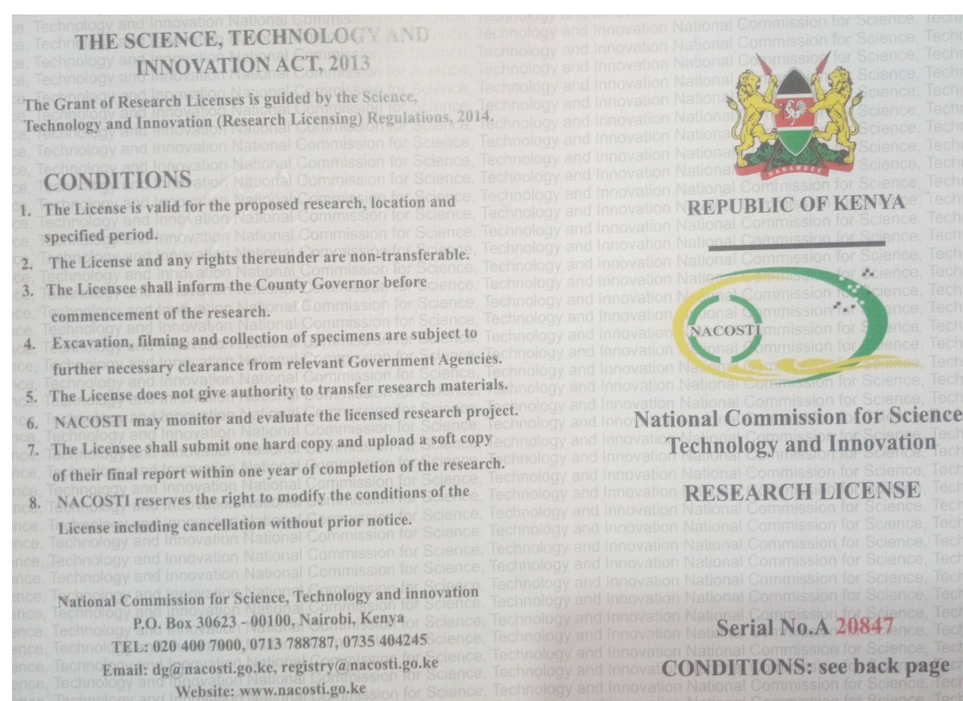
BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Kakamega County.

The County Director of Education
Kakamega County.

Appendix VI: NACOSTI Research licence



THE SCIENCE, TECHNOLOGY AND INNOVATION ACT, 2013

The Grant of Research Licenses is guided by the Science, Technology and Innovation (Research Licensing) Regulations, 2014.

CONDITIONS

1. The License is valid for the proposed research, location and specified period.
2. The License and any rights thereunder are non-transferable.
3. The Licensee shall inform the County Governor before commencement of the research.
4. Excavation, filming and collection of specimens are subject to further necessary clearance from relevant Government Agencies.
5. The License does not give authority to transfer research materials.
6. NACOSTI may monitor and evaluate the licensed research project.
7. The Licensee shall submit one hard copy and upload a soft copy of their final report within one year of completion of the research.
8. NACOSTI reserves the right to modify the conditions of the License including cancellation without prior notice.

National Commission for Science, Technology and innovation
P.O. Box 30623 - 00100, Nairobi, Kenya
TEL: 020 400 7000, 0713 788787, 0735 404245
Email: dg@nacosti.go.ke, registry@nacosti.go.ke
Website: www.nacosti.go.ke

REPUBLIC OF KENYA

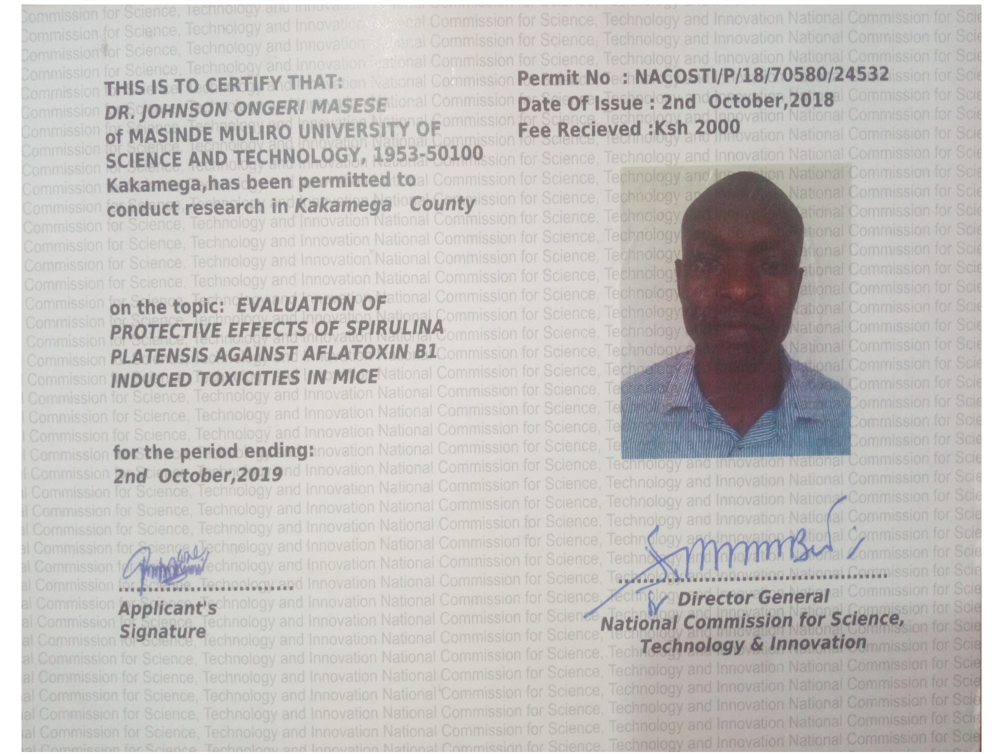
NACOSTI

National Commission for Science, Technology and Innovation

RESEARCH LICENSE

Serial No.A 20847

CONDITIONS: see back page



THIS IS TO CERTIFY THAT:
DR. JOHNSON ONGERI MASESE
of MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY, 1953-50100 Kakamega,has been permitted to conduct research in *Kakamega County*

on the topic: *EVALUATION OF PROTECTIVE EFFECTS OF SPIRULINA PLATENSIS AGAINST AFLATOXIN B1 INDUCED TOXICITIES IN MICE*

for the period ending:
2nd October,2019

Permit No : NACOSTI/P/18/70580/24532
Date Of Issue : 2nd October,2018
Fee Recieved :Ksh 2000

Applicant's Signature

Director General
National Commission for Science, Technology & Innovation

Appendix VII: Research authorization by Ministry of Interior & Co-ordination of National Government

REPUBLIC OF KENYA

THE PRESIDENCY
MINISTRY OF INTERIOR & CO-ORDINATION OF NATIONAL GOVERNMENT

Office Mobile No: 0707 085260
Email-cckakamega12@yahoo.com

When replying please quote

Ref No: ED/12/1/VOL.IV/19

COUNTY COMMISSIONER
KAKAMEGA COUNTY
P O BOX 43-50100
KAKAMEGA.

Date: 31st October, 2018

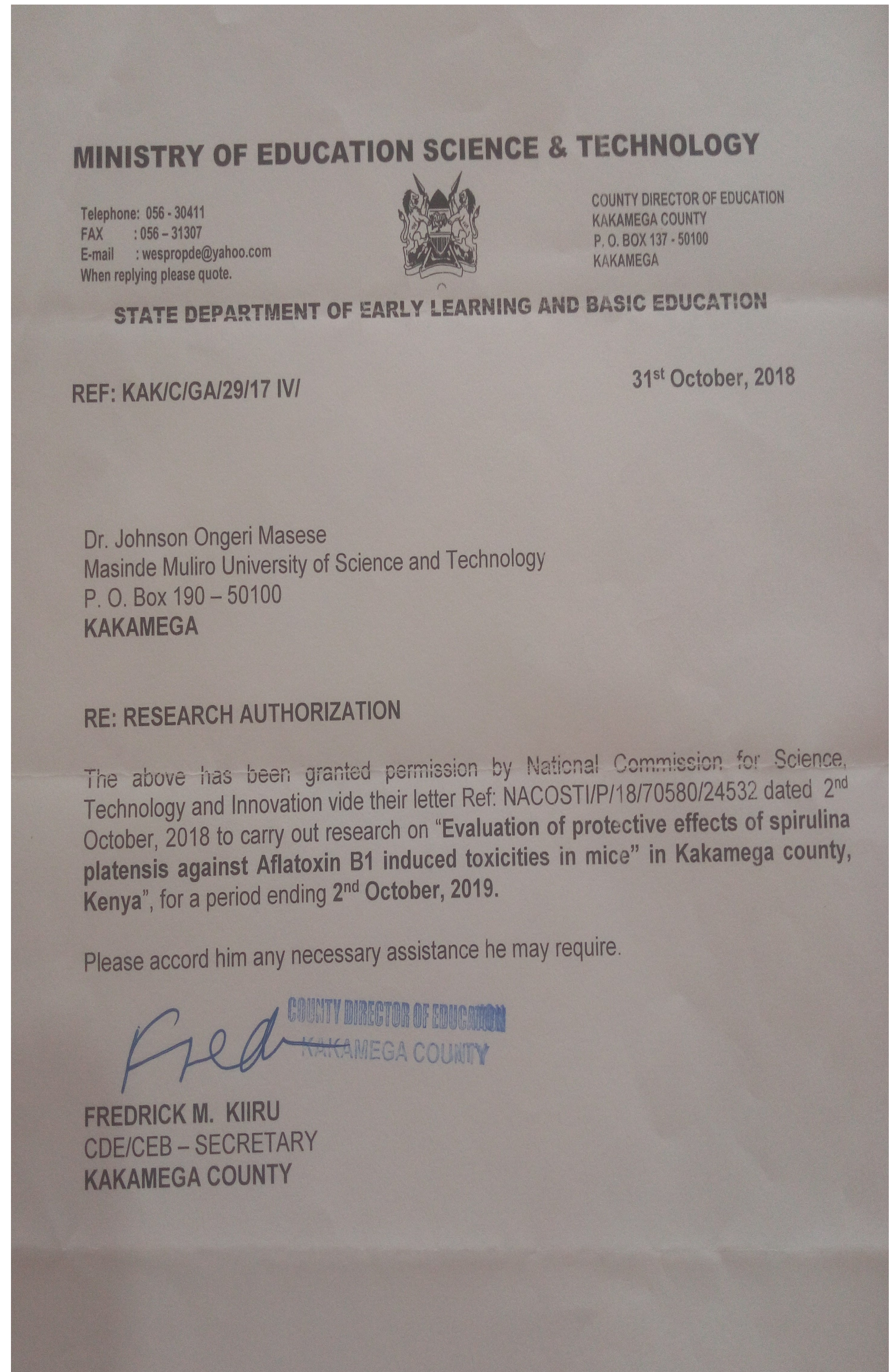
DR. JOHNSON ONGERI MASESE
MASINDE MULIRO UNIVERSITY
OF SCIENCE AND TECHNOLOGY
P O BOX 190-50100
KAKAMEGA

RE: RESEARCH AUTHORIZATION

Following your authorization vide letter Ref: NACOSTI/P/18/70580/24532 dated 2nd October, 2018 by NACOSTI to undertake research on ***"Evaluation of protective effects of sirulina platensis against Aflatoxin B1 induced toxicities in mice in Kakameg County".*** I am pleased to inform you that you have been authorized to carry out the research on the same.

P. K. MARACHI
FOR: COUNTY COMMISSIONER
KAKAMEGA COUNTY

Appendix VIII: Research authorization by the State Department of Early Learning and Basic Education



MINISTRY OF EDUCATION SCIENCE & TECHNOLOGY

Telephone: 056 - 30411
FAX : 056 – 31307
E-mail : wespropde@yahoo.com
When replying please quote.

COUNTY DIRECTOR OF EDUCATION
KAKAMEGA COUNTY
P. O. BOX 137 - 50100
KAKAMEGA

STATE DEPARTMENT OF EARLY LEARNING AND BASIC EDUCATION

REF: KAK/C/GA/29/17 IV/

31st October, 2018

Dr. Johnson Ongeri Masese
Masinde Muliro University of Science and Technology
P. O. Box 190 – 50100
KAKAMEGA

RE: RESEARCH AUTHORIZATION

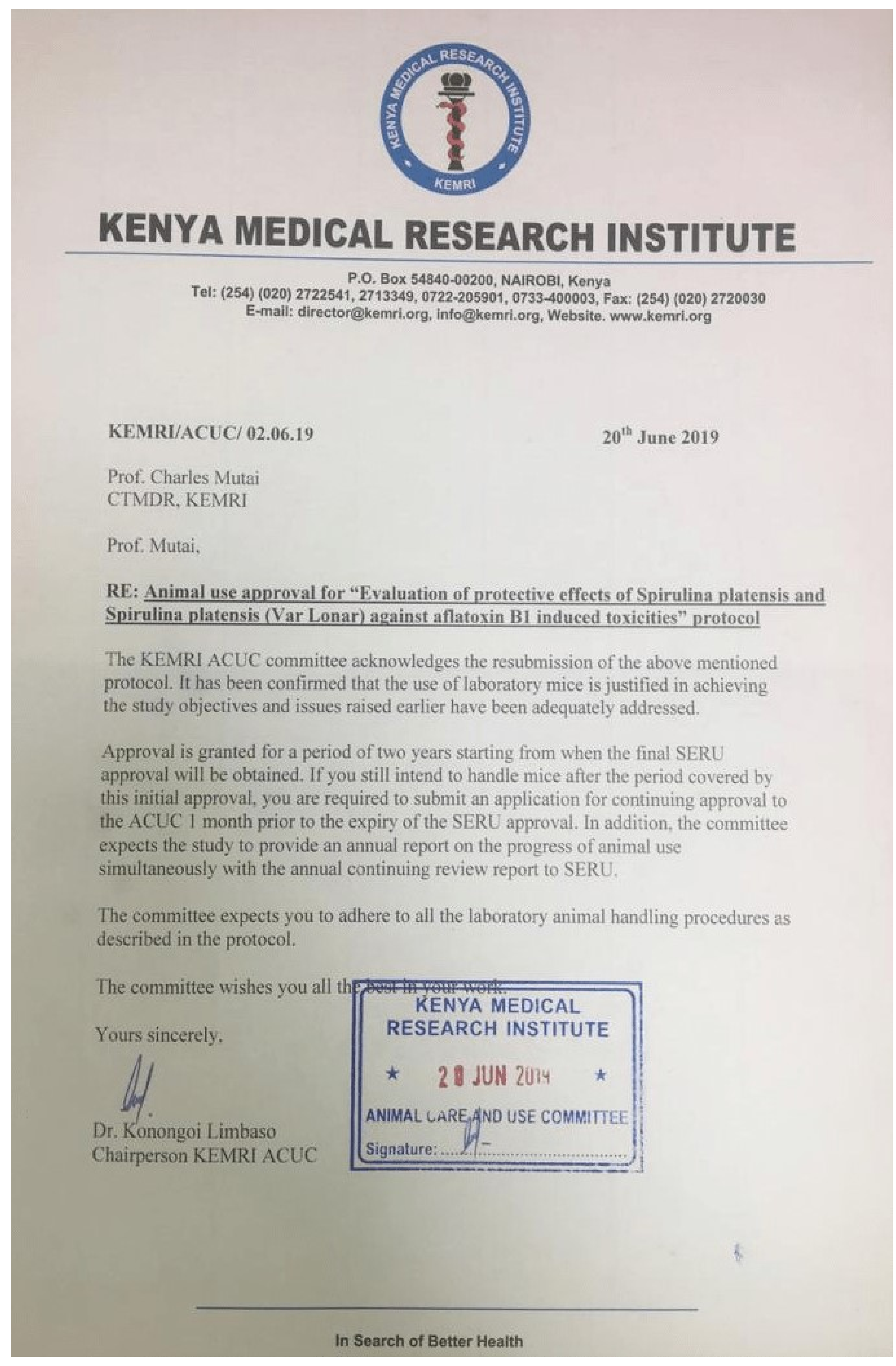
The above has been granted permission by National Commission for Science, Technology and Innovation vide their letter Ref: NACOSTI/P/18/70580/24532 dated 2nd October, 2018 to carry out research on "**Evaluation of protective effects of spirulina platensis against Aflatoxin B1 induced toxicities in mice" in Kakamega county, Kenya**", for a period ending **2nd October, 2019.**

Please accord him any necessary assistance he may require.

COUNTY DIRECTOR OF EDUCATION
KAKAMEGA COUNTY

FREDRICK M. KIIRU
CDE/CEB – SECRETARY
KAKAMEGA COUNTY

Appendix IX: Study approval by KEMRI Animal Use and Care committee



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
20 JUN 2019
ANIMAL CARE AND USE COMMITTEE
Signature: ...

In Search of Better Health

Appendix X: Experimental Animals Request Form

In Search of Better Health

KENYA MEDICAL RESEARCH INSTITUTE

ANIMAL FACILITY

EXPERIMENTAL ANIMALS REQUEST FORM

User name JOHNSON ONGERI MASESE Centre/Department CTMDR

Animal species MICE Strain Swiss Albino Date required 16/10/2020

Sex Male Age/Wt. 8 weeks Number 25

Procedures to be performed on the animals Evaluation of Protective effects of spirulina Plantesis against Aflatoxin B_1 induced Toxicities

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

If yes, name of the pathogen ....

Study date: Start 16/10/2020 End 18/11/2020

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 25

Issued by Sarah Awuor. signature .... Date 16/10/2020

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

Appendix XI: A 96 well plate template used to record the layout of the experimental specimen before reading of absorbance using Multiskan GO® equipment

AMPLITAQ GOLD®

Applied Biosystems

96-Well Plate Template

Date: __/__/__ Experiment/Plate #: ________________________

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Date: __/__/__ Experiment/Plate #: ________________________

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Appendix XII: Kemri Waste Tracking document

FORM NEMA/WM/3
Adopted from Regulation 8 of Waste Management Regulations, 2006
(Legal Notice 121)

Serial Number:

KENYA MEDICAL RESEARCH INSTITUTE

KEMRI WASTE TRACKING DOCUMENT
To be filled in DUPLICATE

A. Transporter	Name of staff transporting the waste ...Johnson Masese P/N0..........................Signature...Masese Job Description...Student Name of person who authorized waste disposal...Stephen Signature of person authorizing waste disposal...[signature]
CONSIGMENT NOTE FOR THE CARRIAGE AND WASTE DISPOSAL OF SOLID WASTE	
B. Description of waste	1) Area collected...Animal tissue house 2) Type of waste ...Mice tissue 3) Description and physical nature of waste (solid/liquid) ...solid 4) Quantity/size of waste (Kgs/liters) ...2kg 5) Number of containers/bags...1
C. Disposer's Certificate (To be filled by the Incineration staff)	I certify that I have received the waste as described in A and B above. The waste was delivered in vehicle/by hand 1426hrs on (Registration No.) at 1426hrs (Time) on 8/12/2021 (date) and the carrier gave/her name as Johnson Masese on behalf of Animal house The waste shall be disposed off as per KEMRI Waste Disposal Guidelines and Waste Management Regulations (Legal Notice 121) of Kenya. Signed: [signature] Name: BOSIBORI MERCYLINE Position: INTERN Date: 8/12/2021 On behalf of : *HSE CORDINATOR*

RECEIVED
Name:...
Signature:...
Date:...

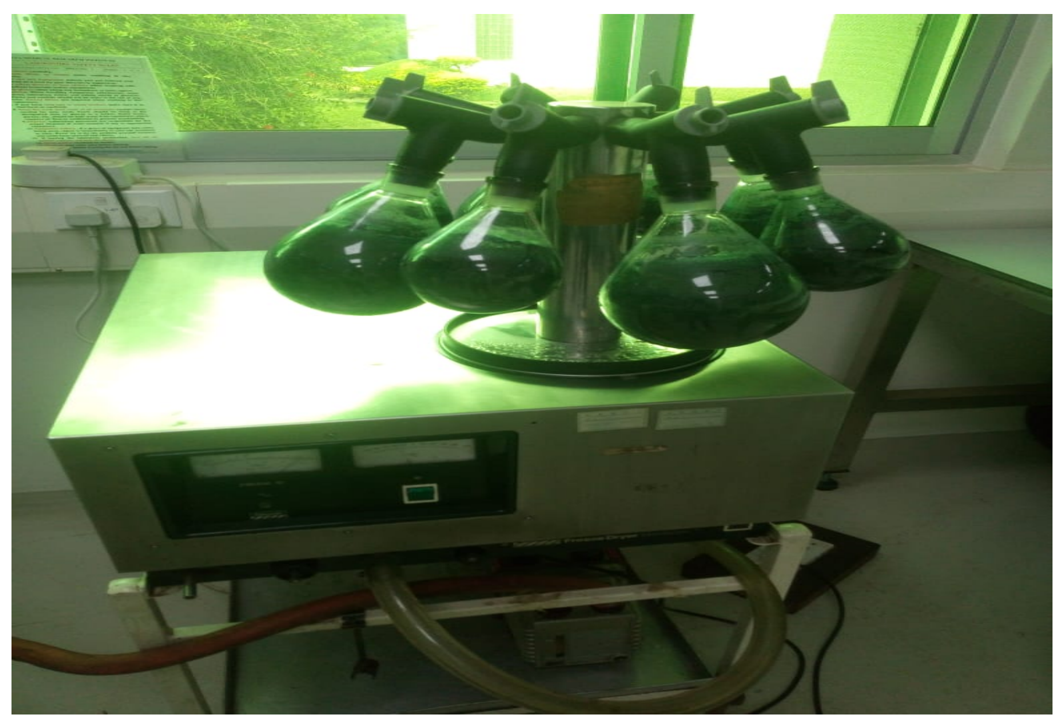
After signing Part C one FORM will be retained at the Incinerator and the other FORM will be taken back by the transporter

NOTE: This is a CONTROLLED document. **The user of this document is responsible for ensuring that they are using the latest revision of the document.**

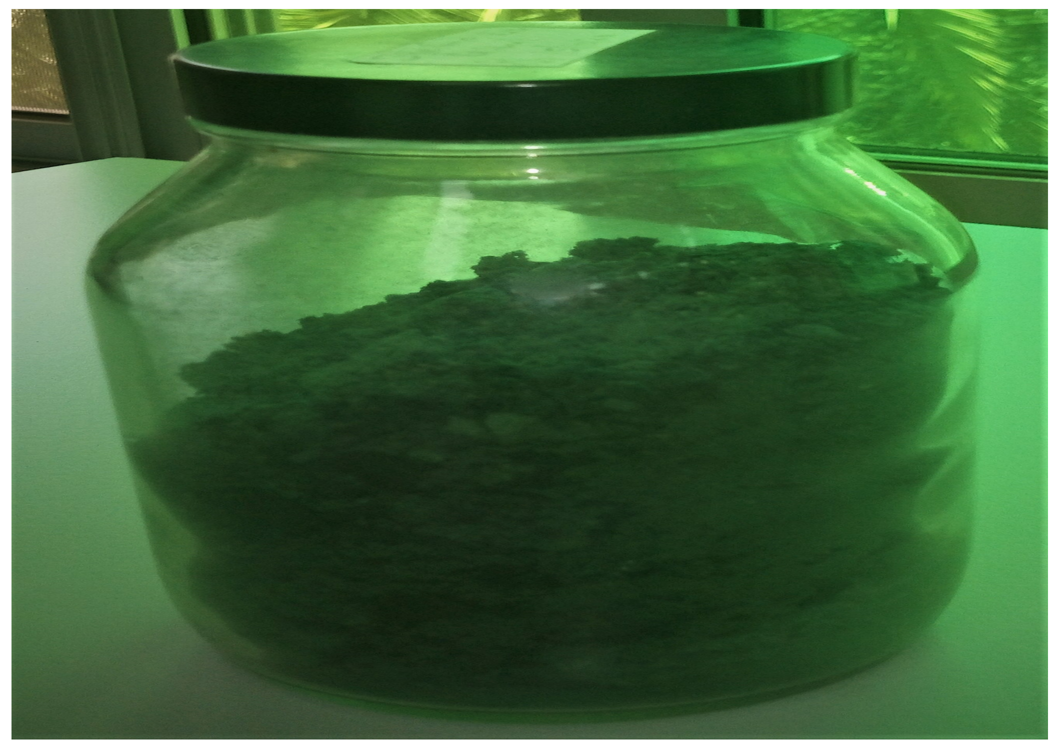
Appendix XIII: Study Photographs

(I) MMUSTMUG SPIRULINA®

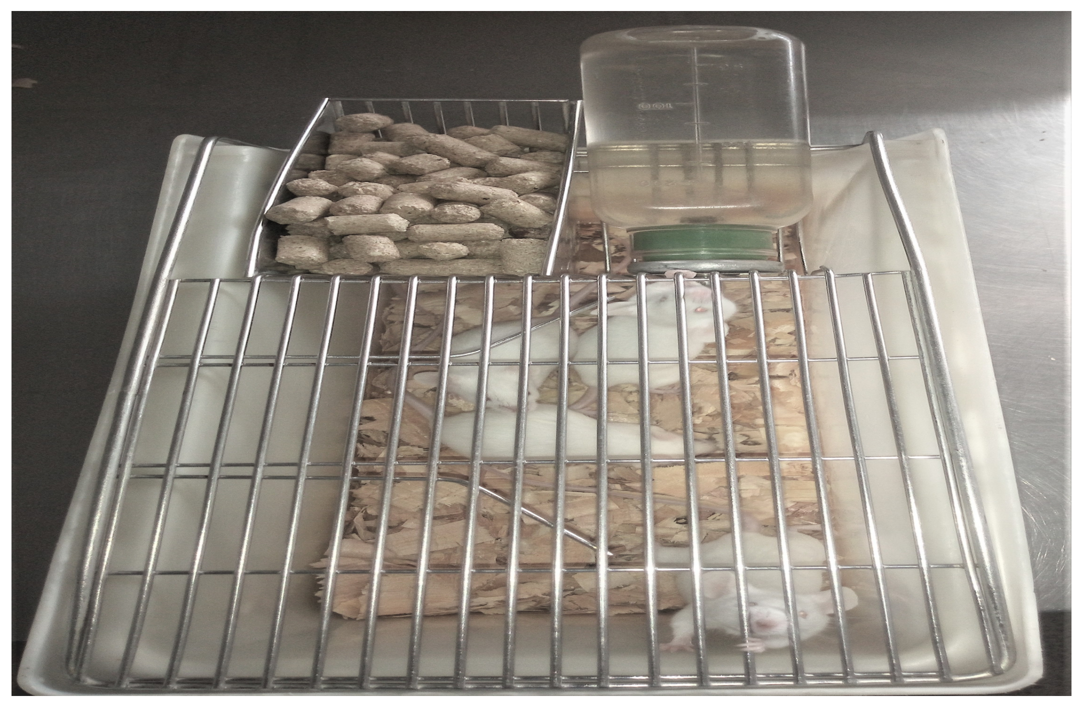
(II) Coating of Spirulina platensis filtrate with dry ice and acetone



(III) Freeze drier (Edwards® freeze dryer modulyo, Kemri No: JJK0035)

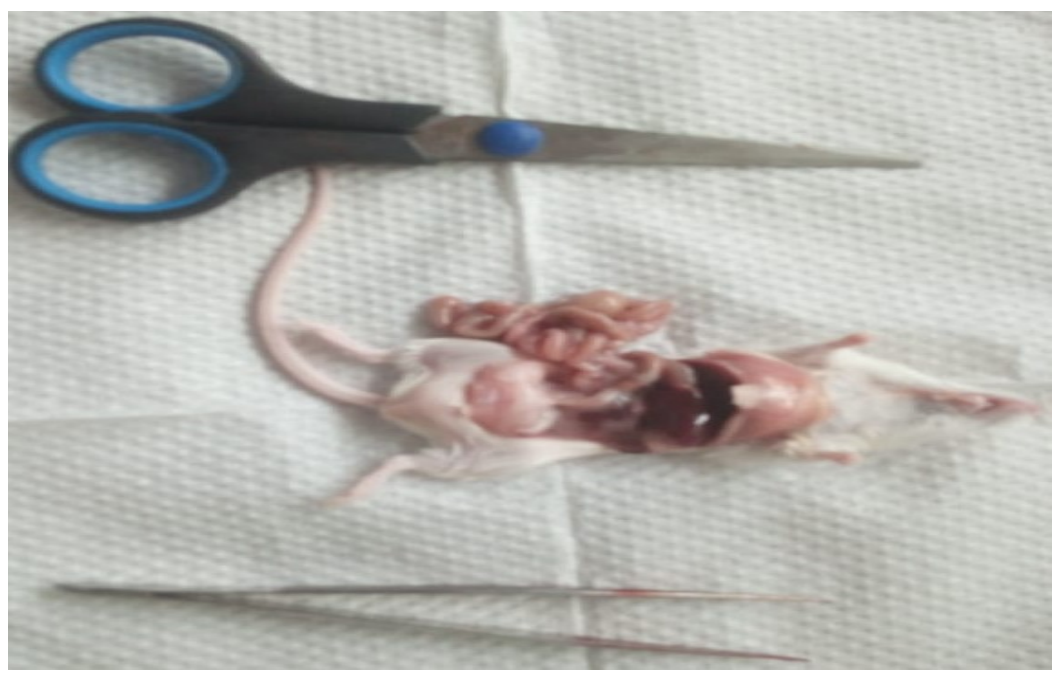


(IV) Freeze dried *Spirulina platensis* powder



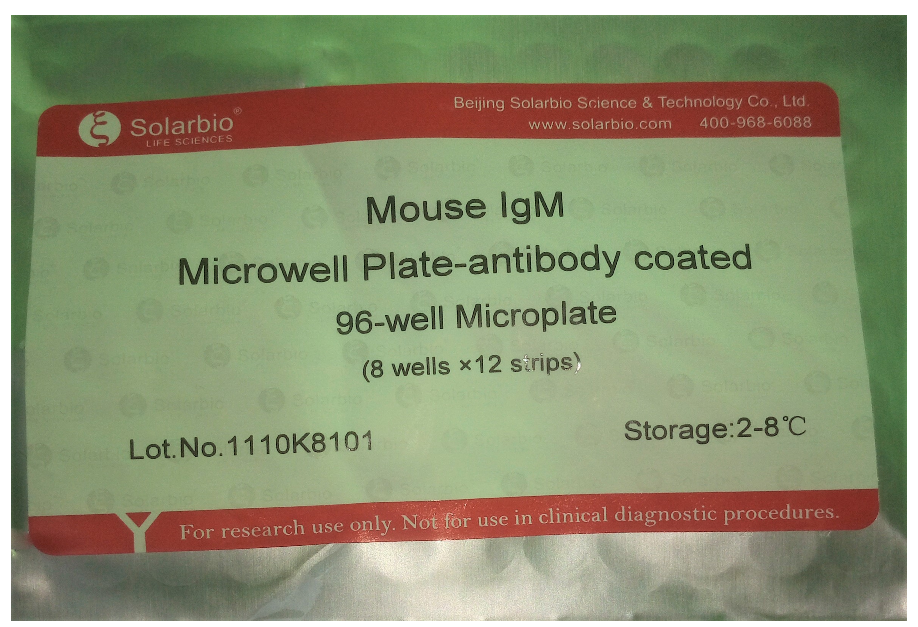
(IV) Male Swiss albino mice in polypropylene cages having a metallic top.

(V) Labelled polypropylene cages for the 5 experimental groups



(VI) Harvesting of sacrificed mice tissues at the end of the experimental period

(VII) Processing of the blood samples before assay procedures



(VIII) ELISA kits used in the quantitative determination of immunoglobulin biomarkers in serum

Appendix XI: List of Study Publications

1. *Spirulina platensis* inhibits aflatoxin B_1 induced biochemical changes in male Swiss albino mice.
2. Protective effect of *Spirulina platensis* extract on aflatoxin B_1 immunotoxicities in mice.
3. Evaluation of effects of *Spirulina* extracts on immunologic dysfunction and inflammation associated with aflatoxin B1 induced toxicity in mice.