EFFICACY AND SAFETY, NUTRITIONAL ANDANTIOXIDANT ACTIVITYOF KENYAN *ANNONA MURICATA* (L.) AND *ANNONA SQUAMOSA* (L.) FRUITSEXTRACTS IN BALB/C MICE MODELOF *L. MAJOR* LEISHMANIASIS

LENNY MWAGANDI CHIMBEVO

DOCTOR OF PHILOSOPHY

(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2019

Efficacy and Safety, Nutritional andAntioxidant Activityof Kenyan *Annona muricata* (L.) *and Annona squamosa* (L.) Fruits Extracts in BALB/c Mice Model of *L. major* Leshmaniasis

Lenny Mwagandi Chimbevo

A thesis submitted in partial fulfilment for the Degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology

2019

DECLARATION

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

Signature…………………………………… Date……………………….

Lenny Mwagandi Chimbevo

This thesis has been submitted for examination with our approval as university supervisors.

Signature……………………………… Date…………………………

Prof. Simon M. Karanja, (PhD)

JKUAT

Signature……………………………… Date…………………………

Prof. Jennifer A. Orwa, (PhD)

KEMRI, Kenya

DEDICATION

To my loving parents; my late father Chimbevo Mwagambo Gambo and my mother Mbodze Nyale Mpunga, my uncle Joseph Bekwekwe Mwangala my wife Anita Dzame Goeand my daughters Lizzy Mlongo Mwagandi and Lynne Mbodze Mwagandi.

ACKNOWLEDGEMENT

In the realization of the awesome task of writing this thesis, I take this opportunity to acknowledge the contribution of various parties and persons. First, I amindebted to God, the creator and redeemer, for the directly or indirectlyprovisions through persons every other time for all the needs that arose during the study period. Acknowledgement, respectful appreciations and gratitudes goes to my supervisors: Prof. Simon M. Karanja of JKUATand Prof. Jennifer A. Orwa of Centre for Traditional Medicine and Drug Research (CTMDR) – KEMRI and he late Dr. Christopher O. anjili of centre for Biotechnology Research and Development (CBRD) - KEMRI for their constant advice, guidance, suggestions, encouragement and follow-up throughout my study period. Also, my sincere gratitude goes to Food Biochemistry Laboratory Departments of Food Science and Technology (JKUAT), Centre for Traditional Medicine and Drug Research (CTMDR) laboratories (KEMRI), Centre for Biotechnology and Research Development (CBRD) laboratories (KEMRI) and Directorate of Research and Innovation laboratories (Mount Kenya University) and their technical staffs where the study was undertaken who have been very cooperative and helpful in technical guidance during the study. My special thanks go to the National Commission for Science Technology and Innovation (NACOSTI) - Kenya for funding my project. Finally, I owe a great debt to the family for instilling a sense of hope in my work. As always, they provided love, encouragement and support. To God be the glory.

TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENT iii

TABLE OF CONTENTS iv

LIST OF TABLES ix

LIST OF FIGURES xii

LIST OF PLATES xiv

APPENDICES xv

ABBREVIATIONS AND ACRONYMS xix

ABSTRACT xx

CHAPTER ONE 1

1.0 INTRODUCTION 1

1.1 Background information 1

1.2 Statement of the problem 3

1.3 Justification 4

1.4 Research questions 5

1.5 Objectives of the study 5

1.5.1 Overall objective 5

1.5.2 Specific objectives 5

1.6 Variables 6

1.6.1 Independent variables 6

1.6.2 Dependent variables 6

CHAPTER TWO 7

2.0LITERATURE REVIEW 7

2.1 The leishmaniases and their global distribution 7

2.2 Economic importance of leishmaniasis 11

2.3 Biology and life cycle of Leishmania 11
2.4 Aetiology, clinical and pathological manifestations of leishmaniasis 14
2.5 BALB/c mice as a model of leishmaniasis study 17
2.6Malnutrition and Leishmaniasis 18
2.7 Chemotherapy of leishmaniasis 19
2.8 Botanicals as alternatives in leishmania management 22
2.8.1 Taxonomy and geographical distribution of Annonaceae spp 22
2.8.2 Future of natural products from folk medicines in treatment of leishmaniasis 23
2.8.3 Annonaceae spp as alternative source of neutraceutical substances 25
2.8.4 Antioxidant, DNA and protein inhibitory activity in leishmaniasis 26
CHAPTER THREE 28
3.0 MATERIAL AND METHODS 28
3.1 Study area 28
3.2 Collection of Plant Materials 28
3.3 Study design 29
3.4 Extraction of phytochemicals from pulp, peel and seeds of *A. muricata* and *A. squamosa*fruits 29
3.5 Phytochemical analysis 31
3.5.1 Qualitative phytochemical analysis 31
3.4.1.6 Test for terpenoids 33
3.5.2 Quantitative phytochemical analysis 34
3.6 Proximate and nutritional composition analysis 38
3.6.1 Moisture content 38
3.6.2Dry matter 38
3.6.3 Ash content 38
3.6.4 Crude fat content 39
3.6.5 Crude protein 39
3.6.6 Crude fiber 40
3.6.7 Carbohydrate content 41
3.6.8 Titratable acidity 41

3.6.9 Reducing sugars 41
3.6.10 Determination of mineral composition 41
3.6.11 Fatty acid composition 42
3.6.12 Vitamin C (Ascorbic acid) 43
3.6.13 Tocopherol 44
3.6.14 Total carotenoids analysis and quantification 44
3.7 Antioxidant assays **........ 46**
3.7.1 In vitro antioxidant assays 46
3.7.2 In vivo antioxidant assays 49
3.8 Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet 50
3.9 Antileishmanial and safety assays 51
3.9.1 *In vitro* antileishmanial assays 51
3.9.2 *In vivo* antileishmanial assay 52
3.9.3 Safety assays 54
3.10 DNA-binding and DNA Topoisomerase I inhibitory activity assays 55
3.10.1 DNA-binding interactions assays 55
3.10.2 DNA Topoisomerase I Inhibitory Activity Assay 56
3.11 Ethical consideration 56
3.12 Data analysis 56
CHAPTER FOUR 58
4.0 RESULTS 58
4.1 Phytochemical analysis 58
4.1.1 Appearance and percentage yeilds of the extracts 58
4.1.2 Qualitative phytochemical analysis 59
4.1.3 Quantitative phytochemical analysis 61
4.2 Proximate, nutritional compositional and antioxidant analysis 62
4.2.1 Preliminary proximate compositional analysis 62
4.2.2 Fatty acid composition 64
4.2.3 Mineral elements composition 64
4.2.4 Carotenoids analysis and quantification 65

4.2.5 Antioxidant activities 67
4.3 Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet 84
4.3.1 Proximate composition analysis of the experimental feeds 84
4.3.2 Proximate Composition of Muscles of BALB/c mice 84
4.3.3 Body mass developments, growth performance and nutrient utilization.. 86
4.3.4 Haematological changes 89
4.3.5 Liver function tests 91
4.5.6 Kidney function tests and lipid profiles 93
4.3.7 Lesion size and liver and splenic length, size and parasite burden........... 95
4.4 Efficacy and safety of the extracts 98
4.4.1 *In vitro* antileshmanial activity 98
4.4.2 *In vivo* antileishmanial activity 104
4.4.3 Determination of safety of the extracts 118
4.5 DNA Binding and DNA Topo I inhibitory activity 122
4.5.1 DNA Binding activity 122
4.5.2 DNA Topoisomerase I inhibitory activity 123
CHAPTER FIVE 126
5.0 DISCUSSION 126
5.1 Phytochemical screening 126
5.2 Proximate, nutritional composition analysis and antioxidant activities......... 128
5.2.1 Proximate and nutritional composition analysis 128
5.2.2 Carotenoids analysis and quantification 133
5.2.3 *In vitro* and in *vivo* antioxidant activity in relation to leishmaniasis 136
5.3 Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet 140
5.4 Efficacy and Safety of the extracts 144
5.4.1 Antileishmanial activity of the extracts 144
5.4.2 *In vitro* toxicity of the extracts 146
5.4.3 *In vivo* toxicity of the extracts 147
5.5 DNA Binding interactions and DNA Topo I inhibitory activity 151

5.5.1 DNA Binding interactions of the extracts 151
5.5.2 DNA Topo I inhibitory activity of the extracts 153
CHAPTER SIX 155
6.0 CONCLUSSIONS AND RECOMENDATIONS 155
6.1 Conclussions 155
6.2 Recomendations 156
REFERENCE 158
APPENDICES 198

LIST OF TABLES

Table 2.1: Proven vectors of human leishmaniasis 15
Table 2.2: Spectrum of leishmaniasis, aetiological agents and distribution 16
Table 3.1: Abbreviations of different crude extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds 30
Table 4.1: Percentage yield, colour and appearance of different crude extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds 59
Table 4.2: Qualitative phytochemical analysis in pulp, peel and seeds of *A. muicata* fruit 60
Table 4.3: Qualitative phytochemical analysis in pulp, peel and seeds of *A. squamosa* fruit 60
Table 4.4: Quantitative phytochemical analysis of pulp and seeds of *A. muricata* and *A. squamosa* fruits 61
Table 4.5: Proximate composition analysis of pulp, peel and seeds of *A. muricata* and *A. squamosa* fruits 63
Table 4.6: Fatty acids composition of pulp and seeds of *A. muricata* and *A. squamosa* fruits 64
Table 4.7: Mineral composition of different parts of *A. muricata* and *A. squamosa* fruits 65
Table 4.8: The total carotenoid concentration in the peel, pulp and seeds of *A. muricata* and *A. squamosa* fruits (µg/100g DW) 66
Table 4.9: Lipid peroxidation (LPO) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps 68
Table 4.10: Nitrous oxide (NO) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps 70
Table 4.11: Superoxide inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps 72
Table 4.12: Hydroxyl (OH^-) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps 74

Table 4.13: Total antioxidant Activity (Equivalent to µg of α-tocopherol) and total Reducing Power (Equivalent to µg of ascorbic acid) of methanolic and aqueous extracts of *A. muricata* and *A. squamosa* fruit pulp....................... 75

Table 4.14a: Effects of *A. muricata* and *A. squamosa* aqueous extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice.. 77

Table 4.14b: Effects of *A. muricata* and *A. squamosa* methanol extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice 78

Table 4.15a: Effects of of *A. muricata* and *A. squamosa* fruits pulp aqueous extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in *invivo* antioxidant study 82

Table 4.15b: Effects of of *A. muricata* and *A. squamosa* fruits pulp methanol extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in *invivo* antioxidant study.. 83

Table 4.16: Proximate composition analysis of experimental diets used in feeding BALB/c mice (mg/100g) 84

Table 4.17: Proximate composition of muscles of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks (mg/100g)................ 85

Table 4.18: Growth performance and feed nutrient utilization in in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks........ 88

Table 4.19: Hematological changes of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 90

Table 4.20: Liver functions in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 92

Table 4.21: Kidney functions and lipid profile in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 94

Table 4.22: Relative weight (mg) and length (mm) of the liver and spleen in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 97

Table 4.23: Efficacies of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts (100 µg/mL) on intracellular survival of *L. major* 99
Table 4.24: Effects of different concentrations of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts on suppression percentage rate of infected cell of bone marrow macrophages 101
Table 4.25: Effect of mean number of amastigotes per host cell at different concentrations of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts on mean number of parasites per host cell 102
Table 4.26: MIC and IC_{50} of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts.. 103
Table 4.27: Relative weight and length of liver and spleen and LDU in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds extracts 112
Table 4.28: Cytotoxicity activities of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts on normal mammalian cells (vero cells) 118
Table 4.29: Percentage decrease in absorbance of DNA methylene green and IC_{50} values of *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous, methanol, ethyl acetae and hexane extracts.. 123

LIST OF FIGURES

Figure 2.1: Taxonomy of Leishmania 8
Figure 2.2: World map highlighting leishmaniasis endemic areas 9
Figure 2.3 Geographical distribution of leishmaniasis in Kenya 10
Figure 2.4: Leishmania digenetic life cycle 13
Figure 2.5: Characterization of antileishmanial phytochemicals from plants 24
Figure 3.1: A map of Kilifi County and Kwale County showing the areas and sites involved in the study 28
Figure 4.1a: Effects of *A. muricata* and *A. squamosa* pulp aqueous extracts on lesion sizes in *L. major* infected BALB/c mice 80
Figure 4.1b: Effects of *A. muricata* and *A. squamosa* pulp methanol extracts on lesion sizes in *L. major* infected BALB/c mice 81
Figure 4.2: Body weight changes of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 87
Figure 4.3: Lesion sizes in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks 96
Figure 4.4: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts 104
Figure 4.5: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds methanol extracts 105
Figure 4.6: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts 106
Figure 4.7: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts 107
Figure 4.8: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts 108
Figure 4.9: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds methanol extracts 109
Figure 4.10: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts 110

Figure 4.11: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts 111
Figure 4.12: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts........... 114
Figure 4.13: Body weight changes in *L. major* infected BALB/C mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds methanol extracts 115
Figure 4.14: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts.... 116
Figure 4.15: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts 117
Figure 4.16: DNA Topo I inhibitory activities of Extracts at 100 μM 124
Figure 4.17: DNA Topo I inhibitory activities of Extracts at 25 μM 124
Figure 4.18: DNA Topo I inhibitory activities of Extracts at 5 μM 125

LIST OF PLATES

Plate 2.1: Annona muricata and Annona squamosa fruits 22

Plate 4.1: Colour of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds packed in airtight containers 58

Plate 4.2: (A) Untreated macrophages with amastigotes occupying the parasitophorus vacuoles (black arrows) in the cytoplasm. Macrophages are seen with their large nuclei, which are eccentrically placed while amastigotes are small round or ovoid structures. (X1000 Giemsa stain). (B) Treated macrophages with parasitophorus vacuoles (black arrows) not occupied by amastigotes. Macrophages are viable with cytoplasm and pseudopods. (X1000 Giemsa stain). 100

Plate 4.3: Impression smears of liver (A) and spleen (B) on 12th week showing dissemination of *L. major* amastigotes inside and outside macrophages 113

Plate 4.4: Enlarged liver (A), Enlarged spleen (B) with weight (3.16±0.06 gm) and length (51.10±0.38 mm) respectively in BALB/c mice on 12th week 113

APPENDICES

Appendix I: Properties of different solvents used in extraction of phytochemicals...... 198

Appendix II: HPLC chromatogram of a sample showing different carotenoids eluted at different retention times 199

Appendix III: Different carotenoids from *A. muricata* and *A. squamosa* fruits peel, pulp and seeds separated on a reverse-phase C_{30} HPLC system and spectral characteristics used in identification from photodiode array detection 200

Appendix IV: (A) HPLC chromatogram for carotenoid standards; (B) HPLC chromatogram for carotenoids from *A. squamosa* (custard apple) 201

Appendix V: Effects of A. muricata pulp fruit aqueous, methanol, ethyl acetate and hexane extracts of on body weight of BALB/c mice in sub-acute toxicity study 202

Appendix VI: Effects of A. muricata peel aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study 203

Appendix VII: Effects of A. muricata seeds aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study 204

Appendix VIII: Effects of *A. squamosa* pulp aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study 205

Appendix IX: Effects of *A. squamosa* peel aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study 206

Appendix X: Effects of *A. squamosa* seeds aqueous, methanol, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study 207

Appendix XIa: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit pulp on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study 208

Appendix XIb: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit peel on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study.................................. 209

Appendix XIc: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit seed on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study........................ 210

Appendix XIIa: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit pulp on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study........................ 211

Appendix XIIb: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit peel on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study........................ 212

Appendix XIIc: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit seeds on relative organ weights (organ [mg]/body weight [g]) of BALB/c mice in sub-acute toxicity study........................ 213

Appendix XIIIa: Effects of of *A. muricata* fruit pulp aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study 214

Appendix XIIIc: Effects of of *A. muricata* fruit seeds aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study 216

Appendix XIVa: Effects of *A. squamosa* fruit pulp aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study 217

Appendix XIVb: Effects of *A. squamosa* fruit peel aqueous, methanol, ethyl acetate and hexane extracts on Haematological profile of BALB/c mice in sub-acute toxicity study 218

Appendex XIVc: Effects of *A. squamosa* fruit seeds aqueous, methanol, ethyl acetate and hexane extracts on Haematological profile of BALB/c mice in sub-acute toxicity study 219

Appendix XVa: Biochemical profile of BALB/c mice treated with *A. muricata* fruit pulp aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 220
Appendix XVb: Biochemical profile of BALB/c mice treated with *A. muricata* peel aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 221
Appendix XVc: Biochemical profile of BALB/c mice treated with *A. muricata* seeds aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 222
Appendix XVIa: Biochemical profile of BALB/c mice treated with *A. squamosa* pulp aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 223
Appendix XVIb: Biochemical profile of BALB/c mice treated with *A. squamosa* fruit peel aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 224
Appendix XVIc: Biochemical profile of BALB/c mice treated *with A. squamosa* seeds aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study 225
Appendix XVII: A grant ward letter from National Commission for Science Technology and Innovation (NACOSTI) for funding the study 226
Appendix XVIII: A letter of approval from Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (SSC) (KEMRI-SCC) 227
Appendix XIX: A letter of approval from Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) (KEMRI-ERC) 228
Appendix XX: A letter of approval from Kenya Medical Research Institute (KEMRI) - Animal Care and Use Committee (ACUC) (KEMRI-ACUC) 229
Appendix XXI: A letter seeking for approval to carry out research in the Food Biochemistry Laboratory in the Department of Food Science and Technology at Jomo Kenyatta University of Agriculture and Technology (JKUAT) 230

Appendix XXII: A letter of approval to carry out research in the Food Biochemistry Laboratory in the Department of Food Science and Technology at Jomo Kenyatta University of Agriculture and Technology (JKUAT).... 231
Appendix XXIII: A letter of approval to carry out research in the Directorate of Research and Innovation laboratories (Mount Kenya University) 232
Appendix XXIV: List of publications arising from the study 233

ABBREVIATIONS AND ACRONYMS

AFPP	Annonaceae fruit pulp pellets
AL	Anthroponotic leishmaniasis
ALC	Apparent lipid conversion
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BMG	Body mass gain
DCL	Diffuse cutaneous leishmaniasis
FBL	Final body lipids
FBM	Final body mass
FBP	Final body protein
FCR	Feed conversion ratio
IBL	Initial body lipids
IBM	Initial body mass
IBP	Initial body protein
INTC	Infected none treated control
ITC	Infected treated control
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MGR	Metabolic growth rate
MGR	Metabolic growth rate
MUFA	Monounsaturated fatty acids
PER	Protein efficiency ratio
PKDL	Post-kalaazar dermal leishmaniasis
PPV	Protein productive value
PUFA	Polyunsaturated fatty acids
RL	Recidivans leishmaniasis
RP	Rat pellets
SFA	Saturated fatty acids
SGR	Specific growth rate
VL	Visceral leishmaniasis
ZL	Zoonotic leishmaniasis

ABSTRACT

Leishmaniasis is classified by the WHO as an emergent category of one of the uncontrolled and NTDsco-exist with malnutrition and HIV/AIDS. Due to lack of vaccine, control relies on ineffective, toxic and expensive chemotherapy and vector control, which are not sustainable. The study was aimed at determination of efficacy and safety, nutritional and antioxidant activity of Kenyan *A. muricata* (L.) *and A. squamosa* (L.) fruits extracts in BALB/c mice model of *L. major*leishmaniasis to develope supportive therapy and efficacious and safe antilrishmanicidal agents. Ripe fresh fruitswere collected from farms in Kilifi and Kwale Counties, Coast regionof Kenya. Dried fruits were powdered, subjected to extraction with solvents of increasing polarity (Hexane, Ethyl Acetate, Methanol and Water) for 48 hours. Proximate and nutritional composition of various partsof the plants, phytochemicals screening, carotenoidsanalysis, *in vitro*and *in vivo* cytotoxicity, antioxidant, DNA-binding and DNA Topo I inhibitory activities of the extracts wereassesed. The efficacies of thedifferent extracts against *L. major* parasites (IDUB/KE/83=NLB-144 strain) wasassessed *in vitro* in inbred infected BALB/c mice maintained on AFPP and RP and i*n vitro* using promastigotes. The results were expressed as mean analyzed using mean separation done through Fischer least significance difference by GenStat program. Comparisons between two treatments were done by Student's t-test and significance established by ANOVA. Differences of $P<0.05$ were considered statistically significant.Majority of the phytochemicalswere detected inthe extracts.The fruitdemonstrated high dry matter, moisture content, crude fat, crude protein, crude fibre, total carbohydrates, oil content, reducing sugar, TSS, ascorbic acid, tocopherol, titratable acidity and ash content. Fatty acid profile showed presence of SFA, MUFAand PUFA. Different parts revealed varying amounts of P, Na, Ca, Mg and P whereas Cu, Fe, Zn and Se were detected in trace amounts. The HPLC profilingof carotinoids revealed presence of neoxanthin, violaxanthin and zeaxanthin, α-carotene, β-carotene, γ-caroteneand chlorophyll. The extracts showed *in vitro* and *in vivo*antioxidant activitiesagainst non-enzymatic and enzymatic antioxidants. The BALB/c mice fed with AFPP showed improve growth, metabolic efficiency and feed utilization.The extracts showed a dose dependent *in vitro* antileishmanial activity with MIC values ranged between 12.50±1.03 µg/mL to 55.0±2.97 µg/mL. The extracts showed antileishmanial activity *in vivo* through reduction ofLHFD swelling and lesion sizes. The extracts ranged from highly toxic (IC_{50} 2.54±0.44-7.55±1.19µg/mL), toxic (IC_{50} 12.85±2.80-94.07±5.81µg/mL) and moderately toxic (IC_{50} 104.81±1.16-292.94±10.10µg/mL). No mortality was observed even at highest dose of 2500 mg/Kg suggesting LD_{50}>2500mg/Kg. The extractsshowed DNA binding interaction via displacement of methyl green in DNA methyl green test and DNA Topo I inhibitory activity by inhibiting relaxation of supercoiled DNA pBR322 at 5, 25 and 100 µM. It can be concluded that the nutritional components of the fruits were responsible for the improve growth, metabolic efficiency and feed utilization of BALB/c mice fed with AFFP. Thephytochemicals detected were responsible for the *in vitro* and *in vivo* antioxidant and antileishmanial activities besides DNA binding interaction and DNA Topo I invibitory activity. Low IC_{50} and lack of mortality in extracts treated mice suggests safety. The study recommends further investigation using purified fractionsof these extracts on higher animal model of the lishmaniasis.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

The protozoan parasite in the genus Leishmania causes the leishmaniases diseases, which invade macrophages of host organisms (Marsella &de Gopegui, 1998; Basu & Lala, 2004; Awasthi *et al*., 2004; Monzote, 2009; Khademvatan *et al*., 2010). The disease is broadly, classified into two types; zoonotic leishmaniasis (ZL), with reservoir hosts being wild animals, commensals or domestic animalsand Anthroponotic leishmaniasis (AL) with humans as the reservoir host (WHO, 1990; Asford, 1997; Marsella &de Gopegui, 1998; Aziz *et al*., 2012a; Aziz *et al*., 2012b). Infected sandflies of the genus *Phlebotomous* and *Lutzomyia*are transmitters of these diseases to humans (Heisch *et al*., 1962; wijiers & Minter, 1966; Piscopo & Mallia, 2006; Karimi *et al*., 2012; Yaghoobi-Ershadi, 2012; Ready Ready, 2013; Zahraei-Ramazani *et al*., 2013; Ready, 2014; Zahraei-Ramazani *et al*., 2015).

Based on parasite tropism, different clinical forms exist; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (RL) and post-kalaazar dermal leishmaniasis (PKDL) (Monzote, 2009; Roy *et al*., 2012). There are 2 million new cases of leishmaniasis per year with 1.5 and 0.5million estimated to be of CL and VL respectively resulting into 50,000 to 60,000deaths per year (Lozano *et al*., 2012). Therefore, the disease has been a public health problem especially in endemic areas of developing countries (Desjeux, 1996; Asford, 1997; Alvar *et al*., 2012).

Although drugs from plants natural products are used to treat 87% of all categories of human diseases (Newman & Cragg, 2007; Cragg & Newman, 2013) and about 25% of the prescribed drugs in the world originate from plants (Rates, 2001), leishmanicidal agents are yet to be developed using natural products from plants. High cost, poor compliance, drug resistance, low efficacy and toxicity limit the utility of modern drugs (Nwaka & Hudson, 2006). Further, inadequacy and cost of modern medicine has lead developing countries relying on complementary and

alternative medicine (CAM) for their primary health care need (FAO, 2004; Nwaka & Hudson, 2006).

Despite the great interest in the role of CAM for the treatment of acute and chronic diseases, leishmaniasis has not been in the consideration. Toxicity and emergence of strains not responsive to the available chemotherapy make the discovery of novel leishmaniasis therapeutics imperative (Iwu *et al*., 1994; Mishra *et al*., 2011b; Roy *et al*., 2012). Among the known plant species growing in world, a significant number are used for nutrition and herbal preparations (FAO, 2004). However, these preparations have not undergone chemical, pharmacological and toxicological investigation for their bioactive compounds (Ghani, 2003). Kenya has long history of documented rich and prestigious heritage of plants with medicinal values (Watt & Breyer-Brandwijk, 1962; Kokwaro, 1993; Owuor & Kisangua, 2005; Muthaura *et al*., 2007) which can be exploited in designing antileishmanial agents.

Annonaceae are tropical and subtropical trees, shrubs and lianas comprising of about 130 genera and more than 2300 species (Hotta *et al*., 1989) and include among other species,*A. cherimola*; Mill (cherimoya), *A muricata*; L (soursop), *A. squamosa*; L (sugar apple), *A. senegalensis*; L (wild soursop) and *A. reticulata*; L (custard apple)(Hotta *et al*., 1989). They have been used as food and nutrition supplements and in traditional medicine preparations for centuries (Maundu *et al*., 1999; Pinto *et al*., 2005; Owuor & Kisangau, 2006; Badrie & Schauss, 2009; Dembitsky *et al*., 2012). Different parts of Annonaceae contain numerous bioactive substances; polyketides, acetogenins, alkaloids, terpenes, flavonoids and oils (Foloronso and Modupe, 2007; Nawwar *et al*., 2012; Onyechi*et al*., 2012; Biba *et al*., 2013; Bhardwaj *et al*., 2013; Florence *et al*., 2014) responsible for the observed insecticidal and pesticidal, cytotoxic, antitumoral, antileishmanial, antivenin, antifeedant, antibacterial, antihelminthic, immuno-suppressant,chemo-preventive, antioxidant, and hepatoprotective properties(Igwe & Onabanjo, 1989; Rupprecht *et al*., 1990; Cassady, 1990; Chang *et al*., 1993; Cortes*et al*., 1993; Abubakar & Aburahman, 1998; Owuor & Kisangua, 2005; George *et al*., 2006; Adewole & Ojewole, 2006; Ajaiyeoba *et al*., 2006; Adewole & Ojewole, 2009; Arthur *et al*., 2011; Kumar *et al*., 2012a;Arthur *et al*., 2012a; Arthur *et al*., 2012b; deLima *et al*., 2012; George *et al*.,

2012; Nandhakumar & Indumathi, 2013; Roopana*et al*., 2012;Samuagam *et al*., 2014; Ravaomanarivo *et al*., 2014; Najmuddin *et al*., 2016).

The fruits are possible contributors of vitamins, mineral salts, fibres and bioactive compounds of diets and provides a delicate balance of food security to the populations in regions where they grow (Maundu *et al*., 1999; Pinto *et al*., 2005; Badrie & Schauss, 2009; Onyechi*et al*., 2012; Dembitsky *et al*., 2012; Othman *et al*., 2014; Baoke *et al*., 2014; Lugwisha *et al*., 2016). The fact that they are used as food and exhibit medicinal potency is a significant aspect of modern trends in research focusing on food-medical interface (nutraceutical). However, the Kenyan varieties have not been fully studied in terms of research to understand their nutritional and phytochemical profiles and antileishmanial properties. It is thus important to undertake studies on the fruits in order to provide their nutritional and phytochemical profiles and aantileishmanial properties. This may have the impact of improving the health sector by strengthening CAM besides providing the base line for the discovery of lead compounds in pharmaceutical industries for the synthesis of leishmanicidal agents.

1.2 Statement of the problem

The Leishmaniases a debilitating disease associated with the poor community is among the most world's prevalent parasitic disease. With annual incidence of 4000 cases in Kenya (WHO, 2017), it constitutes a seriouspublic health and socioeconomic problem not only in endemic areas of developing (third-world) countries but also in developed (first-world) countries having synagistic effect withmalnutrition and HIV/AIDS infections (Alvar *et al*., 1997; Desjeux*et al*., 2000; Anstead *et al*., 2001; Desjeux & Alvar, 2003; Cruz *et al*., 2006; Malafaia, 2009; Malafaia *et al*., 2009; Mishra*et al*., 2011a; Mishra *et al*., 2011b; Roy *et al*., 2012). Increasing cases are due to lack of vaccines, difficulties in controlling vectors, increasing number of parasites resistance to chemotherapy and malnutrition (Anstead *et al*., 2001; Croft & Yardley, 2002; Malafaia, 2009; Malafaia *et al*., 2009; Kedzierski, 2010; Griensven *et al*., 2010; Mishra *et al*., 2011b; Croft& Olliaro, 2011). Few alternative drugs or new formulations of old ones are available as first-line drugs for the treatment of leishmaniasis. However, due tolack of effectiveness, high toxicity,

drug resistance and different strain sensitivity, prohibitive prices, long treatment schedule, empirically drug discovery and inadequate mode of administration not applicable in the field (Iwu *et al*., 1994; Croft & Yardley, 2002; Croft *et al*., 2006a; Croft *et al*., 2006b; Gupta *et al*., 2010; Mishra *et al*., 2011b; Roy *et al*., 2012), none of them is ideal for treatment.

The problem is further complicated by the fact that an effective vaccineremains to be developed thus control measures focus mainly on vector control, chemotherapy and supportive therapy.Althoughplant metabolites from *A. muricata* and *A. squamosa*have been traditionally used to treat human diseases (Pinto *et al*., 2005; Owour & Kisangua, 2006), few studies have forcused in their utilization in the development of antileishmanial agents.The conductedstudies have concentrated on anti-cancer properties (George *et al*., 2006; George *et al*., 2012; Najmuddin *et al*., 2016) reporting activities of roots, leaves, seeds and bark (Pinto *et al*., 2005; Muthaura *et al*., 2007; Ugwu *et al*., 2011; Vila-Nova *et al*., 2011). Further, no studies on the antileishmanial efficacy and safety of the Kenyan varieties have been undertaken making them good candidates in search for antileshmanial agents.

1.3 Justification

Kenya and other developing countries need a re-orientation on the sustainable use of their natural resources in this era of economic recession. This is to harness the abundant flora and fauna for improved primary nutrition and health care delivery and as sources of raw materials for nutraceaticals. Thus, utilization of plants used in folk and traditional medicine as alternative sources of prophylactic and chemopreventive treatment as well as drug discovery and development is paramount.To reduce the mortality and morbidity and bear the cost arising from leishmaniasis, natural products from plants can be utilized. The documented medicinal values of the Kenyan *A. muricata* and *A. squamosa* (Owour & Kisangua, 2006) has not been exploited in the development of antileishmanial agents.This study will help to identify sources and ingredients for the provision of nutraceutical benefits in the synthesis of newer, cheap, safe and efficacious antileishmanicidal agents and food supplements for supportive therapy to be used in management of leishmaniasis.

1.4 Research questions

1. What are the phytochemicals present in *A. muricata* and *A. squamosa* fruits extract(s) from peel, pulp and seeds?

2. What are the proximate and nutritional composition levels and antioxidant activities of *A. muricata* and *A. squamosa* fruits pulp?

3. What are the levels of *in vitro* and *in vivo*antileshimanial efficacy and safety of the extract(s) against *L. major*?

4. What are the levels of growth performance of *L. major* infected BALB/c mice under Annonaceae fruit pulp (AFPP) diet?

5. Do the *A. muricata* and *A. squamosa* fruits peel, pulp and seeds extract(s) poses DNA-bindinginteraction and DNA Topo I inhibitory activities?

1.5 Objectives of the study

1.5.1 Overall objective

To determine the efficacy and safety, nutritional and antioxidant activity of *A. muricata* (L.) andnd *A. squamosa* (L.) fruits extracts couple with DNA binding and DNA Topo I inhibitory activity in BALB/c mice model of *L. major* leishmaniasis

1.5.2 Specific objectives

1. To determine the presence and levels of phytochemicals in *A. muricata* and *A. squamosa* fruits extract(s) from peel, pulp and seeds;

2. To determine the proximate, nutritional composition levels and antioxidant activity of *A. muricata* and *A. squamosa* fruits extracts;

3. To asses the growth performance of *L. major* infectedBALB/c mice under Annonaceae fruit pulp pellets (AFPP) as a diet;

4. To determine the *in vitro* and *in vivo*antileishmanial efficacy and safety of the extract(s) against *L. major*;

5. To determine the DNA-binding interactions and DNA Topo, I inhibitory activity of the extract(s).

1.6 Variables

1.6.1 Independent variables

The parasite inoculum per mouse and the dosage or amount of extracts of *A. muricata* and *A. squamosa* fruit given to each group of BALB/c mice

1.6.2 Dependent variables

Clinical parameters, haematological parameters, biochemical parameters and other measurements; IC_{50} values, parasitaemia levels, Haematocrit (HCT) or packed cell volume (PCV), mean capsular volume (MCV), mean capsular haemoglobin (MCH), mean capsular haemoglobin concentration (MCHC), plasma protein, haemoglobin (HBG), bilirubin and creatine concentration, differential blood cell count, erythrocytes and reticulocytes populations and body weights of the BALB/c mice

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The leishmaniases and their global distribution

The leishmaniases are vector-borne diseases caused by an obligate intracellular species of parasitic protozoan in the genus *Leishmania* (Kinetoplastida: *Trypanosomatidae*) (Figure 2.1) which invade the blood and tissues of the host (Khademvatan *et al*., 2010). Although the hosts of the disease are animals, it finds its way into the human population through co-existence of humans, sandflies and animal reservoirs in the same environment (Mutinga 1975a; Mutinga 1975b; WHO, 1990; Sang & Chance, 1993; Jarallah, 2015; Jarallah, 2016). Further, the disease is one of the world's most neglected tropical diseases (NTDs) (WHO, 2010; Alvar *et al*., 2012; WHO, 2016) based on the limited resources invested in diagnosis, treatment, control and its strong association with poverty (Alvar *et al*., 2006). The disease is second in mortality and fourth in morbidity among all tropical diseases (Bern *et al*., 2008; WHO, 2010; WHO, 2016).

Reports indicates that leishmaniasis occurs in 88 countries in five continents; Africa, Asia, Europe, North America and South America,16 developed countriesand 72 developing countries (Desjeux, 2001; Karimi *et al*., 2012;WHO, 2014; WHO,2016; WHO, 2017). About 350 million people are considered to be at risk of contracting leishmaniasis especially among people living in developing countries (WHO, 2007; WHO, 2010; WHO, 2016; WHO, 2017). Approximately 14 to 20 million people are infected and about 12 million cases are reported worldwide with an estimated rate of 2.0 million new cases reported annually (WHO, 2010; Lozano *et al*., 2012; Luciano *et al*., 2012; WHO, 2016; WHO, 2017). Among the reported cases, 1.5 million are of disfiguring CL and 0.5 million of potentially fatal VL, resulting into 0.06Million deaths per year (WHO, 2010; Lozano *et al*., 2012; WHO, 2016; WHO, 2017). However, with increasing travel to and from endemic regions, more patients with leishmaniasis are detected in western countries (Herwaldt, 1999; Murray, 2000; Guerin *et al*., 2002; Mishra *et al*., 2011a).

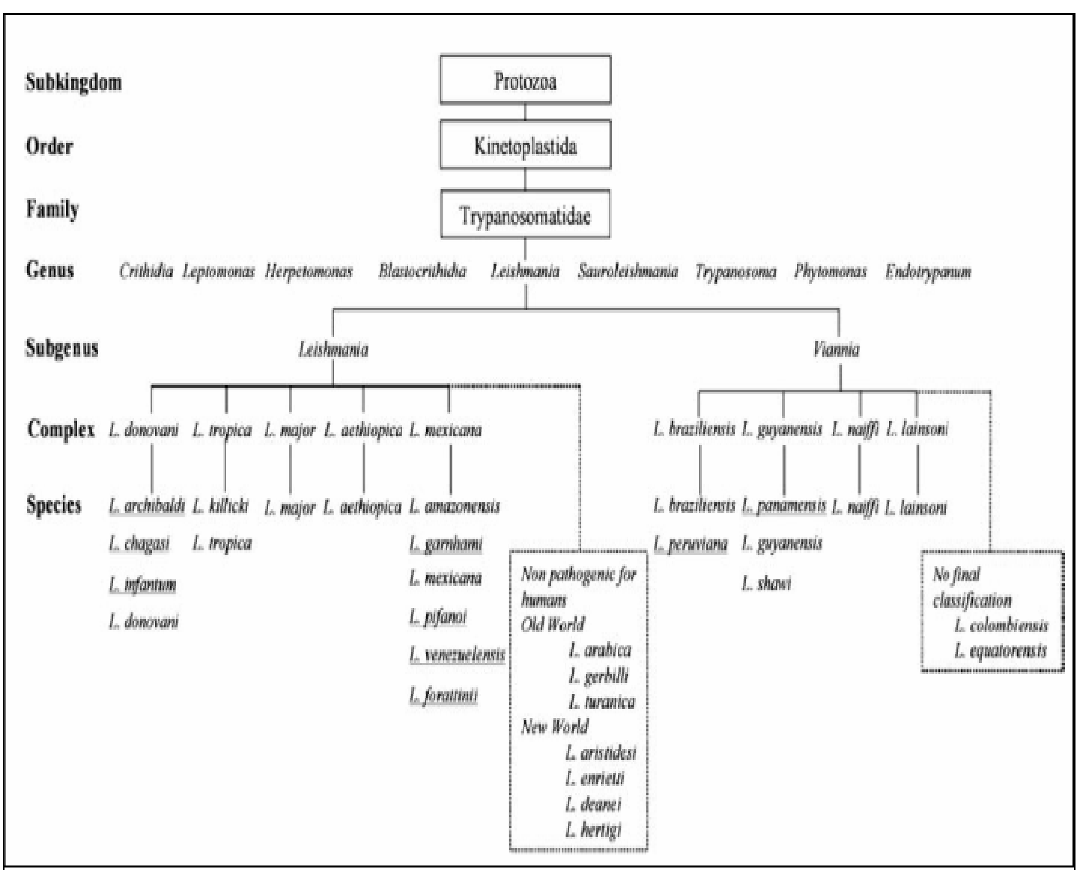


Figure 2.1: Taxonomy of Leishmania based on the scheme published by WHO, (1990)

The disease is a well-recognized endemic andepidemics throughout parts of Africa, India, Middle East, southern Europe, and Central and South America (Figure 2.2). In Africa, Asia Minor and Europe, the causative agent for CL are *L. major*, *L. ethiopica*, *L. tropica, L. infantum* and *L. killicki* (Reitinger *et al*., 2007; Aoun *et al*., 2008; WHO, 2014). Most of CL cases have been reported in 10 countries; Afghanistan, Algeria, Brazil, Costa Rica, Colombia, Ethiopia, Iran, Peru, Syria, and Sudan contribute 75% of the global incidence of CL (WHO, 2001; WHO, 2010; Alvar *et al*., 2012; Karimi *et al*., 2012; WHO, 2016; WHO, 2017). However, Algeria lacked regions of complete evidence consensus due to incomplete and non-contemporary case data as a results of civic war (Alvar *et al*., 2012).The MCL is endemic in 18 out of the 20 American countries where it occurs with different transmission intensities (PAHO, 2017) affecting 72,800 to 119,600 people annually in Brazil (Alvar *et al*., 2012). In the period from 1990 to 2016, 687,780 cases of MCL have been reported

with low mortality (MS, 2016a). The fatal VL is present in 12 countries in the Americas (PAHO, 2017). Majority of VL cases are found in 6 countries; Brazil, Ethiopia, Sudan, South Sudan, India, and Bangladesh (WHO, 2001; WHO, 2010; Alvar *et al*., 2012; Karimi *et al*., 2012; WHO, 2016). Most of the VL cases occur in Brazil with 4,200 to 6,300 cases representing 96% of the cases reported annually (Alvar *et al*., 2012). Further, in Brazil, 84,922 cases of VL were confirmed from1990 to 2016 with case fatality rate reaching 7.4% in 2016 (MS, 2016b).

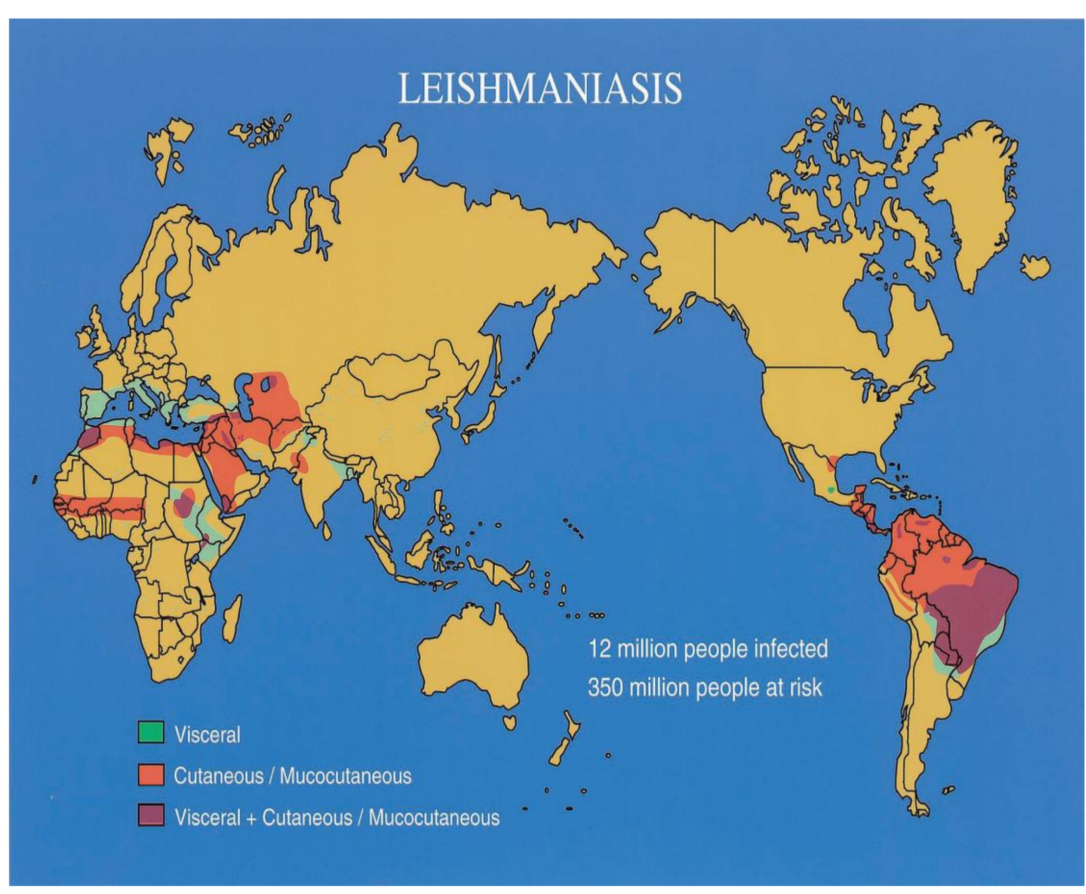


Figure 2.2: World map highlighting leishmaniasis endemic areas (Handman, 2001).

The disease clusters around areas of drought, famine, and high population density (Desjeux, 1996; Alvar *et al*., 2006). In Eastern Africa, common infection centers are in Sudan, Kenya, and Somalia (WHO, 2014).Leishmaniasis has been endemic in many parts of Kenyafrom as back as early 19th century where nine sporadic cases were reported between 1991 and 1939 (Fendall, 1961). The main endemic foci of VL

in Kenya are Eastern and the Rift valley provinces (Muigai *et al*., 1987). The CL occurs on the eastern slopes of Mount Elgon in Western Kenya, parts of the Rift valley and some parts of Central Kenya among other areas (Mutinga, 1975a; Mutinga, 1975b). Some of the districts affectedinclude Baringo, Isiolo, Meru, Turkana, Laikipia, Kajiado, Machakos, Kituiand Wajir among others (Figure 2.3). In Kenya CL is commonlyfound in Baringo, Kitui, Kiambu, Laikipia, Samburu, Nakuru, Nyandarua and Mt. Elgon area (Killick-Kendrick, 1999; Tonui, 2006). In Kenya an average of 600 cases were expected annually with case fatality rate of up to 7% observed during out breaks situation (Tonui, 2006). The cases usually rise to over 1,000 in an epidemic year (Tonui, 2006) and currently, 4, 000 are detected annually (WHO, 2017)

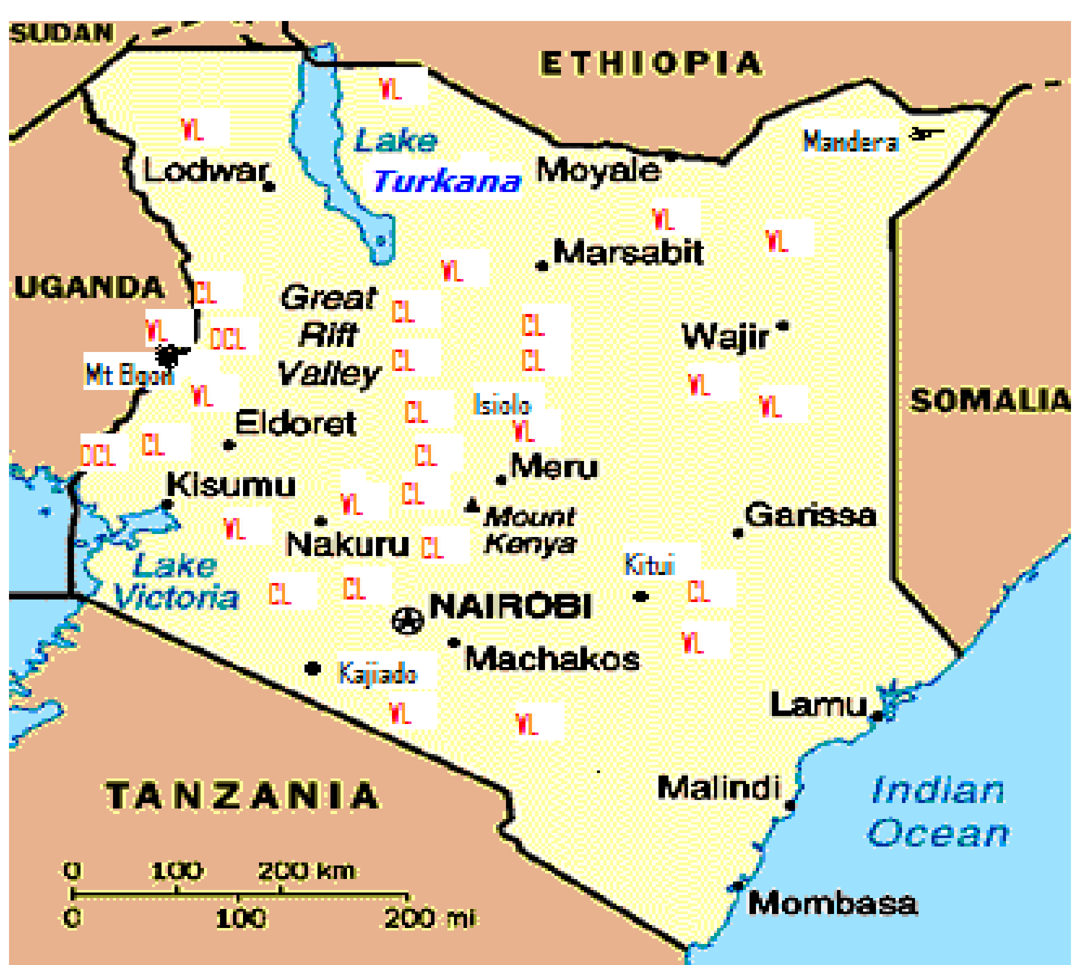


Figure 2.3 Geographical distribution of leishmaniasis in Kenya (Killick-Kendrick, 1999) Key: CL- Cutaneous leishmaniasis; VL- Visceral leishmaniasis; DCL- Diffuse cutaneous leishmaniasis

2.2 Economic importance and burden of leishmaniasis

The leishmaniases diseasescause high morbidity and mortality levels with a wide spectrum of clinical syndromes and was identified as a major public health problem (Desjeux, 1996; Asford, 1997; Desjeux, 2001; Vannier-Santos *et al*., 2002; WHO, 2010; Alvar *et al*., 2012). As one of highlyleading and ranked NTDs in terms of mortality and morbidity, andin 2010 the death caused by the disease was 50,000 to 60,000 (WHO, 2010; Lozano *et al*., 2012; Alvar *et al*., 2012) and 3.3 million disability adjusted life years (Murray *et al*., 2012). The burden is further compounded by inadequacy of low cost treatment and therefore the need for more exploration especially vaccine oriented research (Dumonteil *et al*., 2001; Kedzierski, 2010).

The control of leishmaniasis is complicated and remains a serious problem due to the fact that many species of sandflies and animals are potential vectors and reservoir hosts respectivelly (WHO, 1990; Karimi *et al*., 2012; Yaghoobi-Ershadi, 2012; Zahraei-Ramazani *et al*., 2013; Zahraei-Ramazani *et al*., 2015). As a zoonotic infection, transmission is difficult to interrupt (WHO, 1990; Asford, 1997) although some attempts to reduce vector in mammalian reservoir populations have been successful (WHO, 2010). Collectively, *Leishmania spp*. (Figure 2.1) are responsible for one of the major communicable diseases of the world (Table 2.1), thus was included by WHOto be among the six major diseases targeted for intensive research and control efforts (WHO, 2001; WHO, 2010). Based on overall medical significance, leishmaniasis was regarded second only to malaria (Chang *et al*., 1985; WHO, 2010; Alvar *et al*., 2012).

2.3 Biology and life cycle of Leishmania

Among the ancienteukaryotesis *Leishmania* withdistinctive features includingstructurally and metabolically unusual kinetoplast DNA (kDNA) within the single mitochondrion (Ogbadoyi *et al*., 2003; Roy *et al*., 2012; Aziz *et al*., 2012a; Aziz *et al*., 2012b; Wheeler *et al*., 20016). The kDNA is about 10-15µM in size and arranged in a planar array in a network of thousands of DNA mini-circles topologically interlocked into an elliptical shape (Ogbadoyi *et al*., 2003; Saraiva *et al*., 2005; Aziz *et al*., 2012a; Aziz *et al*., 2012b; Wheeler *et al*., 2016). It appears to

have a regular structure and accounts for 5-20 per cent of the total cellular DNA with a relatively high Adnine-Thymine (AT) content (McConville *et al.*, 2007).Leishmania parasites have a digenetic life cycle alternately hosted by an insect vector and by mammals (Figure 2.4). Two major stages of life cycle thus exist; an intraluminal extracellular developmental stage (flagellated promastigote) in insect vector, a female phlebotomine sandflyand an intracellular developmental stage (replicative amastigotes) in mammals (Roberts, 2006; Kaye & Scott, 2011; Roy *et al.*, 2012).

When a females and fly takes a blood meal from a *Leishmania* infected host, the insect ingests intracellular amastigotes, whichtransform into motilepromastigotes, which escape through the peritrophic membrane enveloping the blood meal(Roberts, 2006; Kaye & Scott, 2011; Roy *et al.*, 2012). The intraluminal development occurs in alimentary canal of sandflywith formation of a motile, flagellated and elongated form, the promastigote. Inside the intestinal tract of the sandfly, promastigotes intensively and successively multiply as free elongated promastigotes (nectomonads) or as attached pro-mastigotes and para-mastigotes (haptomonads) (Walters, 1993; Kaye & Scott, 2011). The parasites subsequently migrate from the multiplication site to the anterior part of the sandfly midgut, where they change into free-swimming metacyclic promastigotes, the stage infective for the vertebrate host (Roberts, 2006; Grimm e*t al.*, 2011; Beattie & Kaye, 2011). During a blood meal by the sandfly, a requirement for oviposition, metacyclic promastigotes are inoculated into the mammalian host, depositing themin the skin (Beattie and Kaye, 2011). Neutrophiles and macrophages among other cells of mononuclear phagocytic system phagocytize the inoculated metacyclic promastigotes transforming themto amastigotes, which multiply by simple mitosis or binary fission (Roberts, 2006; Mishra *et al.*, 2011a; Roy *et al.*, 2012). A morphological change occurs as the parasite takes on an ovoid shape with a short flagellum with a metabolic switch to anaerobic metabolism under acidic conditions found chiefly in the phagolysosome compartment (Naderer & McConville, 2008) anddespite remarkably advances; the complex metabolism ispoorly understood (Roy *et al.*, 2012).

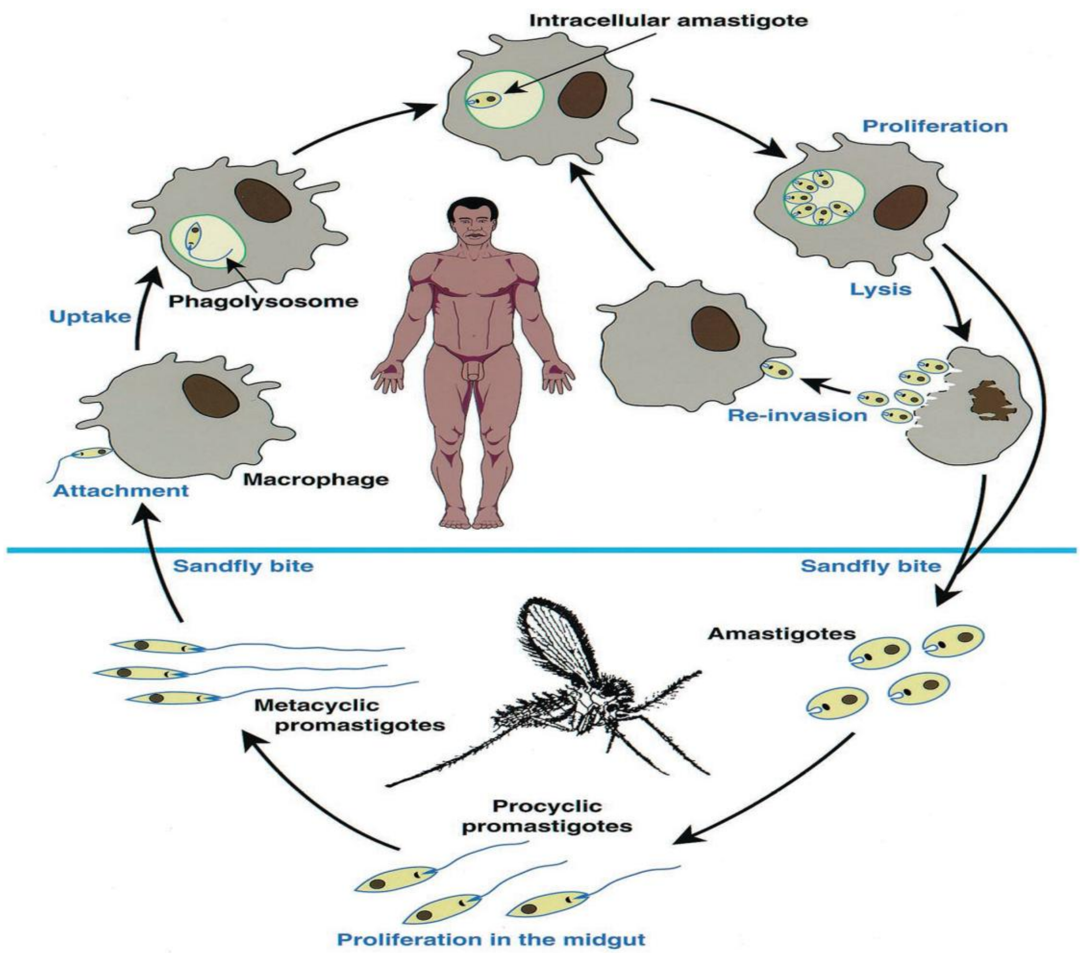


Figure 2.4: Leishmania digenetic life cycle (Handman, 2001)

In order to survive within the host, the parasite evades the host defense system; adopting various mechanisms acquired through evolution interacting with the host defense at molecular levels (Roberts, 2006; Schmid-Hempel, 2009; Roy *et al*., 2010; Xu *et al*., 2011). Feeding habit of specific vector, host genetics and successful inhibition of host defensive oxidative pathways determines the transmission and epidemiology of leishmaniasis (Roberts, 2006; Roy *et al*., 2010; Oliveira *et al*., 2011b). Biochemical mechanisms include inhibition of phagolysosome formation, abnormal activation of protein kinase C, scavenging of the reactive oxygen species (ROS), prevention of apoptosis of infected macrophages, impairment of macrophage antigen presenting cells (APC) function by Major Histocompatible Complex (MHC) molecules and impairment of responsiveness to cytokines (Bogdan & Rollinghoff, 1999; Villa *et al*., 2002; Awasthi *et al*., 2004; Olivier *et al*., 2005;Ameen, 2007;

Oliveira*et al*., 2012a; Oliveira*et al*., 2012b; Roy *et al*., 2012; Kaur *et al*., 2013; Cragg & Newman, 2013).

Additionally, the parasite interfereswith complement activation and humoral immunity mechanisms by blocking protective T-helper cell response within the host's body (Villa *et al*., 2002; Roberts, 2006; Oliveira*et al*., 2012a). The parasite surface and secreted molecules also play a major role in the survival of the parasite in the hostby providing protection to digestive enzymes in the gut of sandfly and to the hydrolase-rich phagolysosomes of host macrophages (Naderer & McConville, 2008; Roy *et al*., 2012). Expression of lipophosphoglycan (LPG), a cell surface glycoconjugate and a stage-specific virulence determinant allows the pathogen to survive and proliferate in the hostile conditions in the host (Chang *et al*., 1990; Quintana *et al*., 2010; Roy *et al*., 2012). The LPG promotes inhibition of parasite-containing phagosome-endosome fusion via alteration of fusion properties of the endocytic system (Desjardins & Descoteaux 1998; Quintana *et al*., 2010; Roy *et al*., 2012) thus protecting the *Leishmania* parasites from digestion in host lysosomes immediately upon invasion. Therepetitive oxidizable phosphorylated disaccharide units of LPG help them to scavengeROS generated during respiratory bursts inside the macrophage cells during phagocytosis (Miao *et al*., 1995; Desjardins & Descoteaux, 1997; Xu *et al*., 2011; Roy *et al*., 2012; Oliveira*et al*., 2012b).

2.4 Aetiology, clinical and pathological manifestations of leishmaniasis

Leishmaniasis reveals diverse clinical manifestations caused by the reaction between the virulence of the parasite species and the host's immune response (Awasthi *et al*., 2004; Boakye *et al*., 2005; Roberts, 2006; Monzote, 2009; Kaye & Scott, 2011; Roy *et al*., 2012). The aetiology of leishmaniasisis attributed to any of the protozoan species of the genus *Leishmania* (Table 2.1) which cause the disease in humans or animals (Marsella &de Gopegui, 1998) transmitted by the bite of an infected female *Phlebotomus* sandfly as the vector (Heisch *et al*., 1962; Wijiers & Minter, 1966; Grimaldi & Tesh, 2012; Yaghoobi-Ershadi, 2012; Zahraei-Ramazani *et al*., 2013; Zahraei-Ramazani *et al*., 2015). About48 species of sand flies exist, 30speicies of the genus *Phlebotomus* and 18 speicies of the genus *Sergentomyia* (Yaghoobi-Ershadi, 2012; Zahraei-Ramazani *et al*., 2013; Zahraei-Ramazani *et al*.,2015).

Table 2.1 Proven vectors of human leishmaniasis (WHO, 1990)

Genus	Subgenus	Parasites transmitted and disease clinical form	Main area of endemic foci
Phlebotomus	*Duboscqui*	*L. major* (ZCL)	Kenya, Senegal
	papatasi	*L. major* (ZCL)	Islamic Republic of Iran, Israel, Jordan, Morocco, Saudi Arabia, Tunisia, USSR
	salehi	*L. Major* (ZCL)	India (Rajasthan)
Paraphlebotomus	*alexandri*	*L. infantum* (ZVL)	China
	sergenti	*L. tropica* (ACL)	Morocco, Saudi Arabia, USSR
Larrousius	*Ariasi*	*L. infantum* (ZVL and CL)	France, Spain
	major neglectus	*L. infantum* (ZVL)	Greece
	perfiliewi	*L. infantum* (CL)	Italy
	perniciosus	*L. infantum* (ZVL and/or CL)	Algeria, France, Italy, Malta, Spain
	Tobbi	*L. infantum* (ZVL)	Cyprus
	Longipes	*L. aethiopica* (ZVL)	Ethiopia
	Pedifer	*L. aethiopica* (ZVL)	Ethiopia, Kenya
Synphlebotomus	*martini*	*L. donovani* (AVL)	Kenya
	Rossi	*L. tropica* (CL)	Namibia
Adlerius	*chinensis*	*L. infantum* (VL)	China
	longiductus	*L. infantum* (ZVL)	USSR
Euphlebotomus	*argentipes*	*L. donovani* (AVL)	

Key: ACL: Anthroponotic cutaneous leishmaniasis, CL: Cutaneous leishmaniasis, VL: Visceral leishmaniasis, ZCL: Zoonotic cutaneous leishmaniasis, ZVL: Zoonotic visceral leishmaniasis.

Four major clinico-pathological categories of leishmaniasis exist: CL, DCL, MCL and VL each caused by distinct species (Table 2.2). The aetiologic agent of VL, *L. donovani* and *L. chagasi* (Hide & Tait, 1991) replicates in mononuclear phagocytic cells of the spleen, liver, lymph glands, and bone marrow producing a chronic disease (Asford, 1997; Jarallah, 2015; Jarallah, 2016; Makwali, *et al*., 2012). The aetiologic agent of CL; *L. tropica*, *L. aethiopica*, *L. major* and *L. Mexicana* (Enrique *et al*., 2005) replicate in dermal histiocytes producing self-limiting skin lesions (Awasthi *et al*., 2004; Roy *et al*., 2012; Shirbazou & Jafari, 2012) The *L. braziliensis* cause CL but also affects macrophages of oro-naso-pharyngeal area thusskin lesions spread to mucous membranes resulting to disfiguring and complication known as MCL or espundia (Pirmez, 1992; Roy *et al*., 2012).

Table 2.2: Spectrum of leishmaniasis, aetiological agents and distribution (Tonui, 2006)

Type of Leishmania	Causative organism	Worldwide distribution
VL	*L. donovani*	China, India, Iran, Sudan and Kenya.
DCL	*L. infantum*	Ethiopia and the Mediterranean basin.
	L. chagasi	Brazil, Colombia, Venezuela &Argentina
CL	*L. tropica*	Mediterranean basin, Afghanistan, Middle East, W. and N. Africa.
	L. major	East Africa.
	L. aethiopica	Kenya and Ethiopia.
	L. mexicana	Central America and Amazon basin
MCL	*L. braziliensis complex*	Brazil, Peru, Ecuador, Columbia &Venezuela

Leishmania aethiopica in eastern Africa and *L. mexicana* species complex in the Americans are the aetiological agents of DCL (Souza *et al*., 2012).Due to disseminated cutaneous lesions resembling leprosy and which do not heal spontaneously,DCL is difficult to treat (Ameen, 2010; Eissa *et al*., 2012). Most DCL patients fail to display *Leishmania* specific cell-mediated immunity due to a defective immune system (Castes, 1984; Vannier-Santos *et al*., 2002; Awasthi *et al*., 2004; Roberts *et al*., 2006; Monzote, 2009; Vinet *et al*., 2010). Clinically, VL is characterized byirregularly high recurringfever, hepatomegaly, splenomegaly, panocytopenia, jaundice, anaemia; weight loss and general weakness, diarrhea, cough, and constantly appearance of hypoalbuminaemia and polyclonal-hypergammaglobulinaemia (IgG and IgM) (Awasthi *et al*., 2004; Monzote, 2009; Roy *et al*., 2012; Sundar & Chakravarty, 2015). In VL, hyperpigmentation of the skin occurs thus giving an alternate name - kalazar or Black fever (Awasthi *et al*., 2004) which is a silent killer, invariably killing almost all untreated patients (Boelart *et al*., 2000). Induced DNA damage in the peripheral blood and spleen cells has been reported due to invasion by *L. chagasi* (Oliveira *et al*., 2011b).

The chronically evolving severe disfiguring MCL is extremely difficult to treat (Masden, 1986; Kaye & Scott, 2011). Disfiguring skin lesions on the face, arms and legs are produced by CL or oriental sores is often self-healing and the initial papule gives rise to an ulcer (Asford & bates, 1998; Eissa *et al*., 2012). One to two lesions or multiple groups of lesionsof varying diameter sizes (0.5 cm to 3 cm) are present on

the exposed sites (Shiraz & syed, 2007; Kaye & Scott, 2011). Some lesions do not ulcerate at all and remain as bluish papules; others develop sporotrichoid nodular lymphangitisand other secondary infections (Shiraz & syed, 2007; Ameen, 2010; Eissa *et al*., 2012). Secondary bacterial associated with painful lesions healing over months or years, leaving an atrophic scar (Shiraz & Syed, 2007; Ameen, 2010; Eissa *et al*., 2012). In general, *L. major*associated lesionsheal in 3 months, *L. tropica* associated leision take about a year while *L. braziliensis* associated leision persists much longer (Kaye & Scott, 2011). Cutaneous leishmaniasis infection induces immunity to re-infection by particular species that cause the disease upon recovery or successful treatment (Asford & bates, 1998; Beattie & Kaye, 2011).

Management of leishmaniasis is more complicated as the parasite invades macrophages and differentiates into proliferative amastigotes (Roy *et al*., 2012). This destroys the host mononuclear phagocytic system making it vulnerable to other common infections (Shiraz & Syed, 2007). Expression of specialized and stage specific molecules links successful invasion of the parasite into host immune system and survival, alterations in macrophage membrane permeability and electrical activity caused by the parasitic infection (Turco & Descoteaux, 1992; Camacho *et al*., 2008; Quintana *et al*., 2010). Molecular specificity is necessary for targeting therapeutic agents to the leishmanial parasite after its successful invasion into the host (Olivier *et al*., 2005; Roy *et al*., 2012).

2.5 BALB/c mice as a model of leishmaniasis study

Animals remain the best model for the characterization of the disease and its impact on to the host. Hamster and mouse are the two well-studied and suitable models for studying the infection and chemotherapy while monkey model is used for the vaccine trial (Awasthi *et al*., 2004). The BALB/c mice model provides a unique opportunity to study CL in human in its active form due to its susceptibility to *L. major* infection, reproducing clinical and pathological features of CL similar to those found in human (Awasthi *et al*., 2004). However, different strains of mice show variability in their susceptibility to *Leishmania* parasite (Bradley & Kirkley, 1977). BALB/c mice have been shown to be highly susceptible to *L. major*, show signs of slow breeding, are small and delicate to breed (Santos *et al*., 2003; Santos *et al*., 2008). The Swiss

Albino mice on the other hand are easy to breed, virtually all are the same in appearance and they are quite resilient but are known to show resistance to *L. major* (Santos *et al*., 2003; Santos *et al*., 2008). Although infection in BALB/c mice is a well-studied susceptible host model, its not suitable for trial of chemotherapeutic purpose due to much higher effective dose to cure *Leishmania* infection compared to human (Croft &Yardley, 2002; Awasthi *et al*., 2004). However, it can form the basis of selecting higher animal models. The matching physiology with human and availability, BALB/c mice have been chosen as a model in this study.

2.6 Malnutrition and Leishmaniasis

Globally, protein energy malnutrition (PEM) also called protein-calorie malnutrition (PCM) and micronutrient deficiencyaffect and cause deaths of millions of people (WHO, 2002; Blossner & Onis, 2005; Gatto *et al*., 2013; Carrillo, 2014). This excess morbidity and mortality is due to the impairment of victims defence mechanisms, which predisposes them to infectious diseases by lowering the immunity of the body (Gross & Newberne, 1980; Carrillo, 2014). Leishmaniasis is one of the world's most devastating NTDs of documented epidemiological and experimental public health importancethat co-exist with chronic malnutrition (Badaro *et al*., 1986; Cerf *et al*., 1987; Maciel *et al*., 2008; Malafaia, 2009; Malafaia *et al*., 2009; Serafim *et al*., 2010; Gatto *et al*., 2013).

Further, the poor understanding of the impact of PEM (PCM) and micronutrient deficiency on immune response against *Leishmania* infection coupled with neglected nutritional status of the host (Malafaia, 2009; Malafaia *et al*., 2009) worsen the situation. The clinical course and progression of leishmaniasis depend on the nutritional status and is associated with the severity and increased mortality (Gatto *et al*., 2013). During infection, there is hypercatabolism aggravated by anorexia, resulting into loss and depletion of body nutrient reserves causing great changes in the host metabolism (Pereira, 2003). Differences in body mass index (BMI), proteins, glucose, triacylglycerols (TAGs) and lipoproteins; high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) are observed in active leishmaniasis (Ghosh *et al*., 2011). Conducted studies have shown high TAGs, low lipoproteins (HDL, LDL) and total cholesterol levels, decreases in

serum albumin, globulin and total protein (Soares *et al*., 2010; Lal *et al*., 2010) which are probably associated with an increased parasite burden(Gatto *et al*., 2013). Increase in TAGs increases body fat and metabolic disorders, including cardiovascular diseases, diabetes, hypertension and dyslipidemia (Gatto *et al*., 2013). Although studies have revealed that good protein energy (PE) also known as protein calorie (PC) promote adaptive immune response in leishmania infection (Anstead *et al*., 2001; Malafaia, 2009; Malafaia *et al*., 2009; Serafim *et al*., 2010;Gatto *et al*., 2013), a few studies have emphasized on the relationship between leishmaniasis progression and malnutrition. Since supportive therapy inform of good PE or PC is one of the management stragtergy of leishmaniasis, studies on nutrional composition of *A. muricata* and *A. squamosa* in BALB/c mice model of the disease is paramount.

2.7 Chemotherapy of leishmaniasis

Pentavalent antimonials (Sb^V) such as sodium stibogluconate (SG) (Pentostam®), and *N*-methylglucamine (Glucantime®), Amphotericin-B (AMB), and pentamidine are the common and mostly used in leishmania chemotherapy (Murray, 2001; Mishra *etal*., 2011a).However, toxicity, high cost and difficult mode of administerationdue to long-term treatment schedules limit the use of these drugs (Roy *et al*., 2012). The antimonials (Sb^V), Pentostam® (sodium stibogluconate, SG) and Glucantime® (meglumine antimoniate) are the leishmanicidal agents with the most favorable therapeutic index (Mishra *etal*., 2011a) and are used as first-line chemotherapeutic agents against all forms of leishmaniasis (Roy *et al*., 2012).

The *in vivo* reduction of Sb^V to a more toxic Sb^{III}in the *Leishmania* amastigotes is thought to be responsible for the antileishmanial action of antimonials (Griensven *et al*., 2010). Due to this mode of action, only amastigotesare susceptible to Sb^V(Roy*et al*., 2012). It has been proposed further thatSb^V can act on several targets including influence on bioenergetics of amastigotes (Chakraborty & Majumder, 1988; Veeken *et al*., 2000; Roy *et al*., 2012), DNA topoisomerase I (DNA Topo I) inhibition(Walker & Saravia, 2004; Wyllie *et al*., 2004; Roy *et al*., 2012) and alteration of thiol-redox potential in both forms of parasite (amastigotes and promastigotes) by actively promoting efflux of thiols, glutathione and trypanothione rendering the parasite more susceptible to oxidative stress (Ameen, 2007; Roy *et al*., 2012). However, due to the

efflux of antimonial from parasite infected host cells via up-regulation and expression of P-glycoprotein multidrug resistance-related protein 1 (P-gp-MDR1), resistance to the parasites has been reported (Roy *et al*., 2012).

The generic sodium antimony gluconate (SAG) is used as a substitute of the high cost branded SG in treatment of leishmanianiasis (Mishra *et al*., 2011a). However, due to the reported serious side effects, declining efficacy and relapse of the disease, SAG is no longer in use (Grogl*et al*., 1992; Thakur *et al*., 2004). Although Sb^V have been used for long, information regarding their chemistry and mode of action is still insufficient (Roy *et al*., 2012). Besides, long term administration and higher doses give rise to toxic effects like increased levels of marker enzymes such as creatine phosphokinase (CK) and alkaline phosphatase(ALkP), hepatomegaly and typical skin reactions for heavy metals(Oliveira *et al*., 2011a; Oliveira *et al*., 2011b; Roy *et al*., 2012).

Pentamidineas an antileshimanial agent (Jha, 1983; Monzote, 2009) hampers replication and transcription at the mitochondrial level in the pathogen through binding to tRNA via non-specific hydrophobic interactions inhibiting aminoacylation and translation of the replicating parasite (Sun & Zhang, 2008; Mishra *et al*., 2011a; Roy *et al*., 2012). It is thought that the charged amidinium groups of pentamidine establish hydrogen bonding with oxygen atom (O_2) of thymine or Nitrogen (N_3) of adenine and form complexes with the minor groove of DNA (Mishra *et al*., 2011a; Oliveira *et al*., 2011b). Pentamidine have been used as drugs of second choice (Thakur *et al*., 1984; Chakraborty & Basu, 1997; Berman *et al*., 1999; Mishra *et al*., 2011a) because of their potential toxicity and adverse side effects such as hypotension, hypoglycemia, leucopenia, thrombocytopenia, cardiac arrhythmia, acute renal failure, elevated serum creatinine level, nausea and fever (Roberts *et al*., 2006; Monzote, 2009; Oliveira *et al*., 2011b; Mishra *et al*., 2011a; Roy *et al*., 2012). Further, the efficacy of pentamidine has gradually declined over the years (Mishra *et al*., 2011a).

Amphotericin B (AmB), a macrolide antibiotic produced by *Streptomyces nodosus* is very successful but highly toxic in leishmanial therapy (Jha, 1983)as it can perturb both parasitic and mammalian cells membranes (Thukar *et al*., 1999; Mishra *et al*., 2011a; Mishra *et al*., 2011b; Roy *et al*., 2012). However, its selective lethalityto

parasitic cells accounting for its efficacyis due to its high affinity towards24-substituted sterols (ergosterol), a major cell membrane sterolforming a binary complex of AmB with membrane sterols (Roy *et al*., 2012). The AmB-membrane sterols complex evokes changesin permeability leading tolipid peroxidation and fragility resulting to uncontrolled ion loss due to formation of barrel shaped transmembrane pores leading to cell lysis (Cohen & Gamargo, 1987; Roy *et al*., 2012). Further, AmB inhibits membrane enzymes H^+-ATPase (Brajtburg *et al*., 1996)and Na^+/ K^+-ATPase (Vertut-Do *et al*., 1988)causing loss in proliferative ability by depletion of cellular energy reserves (Schindler, 1993; Naderer & McConville, 2008). Due to the hydrophobicity of the polyene structure, AmB shows poor gastrointestinal absorption and negligible bioavailability (Gershkovich *et al*., 2009) andcaninteract with the mammalian cell membrane causing cellular dysfunction (Bolard *et al*., 1991; Oliveira *et al*., 2011b). Besides, fungizone, the most commonly used formulation of deoxycholate complexed AmB micellesis toxicoften causing decreased renal function, anaphylaxis, chills, high fever, nausea, phlebitis, anorexia among other adverse effects (Oliveira *et al*., 2011b; Roy *et al*., 2012). Although lipid formulations of AmB reduce toxicity to non-target tissues (Croft *et al*., 2006b), Am B adverse reactions coupled with long therapeutic regimes and development of resistance limits its general usefulness as an anti-infective antileishmanial therapy (Hartsel & Bolard, 1996; Mishra *et al*., 2011a; Mishra *et al*., 2011b; Oliveira *et al*., 2011b; Roy *et al*., 2012).

Paromomycin, an aminocyclitol-aminoglycoside antibioticwas identified as an antileishmanial drug in the 1960s (Monzote, 2009; Mishra *et al*., 2011a; Roy *et al*., 2012). It acts by impairing macromolecular synthesis and altering membrane properties of *leishmania* (Sundar *et al*., 2007; Mishra *et al*., 2011a; Oliveira *et al*., 2011b; Roy *et al*., 2012). Sitamaquine, an orally active analog of 8-aminoquinoline, was under clinical development by the Walter Reed Army Institute in collaboration with Glaxo Smith Kline for use as an antileishmanial drug (Mishra *et al*., 2011a). However, randomized, open label and multicenter Phase II trial conducted in India and Kenya indicates that the drug is efficacious and well tolerated at various dose levels (Wasunna *et al.,* 2005; Sun & Zhang, 2008). Delivery stratergies such as targeting of antileishmanial agents in liposomes, microspheres and nanoparticles

have been successfully in increasing the efficacy and reduce toxicity of antileishmanial drugs (Basu & Lala, 2004; Van de Van *et al*., 2011;Vinet *et al*., 2011; Singodia *et al*., 2011; Gupta *et al*., 2010; Bhowmik *et al*., 2010; Roy *et al*., 2012). However, non-specific accumulation of reticuloendothelial system (RES), instability of liposomal formulations and incomplete degradation of polymeric delivery systems limit these delivery strategies (Basu & Lala, 2004). Therefore, investigations still need to be progressed to discover new, high efficacy and safer antileishmanial drugs.Miltefosine originally developed, as anti-tumor agent is antileshmanial drug approved against VL in India (Mishra *et al*., 2011a). The drug blocks *leishmania* proliferation, alters phospholipid and sterol composition and activates cellular immunity (Mishra *et al*., 2011a). However, prescriptions are avoided due to high cost and serious side effects (Sundar *et al*., 2003; Sun & Zhang, 2008; Oliveira *et al*., 2011a; Khademvatan *et al*., 2011).

2.8Botanicalsas alternatives in leishmania management

2.8.1 Taxonomy and geographical distribution of Annonaceae spp

Annona is a genus of tropical fruit trees belonging to theFamily: Annonaceae, Order: Magnoliales (Hotta *et al*., 1989). More than 119 species exist but only seven species and one hybrid are grown for domestic and/or commercial use (ICUC, 2002). Species include; *A. cherimola*; Mill (cherimoya), *A muricata*; Linn (soursop), *A. squamosa*; Linn (sugar apple) *A. senegalensis*; Linn (wild soursop) and *A. reticulata*; Linn (custard apple) ammong others (Hotta *et al*., 1989) (Plate 2.1).

Plate 2.1: Annona muricata andAnnona squamosa fruits

The fruits are native in tropical North and South America and after arrival of the Spnanish in the Americas; they become widely distributed throughout the tropics (Badrie & Schauss, 2009). The fruits are widespread in the tropics and frost-free subtropics of the world and are found in the West Indies, North and South America, lowlands of Africa, Pasific Islands and Southeast Asia (ICUC, 2002). *Annona muricata* (L.) and *A. squamosa* (L.) are commonly known as soursop and casturd apple respectively in English speaking countries and collectively refered commonly as *Matomoko*in Kenya as *Fenesi* and *Stafeli* respectivellyin Kiswhali. They usually thrive well in the coastal lowlands distributed mostly in Kilifi and Kwale counties. However, they are also found in high altitudes mostly in the central region of Kenya.

2.8.2 Natural products from folk medicines in treatment of leishmaniasis

The effectiveness of natural products as potentially rich sources of new and selective agents for the treatment of leishmaniasis has ben established by ancient records and literature (Mishra *et al.*, 2011a; Mishra *et al.*, 2011b; Wink, 2012; Adebayo *et al.*, 2013). The originally off-patent antibiotic, paromomycin developed from *Streptomyces krestomuceticus*; (Waksman and Henrici) marketed in the United States (US) for the treatment of intestinal parasites is an orphan approved leishmanicidal agentin India (Mishra *et al.*, 2011a).Thourough literature search on natural products has provided a growth on research in plant derived antileishmanial agents. Although several natural products have been discovered with excellent activity against *Leishmania* (Adebayo *et al.*, 2013; Wink, 2012; Mishra *et al.*, 2011a), none of them have been clinically evaluated in studies or projected to reach the clinical applications in near future (Mishra *et al.*, 2011a). Therefore, research focused on leishmanicidal agents from natural products with special attention on structure-activity relationship (SAR) based activity and mechanism of action is crucial (Mishra *et al.*, 2011a).

Although development of highly specific and sensitive non-invasive diagnostic tools could be the newer weapons to combat the spread of leishmaniasis (Guerin *et al.*, 2002), development of effective, convenient, low toxic and low-cost chemotherapy for leishmaniasis is paramount. Different plant-derived natural compounds are in experimental stages of antileishmanial chemotherapies (Tempone *et al.*, 2005;

Mishra *et al*., 2011a; Tempone *et al*., 2011; Roy *et al*., 2012; Wink, 2012; Adebayo *et al*., 2013). Many plants explored contain specific molecules with antileishmanial activity (Shapaz *et al*., 1994; Shapaz *et al*., 1996; Jaramillo *et al*., 2000; Osorio *et al*., 2007; Wabwoba *et al*., 2010; Villa-Nova *et al*., 2011; Mishra *et al*., 2011a; Makwali *et al*., 2012; Al-Musayeib *et al*., 2012; de Lima *et al*., 2012; Roy *et al*., 2012; Kinuthia *et al*., 2013; Alkathiri *et al*., 2017; Iqbal *et al*., 2017; Et-Touys *et al*., 2017) and their compounds have been classified (Figure 2.5). Some of plant derived compounds that are used in antileishmanial therapy include benzylisoquinolines, β-carboline alkaloids, iridoid and steroidal glycosides, terpenoids, flavonoids among metabolites such as acetogenin, aregentilactone (Tempone *et al*., 2005; Gupta *et al*., 2010; Tempone *et al*., 2011; Mishra *et al*., 2011a). The ethanol extracts of *Artemisia indica* leaves (Sen *et al*., 2010), Azole based compounds; 1, 3,4-thiadiazole and thiophenyl azoles (Marrapu *et al*., 2011), nicotinamides (Roy *et al*., 2010;Gazanion *et al*., 2011) have been demonstrated to improve the antileishmanial activity of trivalent antimony in a synergistic manner and exhibit additive effects in combination with Amphotericin (Gershkovich *et al*., 2009; Gazanion *et al*., 2011). Further, many natural products from plants exhibiting antileishmanial activities have been described (Tempone *et al*., 2005; Tempone *et al*., 2011; Mishra *et al*., 2011a; Roy *et al*., 2012; Adebayo *et al*., 2013; Wink, 2012).

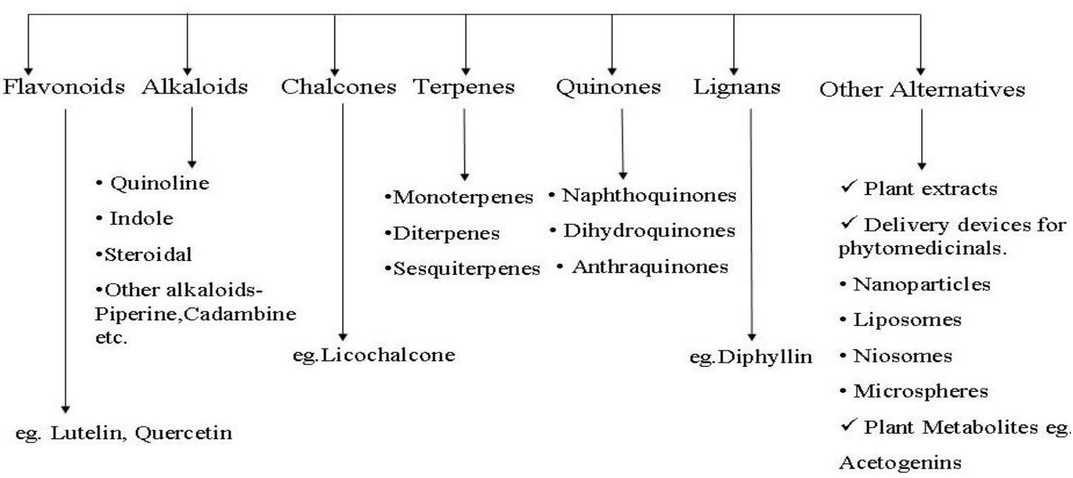


Figure 2.5: Characterization of antileishmanial phytochemicals from plants (Roy et al., 2012)

2.8.3Annonaceae spp as alternative source of neutraceutical substances

Food insecurity coupled with NTDs such as leishmaniasis especially in the developing world, has necessitated research in alternative source of nutraceutical substances using suitable *in vitro* and *in vivo* models. Natural products, notably those derived from plants, have been used to help sustain humankind nutrition and health since the dawn of medicine. The importance of plants in Agriculture and medicine has stimulated significant scientific interest (Moghadamtousi *et al.,* 2013). However, a restricted range of plant species, with regard to their medicinal importance, has experienced detailed scientific inspection, resulting into insufficient knowledge concerning their potential role in medical application (Ghani, 2003; Moghadamtousi, *et al*., 2014). Therefore, to attain a reasonable perception of medicinal values and/or benefits, comprehensive investigations on the role of plants in the management of human leishmania are needed. In a nutraceutical landscape, plants with a long history of use in ethno-medicine are a rich source of nutritional components and active phytoconstituents that provide medicinal or health benefits against various ailments and diseases. The high demand in search for both nutritional and pharmaceutical agents has necessitated the need of experimental work on edible fruits in appropriate animal models.

The Annonaceae fruits are highly perishable and are in abundant in specific regions of Kenya. The perishability of the fruit lowers its shelf life and huge quantities go to wastes reflecting very huge loss to farmers growing these fruits. Further, these fruits are neglected especially as sources of alternative foods and medicine for reasons not well understood. The benefits of this research on these two plants towards the animal use components may be towards the development of food supplements to be used in leishmaniasis supportive therapy and provision of biological active ingredients against leishmaniasis for synthesis of newer, safe and efficacious leishmanicidal compounds. This might reflect on proper utilization of these neglected crops for development of nutraceutical products. It might also contribute towards curbing food insecurity in the region where they are grown bearing in mind that these regions are also leishmania endemic areas.

2.8.4 Antioxidant, DNA binding and protein inhibitory activity inleishmaniasis

During leishmaniasis progression, there is damage of internal organs such as the liver and spleen due to high parasite burden (Makwali *et al*., 2012; Jarallah, 2015; Jarallah, 2016) or metabolic process produced by the host (Oliveira & Cecchini, 2000; Oliveira *et al*., 2011b). Damage of DNA and nitric oxide production has been reported in mice following infection *with L. chagas* (Oliveira *et al*., 2011b; Inacio *et al*., 2014). Besides, adverse effects of leishmania chemotherapy such as AmB interacting with both parasite and host cell membrane induce lipid peroxidation (LPO) of the plasma membrane (Roy *et al*., 2012; Fernandes *et al*., 2013; Alkathiri *et al*., 2017) complicating further the management of the diease. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) aregenerated upon macrophages exposure to *Leishmania* leading to the regulation of the inflammatory response controlled by the cellular antioxidant defense system (Inacio *et al*., 2014; Paiva & Bozza, 2014; Alkathiri *et al*., 2017).

Internal and external pathological factors such as viral, bacterial, and parasitic infections disrupt the body's oxidant/antioxidant balance initiating oxidative stress mechanisms such as oxidation of lipids, proteins, and nucleic acids (Eissa *et al*., 2012; Jafari *et al*., 2014; Aguiar *et al*., 2010). Accumulation of ROS and RNS in cells damage membrane lipids if not prevented by an appropriate antioxidant scavenging system (Alkathiri *et al*., 2017). However, good antioxidant defense system is closely associated with good PE or PCnutrition. Although endogenous antioxidants are available to reduce ROS and RNS accumulation, exogenous antioxidants obtained from appropriate diets can also play a crucial role (Dkhil *et al*., 2016). The exogenous and endogenous antioxidant defense systems act in coordination, with their levels being regulated by each other, to avoid oxidative stress events (Dkhil *et al*., 2013; Dkhil *et al*., 2016).

Further, protein and DNA-binding interaction is important in development of antileishmanial drugs (Wink, 2012). Studies have investigated the inhibitory effect of medicinal plant extracts on protein and DNA sequences (Attard & pacioni, 1996; Peebles *et al*., 2001; Kluza *et al*., 2003; Cao *et al*., 2005; Qin *et al*., 2006; Yamada *et al*., 2006; Correa *et al*., 2007; Lampronti *et al*., 2008; Pommier *et al*, 2012; Kar & Chattopadhyaya, 2016). However, limitations in solubility and low

bioavailabilitynecessitate high doses for effectiveness of most chemotherapeutic agents (Roy *et al*., 2012). Therefore, combination therapy of natural products alongside antibiotics or synthetic drugs is an emerging trend in leishmaniasis therapy (Tiuman *et al*., 2011). Based on the mechanism of action of leishmanicidal such as inhibition of specific enzymes such as DNA Topo I and DNA Topo II (Walker and Saravia, 2004; Wink, 2007; Sen *et al*., 2008; Wink, 2012; Xin *et al*., 2017), the genes coding for these targets can be targeted for development of newer, cheap, safe and efficacious antileishmanials. Although topoisomerase blockers from plant secondary metabolites have been identified (Wink & Schimmer, 2010; Wink, 2012; Xin *et al*., 2017), the search for more compounds with topoisomerase blocking activity forcusing on leishmaniasis is paramount. In the past few decades, antioxidant molecules that are wide spread in plants have come into focus (Alkathiri *et al*., 2017). The Annonaceaefruits are cultivated in several parts of Kenya and have been traditionally used for the treatment of different illnesses including antivenin (Owuor & Kisangau, 2006). Although, few studies have been concentrated on antioxidant, chemopreventive and hepatoprotective properties of *A. squamosa* and *A. muricata* extracts (Arthur *et al*., 2011; Arthur *et al*., 2012a; Arthur *et al*., 2012b; Mariod *et al*., 2012; Nawwar *et al*., 2012; Nandhakumar and Indumathi, 2013; Samuagam *et al*., 2014), the Kenyan varieties have been neglected. Thus theyoffer excellent candidatures for*in vitro* and *in vivo*antioxidantsscreening, DNA binding and DNA Topo I inhibitory activity potentials in relation to leishmaniasis BALB/c mice, which can form the basis of developing efficacious, safe and low costantileishmanial agents.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study area

The samples for the study were collected in Mwarakaya and Chasimba wards of Kilifi County and Gombato-Bongwe and Ukunda Wards of Kwale County. Kilifi County borders Kwale County to the South West

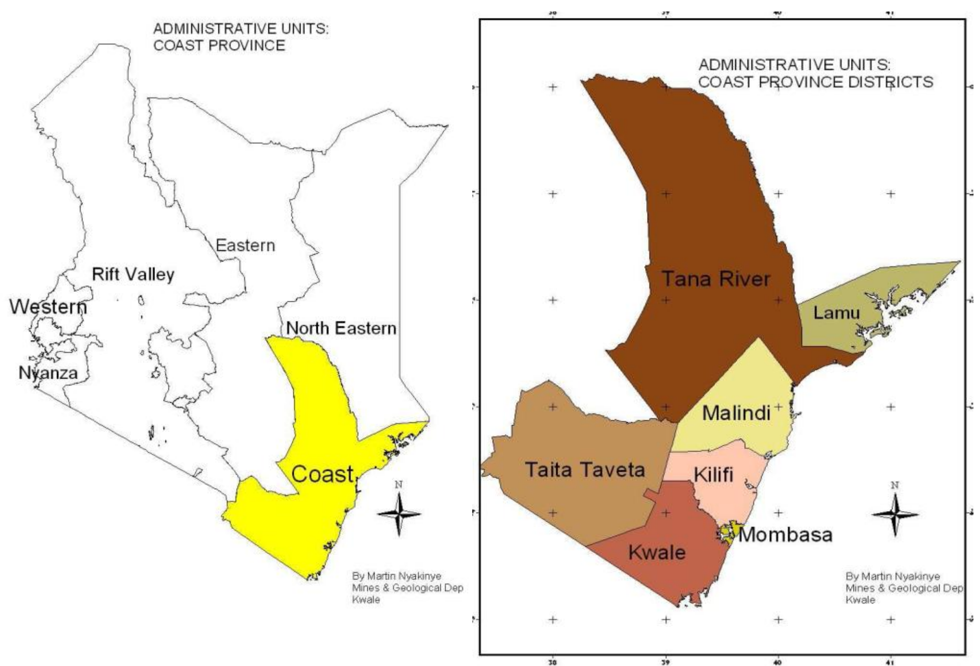


Figure 3.1: A map of Kilifi County and Kwale County showing the areas and sites involved in the study(www.nema.go.ke).

3.2 Collection of Plant Materials

The ripe fresh fruits of *A. muricata* and *A. squamosa*were collected in the period between March and September 2014 from farms in coast province (Kilifi and Kwale Counties) of Kenya. The National Museum of Kenya, Nairobi, identified the species where voucher numbers for *A. muricata* and *A. squamosa* were deposited. The harvested fruits were washed with chlorinated water to retard aging and removal of fungi and bacteria. They were then transported to the laboratory for phytochemicals screening, pellets formulation, proximate and nutrional composition analysisand for the *in vitro* and *in vivo*antileishmanial efficacy, safety and antioxidant activity, DNA binding and DNA Topo I inhibition activity assays. The pulp, peel and seeds from the

fruits were separated from each other and then dried using a constant temperature and humidity chamber (Tokyo Thermo Tech Co. Ltd, Japan) set at 40°C and 95% relative humidity. Dry pulp, peel and seeds were then separately grounded into fine powder using a grinding machine (Mitamura Riken, Kogyo Inc. Tokyo, Japan). The grounded fruits parts inform of powder were weighed using a top-loading balance, transferred into polythene bags, sealed, and stored at 4^{0}C until extraction.

3.3 Study design

Experimental study design was used to determine the *in vitro* and *in vivo*antileishmanial efficacy, safetyand antioxidant activity. Macrophages infected with*L. major* parasite (IDUB/KE/83=LNB-144 strain) and normal mammalian cells; Vero cells (ATCC® CCL-81™)were used todetermine*in vitro* antileishmanial efficacy and safety respectively.*Leishmania major* infected and non-infected BALB/c mice (3 - 4 weeks old) obtained from Animal House Unit, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya were used to determine the*in vivo* antileishmanial efficacy, safety and antioxidant activity.

3.4 Extraction of phytochemicals from pulp, peel and seeds of *A. muricata* and *A. squamosa* fruits

Aqueous extraction was carried out where 50g of each of the dried pulp, peel and seeds from *A. muricata* and *A. squamosa* fruits was macerated in 100 mL sterile distilled water in a Warring blender for 10 minutes. The macerate was first filtered through double-layered muslin cloth and then centrifuged at 4000*g* for 30 minutes there after the supernatant was filtered through Whatman No.1 filter paper. The extracts described and abbreviated in Table 3.1 were finally preserved aseptically in sterile airtight bottle at 4℃ for later use (Biba *et al*., 2013). The organic solvents extraction was carried out using 50g of powdered pulp, peel and seeds from *A. muricata* and *A. squamosa* fruits sequentially using100 mL of solvents of increasing polarity starting with n-hexane followed by ethyl acetate and finally Methanol (MeOH) for 48 hours each with occasional swirling to ensure thorough extraction. The extracts were decanted and filtered through Whatman filter paper and the macerate steeped in solvents (n-hexane, ethyl acetate and MeOH) again for 48 hrs. Extraction process was repeated twice and the filtrates combined and concentrated on

a rotary vacuum evaporator (Bibby Sterilin Ltd, RE 100B, UK) under reduced pressure at a temperature of 50°C and packed and stored in an airtight bottles at 4℃ for later use (Biba *et al*., 2013) as described and abbreviated in Table 3.1.

Table 3.1: Abbreviations of different crude extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds

Extract Abbreviation	Full name of the extract
ASPUAE	*Annona squamosa* pulp aqueous extract
ASPUME	*Annona squamosa* pulp methanol extract
ASPUEAE	*Annona squamosa* pulp ethyl acetate extract
ASPUHE	*Annona squamosa* pulp hexane extract
ASPEAE	*Annona squamosa* peel aqueous extract
ASPEME	*Annona squamosa* peel methanol extract
ASPEEAE	*Annona squamosa* peel ethyl acetate extract
ASPEHE	*Annona squamosa* peel hexane extract
ASSAE	*Annona squamosa* seeds aqueous extract
ASSME	*Annona squamosa* seeds methanol extract
ASSEAE	*Annona squamosa* seeds ethyl acetate extract
ASSHE	*Annona squamosa* seeds hexane extract
AMPUAE	*Annona muricata* pulp aqueous extract
AMPUME	*Annona muricata* pulp methanol extract
AMPUEAE	*Annona muricata* pulp ethyl acetate extract
AMPUHE	*Annona muricata* pulp hexane extract
AMPEAE	*Annona muricata* peel aqueous extract
AMPEME	*Annona muricata* peel methanol extract
AMPEEAE	*Annona muricata* peel ethyl acetate extract
AMPEHE	*Annona muricata* peel hexane extract
AMSAE	*Annona muricata* seeds aqueous extract
AMSME	*Annona muricata* seeds methanol extract
AMSEAE	*Annona muricata* seeds ethyl acetate extract
AMSHE	*Annona muricata* seeds hexane extract

3.5 Phytochemical analysis

3.5.1 Qualitative phytochemical analysis

3.5.1.1Test for alkaloids

Mayer's, Dragendroff's and Wagner's tests were used to test for the presence of alkaloids (Trease & Evans, 1989). Two (2) mg of each of the aqueous and organic pulp, peel and seeds extractsfrom *A. muricata* and *A. squamosa* fruits was mixed with 20 mL of 1% sulphuric acid (H_2SO_4) in a 50 mL conical flask and warmed at 37°C for 2 minutes on a water bath, with intermittent shaking, centrifuged 20,800g for 2 minutes and the supernatant pipetted off into a small conical flask. For Mayer's test, one drop of Meyer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100 mL of distilled water) was added to 0.1 mL supernatant in a semi-micro tube and observed for 2 minutes for the presence of a cream precipitate. For Dragendroff's test, one drop of Dragendroff's reagent was addedto each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*and the mixture observedfor 2 minutes for the formation of reddish orangecoloured precipitate. For Wagner's test, a fraction of each of the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100 mL distilled water) and observed for 2 minutes for the formation of reddish brown coloured precipitate.

3.5.1.2 Test for flavonoids

Aqueous sodium hydroxide (NaOH) test, Sulphuric acid (H_2SO_4) test, ammonium test and Schinodo's test were used in detection of flavonoids (Harborne, 1973). For Aqueous sodium hydroxide (NaOH)test, a fraction of each of the the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*was treated with 1N aqueous NaOH solution and observed for 2 minutes for the formation of yellow-orange colouration. In Sulphuric acid (H_2SO_4) test, a fraction of each of the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*was treated with concentrated sulphuric acid (H_2SO_4) and observed for 2 minutes for the formation of orange colour. Finally, for Schinodo's test a fraction of each of the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*was treated with a piece of magnesium turnings followed by a few drops of concentrated hydrochloric acid

(HCl), heatedslightly and observed for 2 minutes for the formation of dark pink colour. For ammonium test, 5 mL of ammonia solution was added to a portion of the aqueous filtrate of each of the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*followed by addition of concentrated sulphuric acid (H_2SO_4). A yellow coloration observed for 2 minutes which disappeared on standing for 2 minutes indicated the presence of flavonoids.

3.5.1.3 Test for tannin

Ferric chloride ($FeCl_3$) and lead acetate ($Pb(CH_3COO)_2$) tests were used (Trease & Evans, 1989). In ferric chloride test, a few drops of 0.1% ferric chloride ($FeCl_3$) was addedto each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*; a brownish green or a blue-black coloration indicated the presence of tannins. In lead acetate ($Pb(CH_3COO)_2$) test, about 1 mL of each extract from the peel, pulp and seeds of *A. muricata* and *A. squamosa*was diluted with 10 mL of distilled water and 2 to 10 drops of 1% lead acetate ($Pb(CH_3COO)_2$) solution added. The solution was then observed for 2 minutes for the formation of a precipitate, which indicated the presence of tannins.

3.5.1.4 Test for phenolic compounds

Ferric chloride ($FeCl_3$) and lead acetate ($Pb(CH_3COO)_2$) tests were carried out to determine phenolic compounds (Trease & Evans, 1989) where the each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*was diluted to 5 mL with distilled water. For Ferric chloride ($FeCl_3$) test, a fraction of each of the the extracts was treated with 5% $FeCl_3$and observed for 2 minutes for the formation of deep blue-black colour. For Lead acetate ($Pb(CH_3COO)_2$) test, a fraction of each of the extracts was treated with 10% lead acetate ($Pb(CH_3COO)_2$) solution and observed for 2 minutes for the formation of white precipitate.

3.5.1.5 Test for steroids

The method as described by Trease & Evans, (2002) was adopted to detect steroids. About 2 mL of acetic anhydride was added to 2 mL of each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*followed by 2 mL of concentrated sulphuric acid (H_2SO_4). The color change from violet to blue was followed for 2 minutes.

3.4.1.6 Test for terpenoids

Salkowski's test was used to detect terpenoids (Edeoga *et al*., 2005). About 2 mg of each of each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*was dissolved in 2 mL of chloroform taken in a dry test tube. Equal volume of concentrated sulphuric acid (H_2SO_4) was added. The tube was shaken gently. The upper layer of chloroform turning red and lower layer showing yellow green fluorescence indicated the presence of steroids and terpenoids.

3.5.1.7 Test for saponins

Sodium bicarbonate (Na_2CO_3) and froth tests were used (Evans, 2002). For sodium bicarbonate (Na_2CO_3) test, about 5 mL of each of the extracts from the peel, pulp and seedsof *A. muricata* and *A. squamosa* was measured in a test tube and a drop of sodium bicarbonate (Na_2CO_3) added. The mixture was shaken vigorously and kept for 3 minutes. The formation of a honeycomb like froth showed the presence of saponins. In the froth test 10 mL of each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa* was mixed with 5 mL of distilled water and shaken vigorously for about 5 to 10 seconds to form a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously for about 5 to 10 seconds, then observed for 2 minutes for the formation of emulsion.

3.5.1.8 Test for anthraquinones

Anthraquinones were detected as as described by Sofowora, (1993). 2 mg of each of the extractsfrom the peel, pulp and seeds of *A. muricata* and *A. squamosa*was boiled with 10% hydrochloric acid (HCl) for 2 minutes then filtered and allowed to cool. This was then partitioned against equal volume of chloroform. Formation of rose-pink color upon addition of 2 mL of 10% aqueous ammonium solution indicated the presence of anthraquinones.

3.5.1.9 Test for coumarins

Coumarins were detected as as described by Trease & Evans, (2002). About 2 mL of chloroform was added to each of the extracts from the peel, pulp, seeds of *A. muricata* and *A. squamosa* after which the chloroformic phase of each of the extracts were evaporated to dryness, and their respective residues dissolved in pre-heated distilled water and cooled. The cooled solution was divided into two test portions, A

and B. To the test portion A of each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa*, about 0.5 mL of 10% ammonia solution was added. Test portions A and B for each of the extracts from the peel, pulp and seeds of *A. muricata* and *A. squamosa* were observed under UV light for the occurrence of an intense bluish green fluorescence to indicate the presence of coumarins.

3.5.1.10 Detection of glycosides

The method as described by Trease & Evans, (2002), Edeoga *et al*., (2005) and Onike, (2010) was followed. About 2 mL of each of the extracts from the powdered pulp and seeds of *A. muricata* and *A. squamosa* was mixed with about 5 mL of 20% hydrochloric acid (HCl) on a water bath for 2 minutes and then filtered. The filtrates were made alkaline with 2 mL of 20% sodium hydroxide (NaOH) and then 1 mL of Fehling's solution A and B added. The solution was heated on a water bath for 2 minutes and observed for 2 minutes for white coloured precipitate formed.

3.5.2 Quantitative phytochemicalanalysis

3.5.2.1 Determination of alkaloids

Quantification of alkaloids was done by the alkaline precipitation gravimetric method described by Harborne, (1998). To 5g of each of the powdered pulp and seeds of *A. muricata* and *A. squamosa* fruits in 250 mL beaker, 200mL of 10% acetic acid (CH_3COOH) in ethanol (CH_3CH_2OH) was added, covered and allowed to stand for 4 hours at 28^oC. It was later filtered via Whatman No 42 filter paper. The filtrate was then concentrated to one quarter of its original volume by evaporation. Concentrated ammonium hydroxide (NH_4OH) was added drop wise to each of the obtained extracts until the alkaloid precipitated. The alkaloid precipitated was received in a weighed filter paper, washed with 1% ammonia solution and dried in the oven at 80^0C. Alkaloid content was calculated and expressed as a percentage of the weight of powdered sample from peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitsanalyzed.

3.5.2.2 Determination of flavonoids

Quantity of flavonoids was determined according to the method of Harborne, (1998), where 5g of each of the powdered pulp and seeds of *A. muricata* and *A. squamosa*

fruits was boiled in 50 mL of 2M Hydrochloric acid (HCl) solution for 30 minutes under reflux. It was then allowed to cool at room temperature then filtered through Whatman No 42 filter paper. A measured volume of each of the obtained extracts was then treated with equal volume of ethyl acetate starting with a drop. The flavonoid precipitated was recovered by filtration using weighed filter paper. The resulting weight difference gave the weight of flavonoid in the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa* fruits analyzed.

3.5.2.3 Determination of tannins

Tannin content was determined as describe by Kirk & Sawyer, (1998). Five (5) grams of each of the powdered pulp and seeds of *A. muricata* and *A. squamosa*fruitswas dispensed in 50 mL of distilled water and shaken for about 5 to 10 seconds. The mixture was then allowed to stand for 30 minutes at 28°C before it was filtered through Whatman No. 42 filter paper. Two (2) milliliters of each of the obtained extracts and standard tannin solution (tannic acid) 0, 50, 100, 200, 300, 400 and 500 mg/L was dispensed into a 50 mL volumetric flask. Similarly, 2 mL of distilled water was put in separate volumetric flasks as a blank to calibrate the instrument to zero. Two (2) milliliters of Folin-Denis reagent was added to each of the flasks followed by 2.5mL of saturated sadium carbonate (Na_2CO_3) solution. The content of each flask was made up to 50mL with distilled water and allowed to incubate at 28°C for 90 minutes.Their respective absorbance was measured in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan) at 760nm.

3.5.2.4 Determination of phenolic compounds

Total phenolic content was estimated spectrophotometrically using Folin-Ciocalteu reagent, as described by Spanos & Wrolstad, (1990) with slight modification, using gallic acid as a standard. About 2-5g of each of the powdered pulp and seeds of *A. muricata* and *A. squamosa*fruits was homogenized or blended into a puree and passed through cheesecloth to remove debris. It was then centrifuged at 4°C at 12,000g for 20 minutes and the supernatant preserved. The each of the obtained extract was then passed through a 0.45 μM membrane filter. To 0.1 mL of each of the obtained extracts, 5.0 mL of 0.2N Folin-Ciocalteu reagent and 4.0 mL of saturated

sodium carbonate (Na_2CO_3) solution was added. The standard curve was prepared using Gallic acid (0, 50, 100, 200, 300, 400 and 500 mg/L). Each of the prepared mixture from the obtained extracts was allowed to stand for 90 minutes and absorbance measured at 765 nm using UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). The amount of total phenolics was expressed as mg gallic acid equivalents per 100 g of the peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits.

3.5.2.5 Determination of Saponins

Saponin determination was carried out as reported by Ezeonu& Ejikeme, (2016).Exactly 100 $cm^3$of 20% aqueous ethanol (CH_3CH_2OH) was added to 5g of each of the powdered *A. muricata* and *A. squamosa* fruits pulp and seeds in a 250 cm^3 conical flask. The each of the mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55°C. The residue of each of the obtained mixture was re-extracted with another 100 cm^3 of 20% aqueous ethanol (CH_3CH_2OH) after filtration and heated for 4 hours at a constant temperature of 55°C with constant stirring. The each of the obatained extracts was combined and evaporated to 40 cm^3 over water bath at 90°C. Then 20 $cm^3$of diethyl ether was added to each of the obtained concentrate in a 250 cm^3separator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. The purification process was repeated twice after which 60 cm^3 of n-butanol was added and extracted twice with 10 $cm^3$of 5% Sodium chloride (NaCl). The sodium chloride (NaCl) layer was discardeand the remaining solution heated in a water bath for 30 minutes. Each of the obtained solution was transferred into a crucible and dried in an oven to a constant weight. The saponin content was calculated as a percentage:

$$\% \text{ Saponin} = \frac{\text{Wt of sample before drying}}{\text{Weight of sample}} \times 100$$

3.5.2.6 Determination of Glycosides

Glycosides were quantified as described by Ezeonu& Ejikeme, (2016). One (1) gram of each dry powdered pulp and seeds of *A. muricata* and *A. squamosa*fruitswas

weighed into a 250 cm^3 round bottom flask and 200 $cm^3$of distilled water added and allowed to stand for 2 hours for autolysis to occur. Full distillation was carried out in a 250 cm^3 conical flask containing 20 cm^3 of 2.5% sodium hydroxide (NaOH) in each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits after adding an antifoaming agent (tannic acid). About 100 cm^3glycoside, 8 $cm^3$of 6M ammonium hydroxide (NH_4OH) and 2 $cm^3$of 5% potassium iodide (KI) was added to each of the obtained distillate, mixed, and titrated with 0.02M silver nitrate ($AgNO_3$) using a microburette against a black background. Turbidity, which was continuous, indicates the end point. Content of glycoside in the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits was calculated as

$$\% \text{ Glycosides} = \frac{\text{Titre value } (cm^3) \times 1.08 \times \text{extract volume}}{\text{Aliquot volume } (cm^3) \times \text{sample weigh (g)}} \times 100$$

3.5.2.7 Determination of terpenoids

Terpenoids were quantified as reported by Indumathi *et al*., (2014). About 100 mg (Wi) of each of the dried powered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits was taken and soaked in 9mL of absolute ethanol (CH_3CH_2OH) for 24 hours. Each of the obtained extracts after filtration was extracted with 10 mL of petroleum ether using separating funnel. Each of the ontained ether extracts was separated in pre-weighed glass vials and waited for its complete drying (Wf). The ether was evaporated and the percentage yield of total terpenoids contents was measured by the formula

$$\% \text{ Terpenoids} = \frac{Wi - Wf}{Wi} \times 100$$

3.5.2.8 Determination of Steroids

Steroids were quantified as reported by Malik *et al*., (2017). Two milligrams (2 mg) of each of the dried powered pulp and seeds of *A. muricata* and *A. squamosa*was transferred into 10 mL volumetric flasks. 2 mL of 4N Sulphuric acid (H_2SO_4) and 2 mL 0.5% w/vferric chloride ($FeCl_3$) was added, followed by 0.5 mLof 0.5% w/vpotassium hexacyanoferrate (III) [$K_3Fe(CN)_6$] solution. Each of the obtained mixture washeated in a water-bath maintained at 70±2^oC for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was

measured at 780 nm using UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan) against the reagent blank.

3.6 Proximate and nutritional composition analysis

3.6.1 Moisture content

The moisture content was determined as described by AOAC, (2000). About 5g of each fresh peel, pulp and seeds from *A. muricata* and *A. squamosa* fruitswas weighed into a moisture dish and heated in the oven at 130^0C for 2 hours. It was then cooled to room temperature in a desiccator and the finalweight of the sample was taken. Calculations;

$$\% \text{ Moisture} = \frac{\text{Wt of sample before drying} - \text{Wt of sample after drying}}{\text{Wt of sample before drying}} \times 100$$

3.6.2 Dry matter

Dry matter also known as total soluble solids (TSS) were estimated by deducting percentage of moisture from hundred as described by AOAC, (2000).Therefore,% Total solids = 100 – (Percentage of moisture). To confirm the TSS value, it was measured as ^{o}Brix at 20^oC using a refractometer (Atago, N1, brix 0-32, Japan).

3.6.3 Ash content

The method as described by AOAC, (2000) was followed for the determination of ash content. Silica crucibles were heated at 550^oC for 1 hour to obtain the constant weight. They were then cooled to room temperature in a desiccator and weighed accurately. About 5g of each dried powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits was weighed in the silica crucibles. The crucibles with thepeel, pulp and seeds of *A. muricata* and *A. squamosa*fruits were first heated on a heating mantle till all the material were completely charred, followed by incineration in a muffle furnace at 550^oC for 3-5 hours. The crucibles were cooled in a desiccator and weighed. To ensure complete ashing, they were heated again in the furnace for half an hour, cooled and weighed. This was repeated consequently till the weight became constant. The percentage ash content was obtained as follows.

$$\% \text{ Ash content} = \frac{\text{Weight of ash x 100}}{\text{Weight of sample used}}$$

3.6.4 Crude fat content

The n-hexane (b.p 65-70°C) was used for determination of crude fat as described by AOAC, (2000). The extraction flask was heated to constant weight at 105^0C for 1 hour then cooled to room temperature in a desiccator. About 5g of each of the dried powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas weighed into the extraction thimble and stoppered with a cotton wool. The thimble containing thepowdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas dried at 105°C for 1 hour then placed in the extraction apparatus. Hexane was filled to two thirds of the flask, the apparatus was set up, and extraction started. The temperature was controlled so that 80 condensed drops fall down on the thimble per minute. After extraction, hexane was allowed to drain down the flask then the thimble was retrieved quickly with forceps. The collected hexane was then evaporated on a water bath using rotary vacuum evaporator (Bibby Sterilin Ltd, RE 100B, UK). The flask containing extracted fat was dried at 105^0C for 1 hour, and then cooled to room temperature in a desiccator. The weight was finally taken and the % fat calculated as follows:

$$\% \text{ Fat} = \frac{\text{Weight of the extracted fat}}{\text{Weight of the sample used}} \times 100$$

3.6.5 Crude protein

The crude protein was determined using micro Kjeldahl method as describe by AOAC, (2000). Approximately1g of the each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas weighed and transferred to a separate digestion flask. 5 g of catalyst potassium sulphate (K_2SO_4) and 0.5g of copper (II) sulphate ($CuSO_4$) was then added. Fifteen milliliters of concentrated sulphuric acid (H_2SO_4) was added and heated in a MRK fume hood or draft chamber (Mitamura Riken Kogyo Inc. Japan) first with small flame then increasing the temperature gradually. This converted any organic nitrogen to ammonium sulphate ($(NH_4)_2SO_4$) in solution. Each of the obtained contents was heated until the liquid was colorless and continued heating thereafter for another 1 hour. After digesting, the contents of each of the the flask was cooled to room temperature then transferred to a 100mL volumetric flask and filled up to the mark with water. A 10mL aliquot of the

digestion solution was taken into distilling flask and 15mL of 40% sodium hydroxide (NaOH) was added to decompose $(NH_4)_2SO_4$ to alkaline. The ammonia liberated was distilled with 25 mL of 4% boric acid and 2 drops of indicator, which was placed in the receiver flask. The burner was placed under the boiling flask and adjusted so that 60-80 mL of the distillate collects in about 20-30 minutes. Each of the obtained distillate was titrated with 0.02N hydrochloric acid (HCl) solution using double indicator solution (methylene red and methylene blue). The colour change from blue to dirty green to orange indicated the end point. Reagent blank determination was also done. The percentage nitrogen was calculated as follows:

Nitrogen (%) = (V1 – V2) x N x f x 0.014 x 100/v x 100/w

Where:

V1 = Titre for sample (mL)

V2 = Titre for blank (mL)

N = Normality of standard HCl solution (0.02)

F = factor of standard HCl solution

v = volume of diluted digest taken for distillation (10mL)

w = weight of sample taken.

The crude protein content was then obtained by multiplying the percentage nitrogenous matter by a factor of 6.25

3.6.6 Crude fiber

Fiber content was obtained from the loss in weight after ignition of dried residue remaining after digestion of fat-free samples under specified condition. This imitates the gastric and intestinal action in the process of digestion. Two (2) grams of moisture and fat free powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas treated with 200mL of 1.25% sulphuric acid (H_2SO_4). After filtration and washing, the residue was treated with 1.25% sodium hydroxide (NaOH), filtered, washed with hot water and then 1% nitric acid (HNO_3) and again washed with hot water. The residue was finally ignited and the ash weighed. The percentage crude fiber was obtained as described in the AOAC, (2000) as follows:

$$\% \text{ Crude fibre} = \frac{\text{Loss of weight after ignition}}{\text{Weight of sample used}} \times 100$$

3.6.7 Carbohydrate content

Carbohydrate was determined by subtracting the total ash content, crude fat, crude protein and crude fiber from the total dry matter content as described by AOAC, (2000).

3.6.8 Titratable acidity

Titratable acidity was determined as described by AOAC, (2000). Ten grams (10g) of each of the minced fresh peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas mixed with 200 mL of distilled water and boiled for 1 h. each of the obtained mixture was cooled, filtered and the filtrate transferred to a 250 mL volumetric flask and made up to the mark. 10 mL of the filtrate was titrated with 0.1 M NaOH using 1% phenolphthalein solution as indicator. The results were expressed as percentage of citric acid

3.6.9 Reducing sugars

The reducing sugar content was determined as describe by AOAC, (2000). One gram (1g) of each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas mixed with 1.0 mL of copper reagent comprised of 4.0 mL of KNa tartarate:Na_2CO_3: Na_2SO4: $NaHCO_3$ (1:2:12:1.3, w/w, dissolved in 75 mL distilled water) and 1.0 mL of $CuSO_4.5H_2O$: Na_2SO_4 (1:9, w/w, dissolved in 50 mL distilled water) and 1.0 mL of arsenomolybdate reagent (25 g ammonium molybdate in 450 mL H_2O + 21 mL H_2SO_4 + 3g $Na_2HASO_4.7H_2O$ in 25mL H_2O) in a test tube. Each of the test tubes was vortexed thoroughly and boiled in a water bath for 10 minutes. After cooling, the tube was adjusted to 25 mL with distilled water. After 10 minutes, each of the diluted peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitsextracts was filtered through a filter paper and the absorbance was measured at 600 nm in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). Distilled water was used as a blank and glucose as standard for calibration.

3.6.10 Determination of mineral composition

Minerals were determined after dry ashing according to the method described by AOAC, (2000). The total ash obtained after ashing powdered pulp and seeds of *A. muricata* and *A. squamosa*fruitswas boiled with 10mL of 20% hydrochloric acid

(HCl) in a beaker and then filtered into a 100 mL standard flask then made up to the mark with deionized water. The minerals Na, Ca and K were determined from the resulting solution using emission flame photometer. The standard solutions of 100 mg/mL of Na and K were prepared from NaCl and KCl salt. The levels of Mg, Fe, Zn, Mn and Cu were determined through atomic absorption spectrophotometer (AAS) using standard methods. Working standards of 0, 2, 4, 6, 8 and 10mg/L were prepared from the standard solution by serial dilution. Each standard was aspirated into the flame photometer or AAS and its emission and absorption, respectively was recorded to prepare a standard curve. The same procedure was applied for each of the prepared pulp and seeds of *A. muricata* and *A. squamosa*fruits solutions for each of the extracts and results recorded. The mineral concentrations were calculated from the standard curve.

3.6.11 Fatty acid composition

Fatty acid composition was determined according to AOAC, (2000) method. Each of the extracted fat (section 3.6.4) from pulp and seeds of *A. muricata* and *A. squamosa*fruitswas dissolved in 4 mL hexane, transferred to a conical flask and evaporated on a hot plate. 4 mL of 95% methanolic HCl solution was added and heating done under reflux for 1½ hour. Each of the obtained digest was cooled under tap water. Methyl esters were extracted by transferring each of the resulting solution into a separating funnel and 4 mL of hexane added. Each of the obtained funnel contents was placed on a shaker and shaken vigorously at room temperature and let to stand for 10 minutes (Shaker Model KS 250 basic, Germany). The hexane layer was collected, the aqueous layer returned and extraction repeated again. Each of the hexane fractions was combined and washed with 3-4 portions of distilled water to remove acid. Anhydrous sodium sulphate (Na_2SO_4) was added in sufficient quantities to remove water. The obtained filtrates were concentrated using nitrogen gas to about 0.5 mL and the sample was injected into the gas chromatography alongside with standards.

3.6.12 Vitamin C (Ascorbic acid)

3.6.12.1 Reagent preparation

The amount of vitamin C (Ascorbic acid) in each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas determined by redox titration method as described by AOAC, (2000). This involved the reaction between ascorbic acid in the sample and 2,6-dichloroindophenol (DCIP). 10% Trichloroacetic acid (TCA) reagent was prepared by dissolving 10g of TCA in 100 mL of distilled water. Standard ascorbic acid (1mg/mL) was then prepared. The DCIP solution was prepared by dissolving 0.25g of DCIP in about 500mL of distilled water. 0.21g of $NaHCO_3$ was then added and dissolved to the DCIP solution. The resulting solution was finally diluted to 1L with distilled water to make approximate concentration of 250mg DCIP/L.

3.6.12.2 Assay of vitamin C (Ascorbic acid)

Five grams of each of the powered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas ground in a mortar with acid washed sand using a suitable volume of 10% TCA. It was then transferred into a 100 mL volumetric flask and made up to the mark with the 10% TCA reagent, then immediately filtered through a fluted filter paper. Ten millimeters (10 mL) of the sample, (0.05mg/mL) was pipetted into a 100mL conical flask. Two millimeters of the H_2SO_4 mixture and about 25mL of distilled water were then added to the flask. The flask was swirled to mix the solution. A 50 mL burette was filled with the DCIP solution, which was then used to titrate the sample solution until a permanent light red or pink color appeared. The volume of DCIP needed to oxidize all of the ascorbic acid was recorded and the procedure was repeated. A blank determination was also carried out with 10% TCA. The ascorbic acid content was calculated using the dye factor. This was determined by the titration of the standard ascorbic acid solution with DCPIP dye using the balanced equation for the oxidation-reduction reaction between ascorbic acid and DCIP. Vitamin C content was then obtained as follows:

$$\text{Vitamin C content (mg/100g)} = \frac{(A-B)\ C}{W} \times 100$$

Where:

A= Volume in mL of the Indophenol solution used for sample titration

B= Volume in mL of the indophenol solution used for sample blank titration

C= Mass in mg of ascorbic acid equivalent to 1 mLof indophenol standard solution

W= weight in g of sample taken for sample preparation.

3.6.13 Tocopherol

The levels of tocopherol in each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruits was estimated as reported by AOAC, (2000). About 2.5 g of each of the powdered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas homogenized in a small volume of 0.1N H_2SO_4 and the volume made up to 50 mL by adding 0.1N H_2SO_4 slowly, without shaking and the contents allowed to stand overnight. The contents of the flask were then shaken vigorously and filtered through Whatman No.1 filter paper and aliquots of the filtrate were used for the estimation of tocopherol. The plant extract (test), standard and water of 1.5 mL (blank) were pipetted out into three different centrifuge tubes. To all the tubes, 1.5 mL each of ethanol and xylene were added, stoppered, thoroughly mixed and centrifuged. After centrifugation, the xylene layer was transferred into another tube, taking care not to include any ethanol or protein. To 1.0 mL of xylene layer, 1.0 mL of 2, 2-dipyridyl reagents was added, stoppered and mixed. This reaction mixture was taken in the spectrophotometric cuvettes and the extinctions of the test and the standard were read against the blank at 460 nm in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). Then, in turn, beginning with the blank, 0.33 mL of ferric chloride solution was added, mixed well and after exactly 15 minutes, the optical densities (ODs) of the tests and the standard were read against the blank at 520 nm in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). The levels of tocopherol were calculated using the formula;

Tocopherol (mg) = {(A520 - A450)/(StdA520)} x (0.29 x 15)

3.6.14 Total carotenoids analysis and quantification

3.6.14.1 Extraction of carotenoids from different parts of Annonaceae fruits

The extraction of carotenoids for HPLC analysis was performed as describe by Samuagam *et al*., (2014) with modification. Brief, about 200 mg of each of the

powered peel, pulp and seeds of *A. muricata* and *A. squamosa*fruitswas weighed and transferred into 2 mL Eppendorf tubes containing 2 beads. In each tube, 50 µL of 3 mg/mL magnesium carbonate ($MgCO_3$) suspension and 300 µL of tetrahydrofuran (THF) added. Homogenization was done in FastPrep machine set at 5.0 speed for 45 seconds followed by incubation at 4^oC for 20 minutes in dark. Also added to the two tubes was 300 µL of MeOH before homogenization and incubation at 4^oC for 10 minutes. The homogenate was transferred to Spin-X filter, centrifuged for 1 minute at a speed of 4,000 rpm set at 4^oC. Two equal volume (150 µL THF and 150 µL methanol) were pippeted into original extraction tube then followed by vortexing. All THF/methanol/debris was pipetted into spin-X filter and centrifuged again. The filtrate was then transferred to an empty clean 2 mL tube before 450 µL of THF was added to debris pellet in Spin-X filter and incubated on ice for 15 minutes in the dark and centrifuged at maximum speed for 5 minutes. The filtrates were combined and 375 µL petroleum ether and 150 µL of 25% NaCl added to each combined extract and vortexed vigorously before being centrifuged at a maximum speed for 3 minutes at 4^oC for phase separation. The upper phase was then transferred to new 2 mL tube. Second extraction was done with 500 µL petroleum ether before the upper phase being carefully removed and mixed with the initial filtrate. To concentrate the petroleum ether extract to near dryness, the extract was rotor evaporated at 45^oC for 20 minutes. The dried extracts were stored in the Nitrogen (N_2) refrigerator at -80^oC (dark) whenever samples were not analyzed by HPLC immediately after extraction. To resuspended carotenoids; 500 µL ethyl acetate was added then incubated at room temperature for 15 minutes followed by vortexing and filtering the suspension filtered through 0.45 µM nylon syringe filter (Cameo 3N syringe filter, GE Water & Process Technologies, USA).

3.6.14.2 HPLC analysis using YMC C_{30} column

Carotenoid analysis was carried out in a defined YMC C_{30} column using a Dionex HPLC machine (P680 HPLC pump, ASI-100 Automated Sample Injector; PDA-100 Photo Array Detector) and Chromeleon (v6.40 software package). Carotenoids separation was based on polarity gradient (0-5 min 100% methanol: 0.1% ammonium acetate; 6-25 min 4% methanol: ammonium acetate and 96% methyl-tert-

butyl ether; 26-35 ramp to 100% methanol: ammonium acetate) through a guard cartridge (YMC carotenoid S-5, 4.0 mm x 20 mm DC guard, Waters), C_{30} column (YMC carotenoid S-5, 4.6mm x 250mm, Waters) assembly.

3.6.14.3 Identification and quantification of carotenoids

Peak identification was performed as described by Alba *et al*., (2005) with modification where peak areas of the standards were determined at the respective wavelengths providing maximum absorbance. The carotenoids identification was based on the retention time and spectra comparison using respective standards analyzed under identical analytical conditions. Standard Calibration curves for each standard was generated automatically and used to quantify carotenoids.

3.7 Antioxidant assays

3.7.1 *In vitro* antioxidant assays

3.7.1.1 Determination of superoxide scavenging activity

The superoxide (O_2^-) scavenging of *A. muricata* and *A. squamosa*pulp methanol and aqueous extracts was determined based on a riboflavin-light-nitrobuletetrazolium (NBT) system as described by Beauchamp & Fridovich, (1971). The reaction mixture contained 0.5 mL of 50mM phosphate buffer (pH 7.6), 0.3 mL of 50 mM riboflavin, 0.25 mL of 20mM phenazinemethosulfate (PMS) and 0.1 mL of 0.5 mM NBT. Varying concentrations (15-500 µg) of 1 mL of each of the extracts (AMPUAE, AMPUME, ASPUAE and ASPUME) were added to this mixture. Reaction was initiated by using a fluorescent lamp to illuminate the reaction mixtures containing the different concentrations of extracts. After 20 minutes of incubation, the absorbance was measured at 560 nm in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). The absorbance of the control was determined by replacing the sample with methanol. Ascorbic acid was used as a positive control. The percentage inhibition of O_2^- generation was calculated using the formula:

% inhibition = (absorbance of control - absorbance of test sample)/absorbance of control x 100.

3.7.1.2 Determination of hydroxyl scavenging activity

The Hydroxyl (OH^-) scavenging activity of *A. muricata* and *A. squamosa*pulp methanol and aqueous extracts was assayed as described by Halliwell&Gutteridge, (1981). The reaction mixture contained 500 mL of 2.8 mM deoxyribosein a 200 mL of 50mM KH_2PO_4-KOHbuffer (pH 7.4), 200 mL of 100 mM premixed $FeCl_3$and 100mM EDTA solution (1:1; v/v) and 100 mL of 200 mM H_2O_2, with 100 mL of the extracts (AMPUAE, AMPUME, ASPUAE and ASPUME) in different concentrations (15-500 µg). Adding 100 mL of 300 mM ascorbate triggered the reaction, and the mixture was incubated for 1 hour at 37°C. A solution of TBA in 1 mL (1%; w/v) of 50 mM NaOH and 1 mL of 2.8% (w/v; aqueous solution) TCA was added to start the reaction. The mixtures were incubated for 20 minutes in a boiling water bath and then cooled. The absorbance was measured at 532 nm with a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). The absorbance of the control was determined by replacing the sample with methanol. Ascorbate was used as a positive control. The OH^- scavenging activity of the extracts was calculated using the formula:

% inhibition = (Absorbance of control - Absorbance of test sample)/Absorbance of control x 100.

3.7.1.3 Determination of lipid peroxidation inhibition

Lipid peroxidation inhibition of *A. muricata* and *A. squamosa*methanol and aqueous extracts was determined as described by Ohkawa *et al*., (1979). A 10% (w/v) BALB/c mice liver homogenate was prepared using ice cold 0.15M KCl in a Teflon tissue homogenizer, and the protein content was adjusted to 500 mg/mL. In the control system comprising of 1 mL of tissue homogenate, lipid peroxidation was initiated by adding 25 mM $FeSO_4$, 100 mM ascorbate and 10 mM KH_2PO_4. The volume was made up to 3 mL with distilled water and incubated at 37°C for 30 minutes. In the test system, 1 mL of the homogenate was incubated with different concentrations (15-500 µg/mL)of extracts (AMPUAE, AMPUME, ASPUAE and ASPUME). The extent of inhibition of lipid peroxidation was evaluated by estimating the level of thiobarbituric acid reactive substances (TBARS) through measuring the absorbance at 532 nm using a UV-vis spectrophotometer (UV mini

1240 model, Shimadzu Corp., Kyoto, Japan) against a regent blank. Ascorbic acid was used as a positive control. The percentage of inhibition of lipid peroxidation was calculated by using the formula:

% inhibition = (Absorbance of control - Absorbance of test sample)/Absorbance of control x 100.

3.7.1.4 Determination of nitric oxide scavenging activity

Nitric oxide (NO) scavenging activity of *A. muricata* and *A. squamosa*methanol and aqueous extracts was determined by the use of Greiss reagent as described by Green *et al*., (1982). One (1) mL 5 mM Sodium nitroprusside in phosphate buffered saline was mixed with 3 mL of different concentrations (15-500 μg/mL) of extracts (AMPUAE, AMPUME, ASPUAE and ASPUME) dissolved in methanol and incubated at 25°C for 150 minutes. The samples were then allowed to react with Greiss reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was measured at 546 nm using a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan). Ascorbic acid was used as a positive control. The experiments were repeated in triplicate. The percentage scavenging of NO radical activity was calculated using the formula:

% inhibition = (Absorbance of control - Absorbance of test sample)/Absorbance of control x 100.

3.7.1.5 Determination of total reducing power

The reducing property of *A. muricata* and *A. squamosa*methanol and aqueous extracts was determined by assessing the ability of the extracts to reduce $FeCl_3$ solution as described by Yen & Chen, (1995). One (1) mL of different concentration(25-800 μg/mL) of each extracts (AMPUAE, AMPUME, ASPUAE and ASPUME)in water were mixed with 2.5 mL of 0.2 M Na_3PO_4 buffer (pH 6.6) and 2.5 mL of 1% potassium ferric cyanide($K_3Fe(CN)_6$). The mixture was incubated at 50°C for 30 minutes in a water bath. 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture to stop the reaction, and the mixture was then centrifuged at 3000 rpm for 10 minutes. 2.5 mL of the supernatant was mixed with 2.5 mL distilled

water and 0.5 mL of 0.1% $FeCl_3$. The colour developed was read at 700nm using a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan) against a regent blank. The increased absorbance of the reaction mixture is directly proportional to the reducing power of the sample. The reducing power was expressed as microgram equivalents of ascorbic acid as standard.

3.7.1.6 Determination of total antioxidant activity

The total antioxidant activity (TTA) of *A. muricata* and *A. squamosa*methanol and aqueous extracts was evaluated as described by Prieto *et al*., (1999). One (1) mL vitamin E (tocopherol) equivalent to 500 mg was combined with reagent solution (0.6M H_2SO_4, 28 mM Na_2SO_4 and 4mM ammonium molybdate) and the extracts (AMPUAE, AMPUME, ASPUAE and ASPUME). In the case of the control blank, methanol was used in place of the sample. The tubes were capped and incubated in a boiling water bath at 95°C for 60-90 minutes. The samples were then cooled at room temperature, and the absorbance was measured at 695 nm in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan) against a regent blank. The total antioxidant activity was expressed as microgram equivalents of vitamin E.

3.7.2 *In vivo* antioxidant assays

The BALB/c mice were divided into seven groups each group consisting of six mice as follows; Group I: Negative control non infected (NI); Group II: Positive control infected non treated (INT) (50 mg/Kg bwt DMSO); Group III: 20 mg/Kg bwt of pentostam, Group IV: 50 mg/Kg bwt of ASPUAE/AMPUAE, Group V: 50 mg/Kg bwt of ASPUME/AMPUME, Group VII: 50 mg/Kg bwtASPUAE/AMPUAE + 20 mg/Kg bwt pentostam, Group VII: 50 mg/Kg bwtASPUME/AMPUME + 20 mg/Kg bwt pentostam.After the infected animals had developed lesions, the respective groups received oral treatments of the extracts, Pentostam and a combination of the extracts and pentostam for a period of 6 to 8 weeks. After the experimental period, all mice were decapitated and sacrificed, whole blood and serum collected forassays of markers of hepatic damage; Aspartate amino transferase (AST), Alanine amino transferase (ALT), Lactate dehydrogenase (LDH), Creatine Kinase (CK); non enzymatic antioxidants; lipid peroxidation (LPO), Thiobarbituric

acid reactive substances (TBARS) and nitric oxide (NO), enzymatic antioxidantsGlutathione (GSH), Glutathione-s-transferase (GST), Glutathione peroxidase (GSHPx), Superoxide dismutase (SOD) and Catalase (CAT) using an automated Retroflon® Plus automated analyzer as described by Wheeler *et al.*, (1990).Thesizes of Left Footpads(LHFD) swellings were measured using a direct reading using a Verniercaliper whereas the lesion sizes were estimated using the method of Nolan and Farrell, (1987). Liver and spleen impression smears were used to quantitate the parasite loads as described by Bradley and Kirkley, (1977)**.**

3.8 Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet

BALB/c mice same sex were equally and randomly allocated into experiment groups assigned to groups of six mice per group as follows; Non-Infected fed with Rat pellets (NI-RP), Infected Non-Treated control fed with Rat pellets (INTC-RP), Infected Treated Control fed with Rat pellets (ITC-RP), Non-Infected fed with Annonaceae fruit pulp pellets (NI-AFPP), Infected Treated Control fed with Annonaceae fruit pulp pellets (ITC-AFPP), Infected Non-Treated Control fed with Annonaceae fruit pulp pellets (INTC-AFPP). After the different experimental exposures, the mice were closely monitored on a daily basis for agility, hair ruffling, appetite, vomiting, urine colour, skin turgor, ocular tension, limb paralysis, convulsions and roll-over movements as described by Carvalho *et al*.,(2006). Body weight and temperature were measured on a weekly basis. After lesion have developed, density of parasites was determined by counting the number of amastigotes form of parasite from smears made from ulcer stained with Leishman's stain as described by Schnur *et al.,* (1973). The infected footpadswere measured using a direct reading Vernier caliper and lesion size calculations done using the method of Nolan & Farrell, (1987). Means of weekly readings were calculated to facilitate comparison of lesion progression. Growth performance and nutrient utilization was assessed at the end of the experimental period as (12 weeks) described by Kumar *etal*., (2012b) as follows; Body mass gain (BMG) = [(FBM) - (IBM)]/IBM] x 100, Specific growth rate (SGR% per day) = [(FBM) - (IBM)/number of trial days] x 100, Metabolic growth rate (MGR $g/kg^{0.8}/day$) =

(BMG)/{[(IBM/1000)$^{0.8}$ + (FBM/1000)$^{0.8}$]/2}/duration of the trial days, Feed conversion ratio (FCR) = Dry feed fed (g)/BMG (g), Protein efficiency ratio (PER) = fresh BMG (g)/Crude Protein (CP) fed (g), Protein productive value (PPV)% = [(final mice body protein in g – initial mice body protein in g)/total protein consumed in g] x 100, Food consumption (FC) per day = Total amount of food consumed by mice in the experimental period/duration of the trial days and Apparent lipid conversion (ALC)% = [(final mice body lipid in g - initial mice body lipid, g)/total crude lipid consumed in g] x 100. At the end of the experimental period (90 days), the animals were fasted for about 4 hours with free access to water and sacrificed by inoculation with 100µL of pentabarbitone sodium (Sagatal®). Two (2) mL of blood sample was collected for determination of haematological parameters (WBC, RBCs, PCV, HBG, MCV, MCH and MCHC), biochemical parameters parameters (ALT, AST, ALkP, uric acid, cholesterol and glucose), renal and liver functions and blood metabolites using a Retroflon® Plus automated analyzer. Liver and spleen impression smears were used to quantitate the parasite loads as described by Bradley and Kirkley, (1984)**.**

3.9Antileishmanialand safety assays

3.9.1 *In vitro* antileishmanial assays

3.9.1.1 Parasites

Metacyclic promastigotes of *L. major* (IDUB/KE/83=NLB-144 strain) maintained by cryopreservation and *in vitro* culture and periodically passaged in BALB/c mice at KEMRI were used. Parasites were maintained as described by Titus *et al*. (1994). Briefly *L. major* parasites were cultured in Schneider's insect medium supplemented with 20% Fetal Bovine Serum (FBS), glutamine (2 mM), Penicillin G (100 U/mL) and streptomycin (100 µg/mL). Stationary phase amastigotes were harvested in 7 days old cultures. Metacyclic promastigotes were isolated from stationary phase cultures by negative selection using peanut agglutination (Tonui & Titus, 2006).

3.9.1.2 Isolation of macrophagesfor macrophage sensitivity tests

Macrophage sensitivity test was performed as described by Alawa, (2008). Bone marrow, skin lesions or skin ulcers, spleen and lymph nodes cells were harvested from f individual healthy BALB/c mice by injecting 10 mL of bone marrow medium

into the isolated bones in a petri dish. Theextracted cell suspension was incubated at 37°C in 5% CO_2 / 95% air for 7-10days to enable growth, differentiation and adherence of macrophages. The macrophages were then harvested from the petri dishes by dislodging with a cell scraper after refrigerating for 15 minutes. Cell suspension was pooled and pelleted by centrifugation at 3000 rpm for 5 minutes. The cells were then washed twice with 10 mL of incomplete medium and finally resuspended in 10 mL complete medium. Determination of viable cells was done by mixing 15µL of cell suspension with trypan blue dye at a ratio of 1:1. This was loaded onto a haemocytometer and viable cells were counted. Cells were then adjusted to 1x10^{5} cells in appropriate volume of complete medium. Round sterile 13 mm glass coverslips were placed in each well of a 96-well tissue culture plate and the cell suspension was added into each well. Plates were incubated at 37°C in 5% CO_2 / 95% air for a minimum of 1 hour to permit adherence of macrophages.

3.9.1.3 Infection of Macrophages and drug susceptibility assay

This was used to assess the intracellular antileishmanial activity as described by Al-Musayeib *et al*., (2012) with modification as described by Alawa, (2008). Briefly, 1 mg of each of the extracts was weighed and dissolved in 1 mL of PBS and further diluted serially using PBS to obtain concentrations ranging from 0.25-1.0 mg/mL. 3×10^{4} macrophages were seeded in each well of a 96-well plate. After 2 days of outgrowth, 5×10^{5} amastigotes/well, was added and incubated for 2 hours at 37°C. Pre-diluted plant extracts from peel, pulp, and seeds of *A.muricata* and *A.suamosa* fruits were subsequently added, and the plates further incubated for 5 days at 37°C and 5% CO2. The contents of the wells were removed and the cells fixed in methanol and stained with 10% Giemsa stain. Percentage of infected cells was determined microscopically at 1000 magnification and results expressed as percentage (% infection) and percentage reduction (%suppression) of parasite burden as compared to untreated control wells (blank controls without plant extract).

3.9.2 *In vivo* antileishmanialassay

3.9.2.1 Animals

BALB/c mice (3 - 4 weeks old) obtained from Animal House Unit, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya were used. BALB/c mice were

maintained with standard rat pellets (Rate pellets®, Unga Feeds Ltd, Kenya) and *water ad libitum* and kept under standard temperature (26^0C and 60% humidity) and a natural light-darkness cycle throughout the experimental period.

3.9.2.2 Infection of BALB/c mice

BALB/c mice of either sex aged between 3-4 weeks infected of with *L. major* (IDUB/KE/83=NLB-144 strain) as described by Gamboa-Leon *et al*. (2007) were used. The mice were inoculated intradermally with $1x10^6$ metacyclic promastigotes of *L. major* at stationary phase in 40 µL sterile phosphate buffered saline (PBS) in the left footpads while the right footpads were left as a colateral control. Infected mice were kept for 3 to 4 weeks for symptomatic establishment of cutaneous leishmaniasis (CL). After lesion have developed, smears were made from ulcer stained with Leishman's stain and examined under oil immersion of light microscope to determine the density of parasites by counting the number of amastigotes form of parasite (Schnur *et al.,* 1973).

3.9.2.3 Antileishmanial activity screening

The *in vivo* antileishmanial activity screening of the extracts was carried out as describe by Mutiso *et al*., (2011) with modification. The infected mice were left for 3 to 4 weeks for lesion development after which they were divided into four treatment groups consisting of six mice per group as described as follows;Group I: Positive control infected non treated control (INTC) treated with 0.2 mL in 1 mg/mL (50 mg/Kg) DMSO of 5% PBS intraperitoneally (IP) daily, Group II: IT-Pentosam(0.2 mL of 1 mg/mL (20 mg/Kg) pentostam in 5% PBS intradermally (ID) daily,Group III:IT-Extracts (0.2 mL of 1 mg/mL (50 mg/Kg) tested extract in 5% PBS intraperitoneally (IP) daily) and Group IV: Negative control, none infected control (NIC).The mice were monitored on a daily basis for changes in clinical characteristics, body weight and parasite load was determined in any animal that died during the treatment period of 3 to 4 weeks

3.9.2.4 Lesion size and parasite load in liver and splenic impression smear

The infected footpads of all mice groups in different treatment groups were measured using a direct reading Vernier caliper and Left hand footpad (LHFD) sizes determined as described by were Nolan & Farrell, (1987). At the end of the

experimental period, the mice were sacrificed by inoculation with 100µL of pentabarbitone sodium (Sagatal®). Liver and spleen impression smear were made on clean microscopic slides. They were left to dry followed by fixation using absolute methanol. The fixed slides were immersed in a freshly prepared 5% Giemsa stain solution for 20 minutes then flushed with tap water and left to dry. The slides were examined under a compound microscope for enumerating the number of amastigotes per 1000 host nuclei. Bradley & Kirkley,(1977) formula was used to calculate the relative and total numbers of parasites in the spleen in Leishman-Donovan Units (LDU) and total LDU respectively**.**

3.9.3 Safety assays

3.9.3.1 *In vitro* toxicity assay

Normal mammalian cells; Vero cells (ATCC® CCL-81™) were obtained from KEMRI Animal Cell Culture laboratory, Kenya. The cells were routinely sub cultured and maintained in in Dulbecco's medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (50 IU/ml) /streptomycin (50 µg/mL) and L-glutamine (2 mM) at $37^{o}C$ in humidified atmosphere of 5% CO_2,/95% air. Cytotoxicity of the plant extracts was determined using the normal cell line; Vero cells (ATCC® CCL-81™) as described by Wabwoba *et al.*, (2010) with modifications. The cells were cultured for 72 hours at 37°C in 5% CO_2in a humidified incubator, harvested by trypsinization, pooled in a 50mL vial and 100µL cell suspensions, used to test toxicity by adding 150 µL of the highest concentration of the extract and serial diluting. The controls used were cells with no extract and medium alone. The percentage inhibition of cell growth relative to the control was evaluated colourimetrically using Alamar Blue assay where 10µL MTT reagent was added and aspirated off after 4 hours and then 100 µL of dimethylsulfoxide (DMSO) added and the plates shaken for 5 minutes. The absorbance was measured for each well at 562 nm using a micro-titer plate reader.

3.9.3.2 *In vivo* acute and sub-acute toxicity assay

The acute toxicity was determined in BALB/c mice using a modified method of Lorke, (1983) as describe by Adewole& Ojewole, (2009). The extracts were prepared by suspending them either 30% DMSO in distilled water. The mice were

fasted for overnight prior to dosing and randomly divided into five experimental groups each consisting of six mice per group. The mice were subjected stepwise, graded doses of extracts (100, 500, 1000and 2500 mg/Kg) orallydaily as describe by Adewole & Ojewole, (2009) as follows; Group I: Control (3 mL/Kg DMSO), Group II: 100 mg/Kg (Extracts), Group III: 500 mg/Kg (Extracts), Group IV: 1000 mg/Kg (Extracts) and Group V: 2500 mg/Kg (Extracts). After administration ofa single dose of the extract, the mice were then allowed free access to food and drinking water and observed continuously every 30 minutes for 3 hours, there after pobservations were made after 12 hours, 24 hours, 48 hours and finally 72 hours for general and neurological behaviors, autonomic profiles and mortality. The log dose-response plots were constructed from which the LD_{50} were estimated.

The same mice were further subjected to sub-acute toxicity assayas described by OECD, (2008). Changes in body weights were recorded weekly using a sensitive analytical balance during the dosing period. Animals were observed for clinical signs and mortality each day from the first day of dosing (D0) to the last day of experiment (D14). At the end of study period, the animals were extranguinated under Sagatal® anesthesia, blood collected by heart puncture in 2 tubes for hematological parameters (WBC, RBCs, PCV, HBG, MCV, MCH and MCHC), biochemical parameters (ALT, AST, ALkP, uric acid, cholesterol and glucose) and blood metabolites while some organs (Heart, kidney, liver, spleen, and stomach) were obtained for anatomical and histological analysis.

3.10 DNA-binding and DNA Topoisomerase I inhibitory activity assays

3.10.1DNA-binding interactions assays

The DNA-methyl green test was performed as described by Attard & Pacioni, (1996). 50 μL of the extracts were incubated with 200 μL of DNA-methyl green in the dark at 25^0C for 24 hours. The decrease in absorbance at 650 nm was calculated as a percentage of the untreated DNA-methyl green absorbance value. The median inhibitory concentration (IC_{50}) was calculated by regression analysis. Cucurbitacin E and Dexamethasone were used as potent and moderate positive controls, respectively

3.10.2 DNA Topoisomerase I Inhibitory Activity Assay

Calf thymus DNA Topo I was purchased from Sigma and assayed using TopoGen (Columbus, OH, U.S.A.) assay kitas described by Pastor & Cortes, (2002) with modification.Different concentrations (1, 5, 10, 25, 50 and 100 µM) of tested extracts were prepared using DMSO whereas positive control camptothecin (CPT) was prepared at the concentration of 10 µM. The DNA Topo I inhibitory activity was measured by assessing the relaxation of supercoiled pBR322 plasmid DNA. The reaction mixture (20 µL each), containing 35 mM Tris-HCI (pH 8.0), 72 mM KCI, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5 mM spermidine, 0.01% bovine serum albumin (BSA), 0.5 µg pBR322 plasmid DNA, 1.0 U calf thymus DNA Topo I, and 0.2 µL various concentrations of tested extracts, were incubated at 37 °C for 30 min. The reactions were terminated by adding dye solution containing 1% SDS, 0.02% bromophenol blue, and 50% glycerol. The mixtures were applied to 1% agarose gel and subjected to electrophoresis for 1 h in Tris-borate-EDTA buffer (0.089 mM). The gel was stained with Gelred and visualized under UV illumination, photographed with a Gel imaging system.

3.11 Ethical consideration

All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).The KEMRI's Animal Care and Use Committee (ACUC) and Scientific and Ethics Review Unit (SERU) approved all experimental protocol before using them. The ethical issues in animal studies, stress or surffering subjected to the animals through infection with *L. major* ad killing of the animals was addressed by treating the animals with drug/extracts and anaesthetizing them using pentabarbitone sodium (Sagatal®) respectively. For invasive procedure, mice were anaesthetized with (Sagatal®) and those animals, which died because of experimentation, were placed in biohazard bags and stored at -20°C until incineration.

3.12 Data analysis

The different data were analyzed using mean separation by GenStat discovery 14th Edition (Pyne *et al.*, 2011) through Fischer least significance difference.

Comparisons between two treatments were done by means of unpaired Student's t-test and for multiple means by Analysis of variance (ANOVA) followed by Duncan's test, $P < 0.05$ consideredstatistically significant.The results were expressed as mean ± standard error of the mean.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical analysis

4.1.1 Appearance and percentage yeilds of the extracts

Twenty-four (24) extracts were obtained pulp, peel and seeds of *A. muricata* and *A.squamosa*. The percentage yields calculated from each of the extracts are presented in table Table 4.1. From the results, it is clear that the methanol extracts gave highest percentage yields followed by aqueous and ethyl acetate extracts whereas the hexane extracts had the lowest percentage yields. The appearances of the different extracts of the pulp, peel and seeds of *A. muricata* and *A. squamosa*fruits varied in consistency ranging from oily (ethyl acetate and Methanol), caramelized (aqueous or water) and pelleted (hexane) as indicated in Table 4.1. The colour differed significantly ranging from Brownish black to blackis brown (seeds), greenish black (peels) and light brown to brownish yellow (pulps) (Plate 4.1). Further, in terms of consistency, the extracts were found to be sticky in the non-polar to not so polar solvents (Hexane and ethyl acetate) and non-sticky in the polar solvents (aqueous and Methanol)

Plate 4.1: Colour of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds packed in airtight containers.

Table 4.1: Percentage yield, colour and appearance of different crude extracts of *A. muricata*and *A. squamosa*pulp, peel and seeds

Plant Species	% Yield	Colour	Appearance
ASPUAE	14.00±1.04	Light brown	Pellets
ASPUME	24.14±1.27	Brownish yellow	Caramel
ASPUEAE	2.41±0.53	Brownish	Caramel
ASPUHE	1.54±0.16	Yellowish brown	Oily
ASPEAE	6.00±0.87	Golden brown	Pellets
ASPEME	25.06±1.09	Greenish black	Caramel
ASPEEAE	2.51±0.11	Greenish-White	Caramel
ASPEHE	1.63±0.17	Greenish black	Oily
ASSAE	9.00±0.94	Blackish brown	Pellets
ASSME	25.54±1.67	Brownish	Caramel
ASSEAE	2.55±0.49	Blackish brown	Caramel
ASSHE	1.97±0.42	Blackish brown	Oily
AMPUAE	7.00±1.05	Light brown	Pellets
AMPUME	28.69±1.48	Yellowish brown	Caramel
AMPUEAE	2.87±0.27	Yellowish brown	Caramel
AMPUHE	1.75±0.42	Yellowish brown	Oily
AMPEAE	19.00±1.57	Blackish brown	Pellets
AMPEME	22.33±1.29	Blackish green	Caramel
AMPEEAE	2.23±0.39	Greenish	Caramel
AMPEHE	2.13±0.75	Greenish black	Oily
AMSAE	7.00±1.33	Blackish brown	Pellets
AMSME	25.56±1.10	Brownish black	Caramel
AMSEAE	2.56±0.36	Blackish brown	Caramel
AMSHE	1.57±0.10	Blackish brown	Oily

Mean values (n=3) ± SEM

4.1.2 Qualitative phytochemical analysis

The presence and/or absence of phytochemicals (Alkaloids, Flavanoids, Glycosides, Anthocyanin, Saponins, Steroids, Tannin, Coumarins, Terpenoids and Phenols) in hexane, ethyl acetate, methanol and aqueous extracts from pulp, peel and seeds of *A. muricata* and *A. squamosa* using different screening tests is presented in Table 4.2 and Table 4.3.

Table 4.2: Qualitative phytochemical analysis in pulp, peel and seeds of *A. muicata* fruit

Phytochemical	AMPUAE	AMSAE	AMPEAE	AMPUME	AMSME	AMPEME	AMPUHE	AMSHE	AMPEHE	AMPUEAE	AMSEAE	AMPEEAE
Alkaloids	-	-	+	+	+	+	+	+	-	+	+	-
Flavonoids	+	+	+	+	+	+	+	+	-	+	+	-
Glycosides	-	-	+	-	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	-	+	+	-
Steroids	-	-	-	-	-	-	+	+	+	+	+	+
Fats and oils	-	-	-	-	-	-	+	+	+	+	+	+
Tannin	+	+	+	+	+	+	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids	-	+	-	-	+	-	-	+	+	-	+	+
Anthocyanin	+	+	+	-	-	-	-	+	+	-	+	+

Key: (+) = Present; (-) = Absent

Table 4.3: Qualitative phytochemical analysis in pulp, peel and seeds of *A. squamosa* fruit

Phytochemical	ASMPUAE	ASSAE	ASPEAE	ASPUME	ASSME	ASPEME	ASPUHE	ASSHE	ASPEHE	ASPUEAE	ASSEAE	ASPEEAE
Alkaloids	-	-	+	+	+	+	+	-	+	+	+	+
Flavanoids	+	+	+	+	+	+	+	+	+	+	+	+
Glycosides	-	-	-	-	+	-	+	+	-	+	+	-
Phenols	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	-	+	+	+	+	+	+	+	+	+
Fats and oils	-	-	-	+	+	+	+	+	+	+	+	+
Tannin	+	+	-	+	+	-	+	+	-	+	+	-
Coumarins	-	-	+	-	-	+	-	-	-	-	-	+
Terpenoids	+	+	+	-	+	+	+	+	-	+	+	+
Anthocyanin	-	+	-	-	+	-	-	+	-	-	+	-

Key: (+) = Present; (-) = Absent

4.1.3 Quantitative phytochemical analysis

The quantities of the identified phytochemicals obtained in this study are presented in Table 4.4. There was a significant difference ($P<0.05$) between the phytochemical contents of the pulps and seeds of *A. muricata* and *A. squamosa* fruits. Further, the phytochemical contents between pulps and seeds of either *A. muricata* or *A. squamosa* fruit significantly differed ($p<0.05$). The seeds had significantly higher content of phenols (118.23±2.38 mg/100g in *A. squamosa*), saponins (9.56±0.31 mg/100g in *A. squamosa*), terpenoids (19.04±0.05 mg/100g in *A. squamosa*) and alkaloids (23.43±0.60 mg/100g in *A. muricata*) than the pulps. Further, the contents of phenols (111.82±1.75 mg/100g), flavonoids (178.70±1.98 mg/100g in *A. muricata*) and tannins (63.63±1.86 mg/100g) in the pulp of *A. muricata* were significantly higher than in pulp of *A. squamosa* with the opposite relationship observed in the seeds. However, the seeds of *A. squamosa* had higher content of phenols (118.23±2.38 mg/100g). Low content of glycosides, terpenoids and steroids were observed in pulp and seeds of *A. muricata* and *A. squamosa*. However, the seeds of *A. squamosa* had moderate quantity of terpenoids (19.04±0.05 mg/100g).

Table 4.4: Quantitative phytochemical analysis of pulp and seeds of *A. muricata* and *A. squamosa*fruits

Phytochemical	Phytochemical concentration in different plant part and species (mg/100g DWB)			
	A. Squamosa		*A. muricata*	
	Pulp	Seed	Pulp	Seeds
Alkaloids	14.69^{c}±0.32	7.69^{b}±0.32	2.34^{a}±0.12	23.43^{d}±0.60
Flavonoids	26.06^{a}±0.60	48.31^{b}±1.07	178.70^{d}±1.98	80.69^{c}±1.78
Phenols	3.71^{b}±0.27	118.23^{c}±2.38	111.82^{d}±1.75	45.08^{b}±1.16
Saponins	6.05^{a}±0.24	9.56^{b}±0.31	0.61^{c}±0.08	4.77^{d}±0.10
Tannins	30.52^{d}±5.03	16.86^{c}±0.63	63.63^{b}±1.86	1.14^{a}±0.03
Glycosides	0.58^{a}±0.04	0.48^{a}±0.07	0.38^{c}±0.10	0.44^{ab}±0.11
Terpenoids	5.87^{b}±0.04	19.04^{a}±0.05	4.87^{b}±0.77	6.57^{b}±1.24
Steroids	0.46^{a}±0.03	0.26^{b}±0.03	0.49^{a}±0.67	0.44^{a}±0.08

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05).

4.2 Proximate, nutritional compositional and antioxidant analysis

4.2.1 Preliminary proximate compositional analysis

Preliminary proximate compositional analysis revealing the percentages of dry mater, ash content, moisture content, crude fiber, crude protein, crude fats and carbohydrates, reducing sugars, TSS and TBA are presented in Table 4.5. The dry matter, moisture content, ash content and crude proteins in the pulp, peel and pulp of *A. muricata* fruit compared to *A. squamosa*fruitdid not differ significantly ($P>0.05$). However, a significant difference ($P<0.05$) was observed between the pulp, peel and seeds as exemplified by dry matter in *A. squamosa* peel (97.95±0.15 mg/100g)against *A. muricata* pulp (93.09±0.42 mg/100g), moisture content in *A. squamosa*pulp (82.38±1.61mg/100g) against *A. muricata* seeds (44.01±6.93 mg/100g) and crude protein in *A. muricata* seeds (44.01±6.93 mg/100g) against *A. muricata* peel (3.75±0.98 mg/100g). The fiber content, crude fats, oil content, carbohydrates, reducing sugars, TSS, TBA, ascorbic acid and tocopherol differed significantly ($P<0.05$) between *A. muricata* and *A. squamosa* fruits and also between in the pulp, peel and seeds of the fruits (Table 4.5). Crude fats and oil contents were significantly high in the seeds, carbohydrates, reducing sugars, TBA, ascorbic acid and tocopherol were significantly high in the pulps whereas TSS was significantly high in in the pulps (Table 4.5).

Table 4.5: Proximate composition analysis of pulp, peel and seeds of *A. muricata*and *A. squamosa*fruits

Parameter	Proximate percentage composition (mg/100g)					
	A. squamosa			*A. muricata*		
	Pulp	Seeds	Peel	Pulp	Seeds	Peel
Dry matter (%)	$94.51^{a}\pm0.82$	$95.31^{b}\pm0.01$	$97.59^{c}\pm0.15$	$93.09^{a}\pm0.42$	$94.94^{b}\pm0.80$	$95.08^{c}\pm0.23$
Moisture	$82.38^{b}\pm1.61$	$70.02^{a}\pm0.17$	$42.38^{b}\pm1.61$	$81.09^{b}\pm0.67$	$82.38^{b}\pm1.6$	$41.02^{c}\pm0.70$
Ash content	$2.92^{b}\pm0.31$	$3.02^{b}\pm0.40$	$2.88^{b}\pm1.75$	$8.93^{c}\pm0.69$	$0.89^{a}\pm1.57$	$2.96^{b}\pm0.93$
Crude Protein	$26.25^{b}\pm2.15$	$31.28^{c}\pm5.31$	$4.36^{a}\pm0.66$	$35.04^{b}\pm3.18$	$44.01^{c}\pm6.93$	$3.75^{a}\pm0.98$
Fiber content	$43.84^{c}\pm4.12$	$38.69^{a}\pm13.83$	$52.20^{b}\pm1.10$	$72.95^{c}\pm0.86$	$40.48^{a}\pm1.21$	$50.03^{b}\pm1.81$
Crude Fat	$4.30^{a}\pm0.39$	$19.04^{b}\pm4.63$	$4.08^{a}\pm0.33$	$1.89^{a}\pm0.62$	$17.83^{b}\pm9.49$	$4.99^{a}\pm2.01$
Oil Content	26.13±1.05	39.12±2.58	18.58±1.87	33.67±1.97	48.57±2.07	25.97±1.48
Carbohydrate	$38.24^{a}\pm2.18$	$29.88^{b}\pm1.63$	$36.62^{c}\pm1.43$	$2.42^{d}\pm0.88$	$30.68^{b}\pm6.92$	$32.19^{c}\pm4.00$
Reducing sugars	$7.70^{c}\pm0.12$	$0.35^{d}\pm0.77$	$0.19^{a}\pm0.12$	$3.13^{e}\pm1.01$	$0.17^{a}\pm0.02$	$0.57^{b}\pm0.05$
Total soluble solids	$16.77^{d}\pm0.09$	$19.67^{c}\pm1.47$	$28.16^{a}\pm2.47$	$7.21^{e}\pm1.67$	$17.58^{d}\pm1.08$	$26.57^{ab}\pm1.23$
Titratable acidity	$0.18^{a}\pm0.01$	$0.05^{b}\pm0.01$	$0.01^{c}\pm0.00$	$0.78^{d}\pm0.05$	$0.21^{a}\pm0.01$	$0.09^{e}\pm0.01$
Ascorbic acid	$19.60^{a}\pm2.77$	$13.75^{c}\pm0.17$	ND	$37.24^{b}\pm1.77$	$21.85^{a}\pm0.04$	ND
Tocopherol	$17.42^{a}\pm1.25$	$10.15^{c}\pm0.13$	ND	$29.66^{b}\pm1.07$	$16.55^{a}\pm0.19$	ND

Mean values (n=3) ± SEM. Means with different superscript letters within a row are significantly different ($P < 0.05$)

4.2.2 Fatty acid composition

The fatty acid profile of *A. squamosa* and *A. muricata* fruit pulp and seeds showed the presence of saturated fatty acids (SFA), Caprylic (C8:0), Capric (C10:0) (C12:0), Myristic (C14:0), Lauric Palmitic (C16:0) and Stearic (C18:0), monounsaturated fatty acids (MUFA); Oleic (C18:1) and polyunsaturated fatty acids (PUFA); Linoleic (C18:2) and Linolenic (C18:3) as presented in Table 4.6. Unsaturated fatty acids (USFA) were significantly in higher amounts than the SFA attributed to the presence of high contents of the three USFA; linoleic acid (500.16±17.12mg/100g in *A. muricata* seeds), linolenic acid (196.67±17.82mg/100g in *A. muricata* seeds) and oleic acid (75.08±7.83mg/100g in *A. muricata* seeds). Linoleic acid was the major PUFA whereas oleic acid was the major MUFA with significantly higher values inseeds. The predominant SFA was palmitic acid with 457.73±30.16 mg/100g and 349.18±14.31 mg/100g inpulp and seeds of *A. muricata* respectively. The amount of fatty acids in *A. muricata* pulp and seeds were significantly higher ($P < 0.05$)than in the pulp andseeds of *A. squamosa*.

Table 4.6: Fatty acids composition of pulp and seeds of *A. muricata* and *A. squamosa* fruits(mg/100g DWB)

Fatty acid	*A. muricata*		*A. squamosa*	
	Pulp	Seed	Pulp	Seeds
Caprylic (C8:0)	0.01^{c}±0.00	21.46^{a}±5.81	5.87^{b}±0.24	0.27^{c}±0.07
Capric (C10:0)	8.21^{a}±0.83	1.67^{b}±0.14	0.84^{b}±0.09	10.51^{a}±1.76
Lauric (C12:0)	3.53^{ab}±1.46	5.13^{a}±0.27	2.46^{b}±0.32	0.89^{c}±0.46
Myristic (C14:0)	90.14^{b}±8.01	211.47^{a}±3.65	34.94^{c}±0.40	150.44^{ab}±17.68
Palmitic (C16:0)	457.73^{a}±30.16	349.18^{b}±14.31	208.01^{c}±7.03	213.31^{c}±2.08
Stearic (C18:0)	37.67^{b}±5.93	34.27^{b}±0.41	18.47^{c}±0.08	59.15^{a}±1.82
Linoleic (C18:2)	372.45^{b}±24.34	500.16^{a}±17.12	80.13^{d}±3.16	173.22^{c}±8.97
Linolenic (C18:3)	100.12^{b}±2.39	196.67^{a}±17.82	67.33^{c}±10.03	129.99^{b}±2.86
Oleic (C18:1)	38.65^{b}±6.97	75.08^{a}±7.83	1.07^{c}±0.00	50.80^{c}±0.10

Mean values (n=3)± SEM. Means with different superscript letters within a row are significantly different ($P < 0.05$)

4.2.3 Mineral elements composition

The pulp and seeds of *A. muricata* and *A. squamosa* revealed the presence of different minerals elements (Table 4.7). The pulp and seeds of *A. muricataand A. squamosa* fruits revealed the presence of K, Na, Cu, Mg and P. Other mineral elements were detected in trace amounts and included Cu, Fe, Zn and Se.

Generally,there were significant differences (p<0.05) in the concentration mineral elements between *A. muricataand A. squamosa* fruits as examplified by Ca (857.16±6.39 and 454.96±2.88 in *A. muricata* and *A. squamosa* pulp respectvelly). Further, a variation in mineral elements concentration was evident between the pulp and the seeds. Significantly higher concentration of most the identified mineral elements analyzed was recorded in the seeds than in the pulpin *A. squamosa* whereas in *A. muricata*, the pulp had higher mineral element concentration than the seeds.

Table 4.7: Mineral composition of different parts of *A. muricata*and *A. squamosa*fruits(mg/100g DWB)

Mineral	*A. muricata*		*A squamosa*	
	Pulp	Seeds	Pulp	Seeds
K	$322.25^{b}±13.11$	$354.58^{b}±2.17$	$48.03^{a}±0.60$	$23.31^{a}±0.73$
Na	$843.38^{b}±16.25$	$20.99^{b}±1.35$	$10.41^{a}±0.41$	$31.39^{a}±0.56$
Ca	$857.16^{b}±6.39$	$158.37^{b}±2.89$	$454.96^{c}±2.88$	$648.52^{c}±2.48$
Mg	$24.00^{a}±0.77$	$13.71^{a}±0.34$	$395.54^{c}±4.58$	$52.00^{c}±1.01$
P	$32.50^{c}±0.94$	$146.30^{a}±4.02$	$28.71^{b}±0.99$	$22.20^{b}±0.81$
Cu	$1.00^{c}±0.03$	$0.04^{c}±0.00$	$0.10^{a}±0.00$	$0.03^{a}±0.00$
Fe	$1.05^{b}±0.06$	$3.58^{b}±0.12$	$1.65^{a}±0.04$	$2.07^{a}±0.02$
Zn	$0.40^{b}±0.03$	$0.46^{b}±0.04$	$0.32^{a}±0.02$	$0.31^{a}±0.02$
Se	$0.79^{a}±0.07$	$1.51^{a}±0.03$	$0.87^{a}±0.01$	$1.46^{a}±0.04$

Means values (3) ± SEM. Means with different superscript letters within a row are significantly different (P < 0.05)

4.2.4Carotenoids analysis and quantification

TheHPLC chromatograms of identified carotenoids from pulp, peel and seeds of *A. muricata* and *A. squamosa*is presented in (Appendix II).In terms of spectral characteristics, the range of wavelength used to elute the different carotinoids was between 400 nm and 466 nm (Appendix III).The HPLC fingerprinting of the peel, pulp and seeds revealed the presence of neoxanthin, violaxanthin, leutine, lycopene, zeaxanthin, α-carotene, β-carotene γ-carotene, chlorophyll a, chlorophyll b and other unknowns. The carotenoids were identified by comparisons to retention times and UV spectra of authentic standards analyzed under identical analytical conditions. Fifteen (15) peaks were observed (HPLC chromatogram in Appendix II), reflecting 15 different carotenoids as compared to the standards and samples during elution such as leutein, lycopene and β-carotene with retention time of 4 min, 8.67 min and 17.2 min respectively (Appendix IV). These include 10 identified of carotenoids and five 5 unidentified carotenoids eluted at different wavelength and retention times.

Table 4.8: The total carotenoid concentration in the peel, pulp and seeds of *A. muricata* and *A. squamosa* fruits(µg/100g DW)

Carotenoids	Concentration					
	Annona muricata			*Annona squamosa*		
	Pulp	Peel	Seeds	Pulp	Peel	Seed
Neoxanthin	0.677^{c}±0.03	0.32^{a}±0.02	0.56^{bc}±0.03	0.68^{c}±0.01	0.44^{ab}±0.02	0.50^{b}±0.03
Violaxanthin	0.326^{a}±0.05	0.04^{a}0.02	0.18^{a}±0.05	12.44^{a}±0.17	0.842^{a}±0.01	0.31^{a}±0.02
Lutein	2.864^{a}±0.51	315.55b±0.89	7.67^{a}±0.01	1.140^{a}±0.01	217.96^{b}±0.66	6.34^{a}±0.04
Antheraxanthin	2.51^{a}±0.34	78.53^{b}±0.50	20.57^{ab}±0.06	0.20^{a}±0.001	0.03^{b}±0.001	0.09^{ab}±0.01
Zeaxanthin	1.09^{a}±0.12	47.93^{b}±0.32	0.08^{a}±0.01	10.45^{c}±0.04	33.57^{b}±0.37	0.28^{a}±0.01
α-Carotene	8.49^{a}±0.05	949.60^{b}±0.03	75.47^{c}±0.15	16.95^{a}±0.01	1089.76^{b}±0.01	74.60^{c}±0.12
β-Carotene	0.19^{a}±0.01	75.97^{b}±0.39	200.00^{c}±0.39	3.82^{a}±0.01	43.939^{b}±0.19	255.36^{c}±0.20
ɤ-Carotene	1.30^{a}±0.17	15.51^{a}±0.05	108.12^{b}±0.03	1.74^{a}±0.03	0.06^{a}±0.01	132.54^{b}±0.84
Chlorophyll a	0.29^{a}±0.02	3.83^{b}±0.02	3.42^{a}±0.07	0.16^{a}±0.01	10.17^{b}±0.04	0.03^{a}±0.01
Chlorophyll b	2.97^{a}±0.53	68.31^{b}±0.10	5.30^{a}±0.02	4.75^{a}±0.12	129.11^{b}±0.01	0.03^{a}±0.01
Others	1.69^{a}±0.13	37.13^{b}±0.35	247.90^{c}±0.03	4.20^{a}±0.01	18.05^{b}±0.12	312.42^{c}±0.51

Mean values (n=3) ± SEM. Means with different superscript letters within a row are significantly different (P<0.05)

4.2.5Antioxidant activities

4.2.5.1 *In Vitro*antioxidantactivities

4.2.5.1.1 Lipid peroxidation (LPO) inhibition activity

The LPO scavenging activities of ASPUME, AMPUME, ASPUAE and AMPUAE are presented in Table 4.9. The ASPUME and AMPUME registered higher LPO inhibition at a percentage inhibition of 71.93±0.22% and 78.20±0.11% respectively at 500 µg/mL compared to ASPUAE and AMPUAE at a percentage inhibition of 55.27±0.34% and 60.49±0.43% respectively. In all concentration ranges (15 µg/mL – 500 µg/mL), the LPO percentage inhibition of ASPUME and AMPUME were significantly higher ($P<0.05$) compared to ASPUAE and AMPUAE. The IC_{50} ranged from 433.50±7.12 µg/mL (AMPUME) to 475.33±1.50 µg/mL (ASPUAE). The slightly lower IC_{50} value of methanol extracts 433.50±7.12 µg/mL (AMPUME) and 448.39±11.35 µg/mL (ASPUME) in comparison with the aqueous extracts of 465.00±3.33 µg/mL (AMPUAE) and 475.33±1.50 µg/mL (ASPUAE) is an indication that the methanol extracts (AMPUME and ASPUME) have better ability toinhibit LPO as compared to the aqueous extracts (AMPUAE and ASPUAE). However, the IC_{50}values were significantly higher ($P<0.05$) as compared to standard ascorbic acid of (IC_{50}; 121.78±0.93 µg/mL).

Table 4.9: Lipid peroxidation (LPO) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps

Conc (µg/mL)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	15.53^{c}±0.34	11.52^{a}±0.22	17.37^{d}±0.16	11.01^{a}±0.17	9.76^{b}±0.15
30	30.49^{a}±0.16	16.33^{b}±0.35	32.90^{a}±0.15	19.49^{c}±0.19	15.08^{b}±0.18
60	34.35^{b}±0.27	39.45^{a}±0.32	34.54^{b}±0.31	36.45^{c}±0.35	41.14^{d}±0.30
125	66.92^{a}±0.36	51.78^{c}±0.12	65.44^{a}±0.62	56.06^{d}±0.16	63.55^{b}±0.30
250	74.25^{c}±0.12	50.30^{b}±0.20	76.08^{c}±0.26	58.23^{a}±0.22	82.35^{d}±0.38
500	71.93^{a}±0.22	55.27^{b}±0.34	78.20^{c}±0.11	60.49^{d}±0.43	91.04^{e}±0.43
IC_{50}(µg/mL)	448.39^{d}±11.35	475.33^{e}±1.50	433.50^{c}±7.12	465.00^{b}±3.33	121.78^{a}±0.93

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.2.5.1.2 Nitric oxide (NO) scavenging activity

Moderate NO scavenging activities were observed by ASPUME, AMPUME, ASPUAE and AMPUAE (Table 4.10). In all concentration ranges (15 µg/mL – 500 µg/mL), inhibition of ASPUME and AMPUME were significantly higher ($P<0.05$) than ASPUAE and AMPUAE. Theinhibition of ASPUAE (42.38±0.35%) and AMPUAE (46.81±0.17%) were lower than those ofASPUME (63.27±0.38%) and AMPUME (70.78±0.46%) at 500 µg/mL.The IC_{50} values were 354.39±4.98 µg/mL (ASPUME), 370.00±1.67 µg/mL (ASPUAE), 345.50±1.94 µg/mL (AMPUME) and 354.67±1.50 µg/mL (AMPUAE). Although all the extracts (ASPUME, ASPUAE, AMPUME and AMPUAE) exhibited certain degree of NO scavenging, the IC_{50} values were high ranging from 345.67±1.94 µg/mL (AMPUME) to 370.00±1.67 µg/mL (ASPUAE) compared to the standard (Ascorbic acid) value of 124.75±2.93 µg/mL.

Table 4.10: Nitrous oxide (NO) inhibition activity of methanol and aqueous extracts of *A. muricata*and *A. squamosa*fruits pulps

Conc (µg/mL)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	24.52^{b}±0.21	10.71^{a}±0.26	20.89^{c}±0.48	16.52^{d}±0.75	29.64^{e}±0.21
30	42.77^{a}±0.25	32.57^{c}±0.29	44.28^{b}±0.31	36.41^{d}±0.23	41.83^{b}±0.27
60	53.87^{c}±0.68	41.12^{b}±0.28	56.20^{d}±0.31	43.10^{a}±0.32	64.79^{e}±0.23
125	61.51^{d}±0.17	42.93^{a}±0.11	63.94^{e}±0.)22	46.36^{b}±0.24	81.49^{c}±0.19
250	59.36^{a}±0.29	40.16^{b}±0.29	64.51^{c}±0.24	45.41^{d}±0.16	78.75^{e}±0.19
500	63.27^{d}±0.38	42.38^{e}±0.35	70.78^{c}±0.46	46.81^{b}±0.17	79.49^{a}±0.27
IC_{50}(µg/mL)	354.39^{b}±4.98	370.00^{a}±1.67	345.50^{c}±1.94	354.67^{d}±1.50	124.75^{e}±2.93

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.2.5.1.3 Superoxide (O_2^-) scavenging activity

Moderate superoxide (O_2^-) scavenging activities were exhibited by the aqueous extracts (ASPUAE and AMPUAE) than methanol extracts (ASPUME and AMPUME) in all concentration ranges (Table 4.11). The AMPUME and ASPUME displayed a greater degree of inhibition at 65.19±0.28% and 56.16±0.45% respectively, than AMPUAE and ASPUAE, which was at 47.45±0.33% and 49.54±0.38% respectively. The IC_{50} values were lower in AMPUME (155.56±2.62 µg/mL) and ASPUME (185.00±3.333 µg/mL) compared to AMPUAE (300.00±16.67 µg/mL) and ASPUAE (310.00±3.333 µg/mL). However, all the IC_{50} values of all extracts (ASPUME, ASPUAE, AMPUME and AMPUAE) were significantly higher ($P<0.05$) than that of the standard ascorbic acid (131.88±3.13 µg/mL).

Table 4.11: Superoxide inhibition activity of methanol and aqueous extracts of *A. muricata*and *A. squamosa*fruits pulps

Conc (µg/mL)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	8.46^{b}±0.16	4.93^{a}±0.33	9.60^{b}±0.12	6.85^{c}±0.16	12.33^{d}±0.33
30	17.82^{b}±0.10	11.24^{a}±0.27	18.66^{c}±0.19	10.16^{a}±0.26	18.48^{c}±0.18
60	25.77^{b}±0.37	19.57^{a}±0.20	27.43^{c}±0.34	19.61^{a}±0.21	30.56^{d}±0.42
125	35.26^{b}±0.55	31.94^{c}±0.52	39.80^{a}±0.17	33.93^{b}±0.21	49.51^{d}±0.17
250	50.51^{a}±0.29	45.46^{b}±0.26	44.99^{b}±0.26	44.19^{b}±0.25	65.19^{c}±0.29
500	56.06^{a}±0.45	49.54^{b}±0.38	65.19^{c}±0.28	47.45^{d}±0.33	75.25^{e}±0.25
IC_{50} (µg/mL)	185.00^{d}±3.33	310.00^{a}±3.33	155.56^{c}±2.62	300.00^{b}±16.67	131.88^{e}±3.13

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.2.5.1.4 Hydroxyl (OH^-) scavenging activity

The extracts (ASPUME, ASPUAE, AMPUME and AMPUAE) had the potential of scavenging OH^- (Table 4.12), which increased with increasing concentration of the extracts. The OH^-inhibition observed was in the range of 13.59±0.13% – 65.53±0.15% (ASPUME) and 13.28±0.14% – 76.65±0.33% (AMPUME) and 9.88±0.12% – 42.36±0.34% (ASPUAE) and 8.65±0.34 – 60±0.27% (AMPUAE) at a concentrations ranges of 15-500 µg/mL. The OH^- scavenging ability of the methanol extracts (ASPUME and AMPUME) were found to be higher than those of the aqueous extracts (ASPUAE and AMPUAE).Although the extracts exhibited the potential to scavenge OH-, the IC_{50} values were still significantly higher at 300.00±16.67 µg/mL (AMPUME and ASPUME), 311.33±2.36 µg/mL (ASPUAE) and 316.67±5.09 µg/mL (AMPUAE) than that of the standard (ascorbic acid)171.77±4.91 µg/mL.

Table 4.12: Hydroxyl (OH^-)inhibition activity of methanol and aqueous extracts of *A. muricata*and *A. squamosa*fruits pulps

Conc (µg/mL)	Percentage inhibition of different extracts				
	ASME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	13.89^{a}±0.13	9.88^{b}±0.12	13.28^{a}±0.14	8.65^{b}±0.34	20.39^{c}±0.35
30	20.19^{a}±0.25	15.84^{c}±0.07	28.92^{b}±0.11	13.08^{e}±0.21	33.97^{d}±1.19
60	32.27^{a}±0.24	21.93^{c}±0.15	37.39^{e}±0.17	27.89^{d}±0.26	45.95^{b}±0.42
125	30.56^{b}±0.26	24.92^{a}±0.22	54.84^{c}±0.12	37.83^{d}±0.13	64.03^{e}±1.58
250	42.79^{a}±0.30	36.73^{b}±0.09	63.79^{c}±0.26	54.72^{d}±0.24	84.03^{e}±0.80
500	65.53^{a}±0.15	42.36^{b}±0.34	76.65^{c}±0.33	60.13^{d}±0.27	94.65^{e}±0.72
IC_{50}(µg/mL)	300.00^{b}±16.67	311.33^{a}±2.36	300.00^{b}±16.67	316.67^{c}±5.09	171.77^{d}±4.91

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.2.5.1.5 Total oxidant and reducing activities

The total antioxidant activity and reducing powers of ASPUME, ASPUAE, AMPUME and AMPUAE were evaluated as equivalent to µg/mL of α-tocopherol and equivalent to µg/mL of ascorbic acid respectively as presented in Table 4.13. The reducing powers against the standard ascorbic acid were found to be 57.37±0.71 µg/mL (ASPUME) and 62.92±2.50 µg/mL (AMPUME), 44.28±0.58 µg/mL (ASPUAE) and 46.61±0.32 µg/mL (AMPUAE). Total antioxidant activitieswere found to be 201.12±1.02 µg/mL (ASPUME), 199.15±1.07 µg/mL (AMPUME), 183.09^{a}±1.76 µg/mL (ASPUAE) and 181.36±0.87 µg/mL (AMPUAE)

Table 4.13: Total antioxidant Activity (Equivalent to µg of α-tocopherol) and total Reducing Power (Equivalent to µg of ascorbic acid) of methanolic and aqueous extracts of *A. muricata*and *A. squamosa*fruit pulp

Parameter	Solvents used in extraction and species of the plant			
	ASPUME	ASPUAE	AMPUME	AMPUAE
Total antioxidant activity	201.12^{b}±1.02	183.09^{a}±1.76	199.15^{c}±1.07	181.36^{a}±0.87
Total Reducing activity	57.37^{c}±0.71	44.28^{a}±0.58	62.92^{b}±2.50	46.61^{a}±0.32

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05)

4.2.5.2.*In vivo*antioxidant activity

4.2.5.2.1 Markers of liver and cardiac damage

Hepatoprotective role of the extracts were assessed through determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels whereas activities of cardiac markers, lactate dehydrogenase (LDH) and creatinine kinase (CK) depicted cardiac involvement. Infection with *L. major* in BALB/c mice resulted in significant increase ($p<0.05$) in AST, ALT, LDH and CK activities as shown in the positive control group (Group II). After treatment in IT groups (Group III, Group IV, Group V, Group VI and Group VII), there was a significant decrease ($p<0.05$) ALT, AST, LDH and CK activities (Table 4.14a-b). Further, the combined treatment of the extracts and Pentostam (Group VI and Group VII) had the highest significant decrease ($p<0.05$) in AST, ALT, LDH and CK activities compared to the extracts (Group IV and Group VI) and Pentostam separately (Group III).

4.2.5.2.2 Enzymatic and non enzymatic antioxidant levels

Antioxidant activity was analyzed through the level of non-enzymatic antioxidants (NO, TBRS (MDA), LPO and ROS) and enzymatic antioxidants (GSH, GST, GSHPx, CAT and SOD). The infection with *L. major* caused asignificant (*p<0.05*) elevation ofNO, TBRS (MDA) LPO and ROSlevels (Table 4.14a-b). In all theinfected treated (IT) groups of BALB/c mice (Group III, Group IV, Group V Group VIand Group VII), a significant (*p<0.05*) decrease in the NO, TBRS (MDA),LPO and ROS levelswas observed after treatment compared tonegative control (Group I) and positive control (Group II). The combined treatment of the extracts and Pentostam (Group VI and Group VII) showed the highest significant decrease in NOandTBRS (MDA) levels (*p<0.05*) compared to the extracts (Group IV and Group V) and Pentostam separately (Group III).However, the ROS increased significantly (*P<0.05*) in all the infected treated groups of mice (Group III, Group IV, Group V Group VIand Group VII) compared to the negative control group (Group I) and the positive control group (Group II).

The alteration of GSH, GST, GSHPx, SOD and CAT were used to prove the effect of leishmaniasis on the oxidant/antioxidant imbalance. The BALB/c infection with *L. major* caused a significant (*p<0.05*) decreaseinGSH, GST, GSHPx, SOD and CAT activities (Table 4.14a-b). Compared to the negative control group (Group I) and positive control group (Group II), the IT groups (Group III, Group IV, Group V Group VI and Group VII), showed a significant increase inGSH, GST, GSHPx, SOD and CAT activities (*p<0.05*) after or concurrently with the treatment. Further, the combined treatment of the extracts and Pentostam (Group VI and Group VII) showed the highest significant increase inGSH, GST, GSHPx, SOD and CAT activities (*p<0.05*) compared to the extracts (Group IV and Group VI) and Pentostam separately (Group III).

Table 4.14a: Effects of *A. muricata* and *A. squamosa* aqueous extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice

Antioxidant	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
GSH(IUg^{-1} of Hb)	4.18±0.28	1.43±0.15***	4.02±0.55	4.82±0.75*	4.21±0.47	8.27±1.15**	9.68±1.12**
GST (IU g^{-1} of Hb)	1.62±0.02	1.07±0.04*	2.08±0.09*	3.28±0.09*	3.32±0.05*	7.00±0.44***	10.90±1.95***
GSHPx(IU g^{-1} of Hb)	3.55±0.13	2.02±0.17*	4.22±0.74*	3.92±0.14*	4.08±1.08**	8.23±1.06	7.17±2.28***
CAT(μMmg^{-1} Hb)	7.58±0.06	2.15±0.03***	3.83±0.17*	6.93±0.47*	6.05±0.03*	9.90±0.06*	8.97±0.11*
TBARS/MDA (nMmL^{-1})	0.88±0.02	1.20±0.01*	0.76±0.02*	1.03±0.00*	0.86±0.02	0.75±0.02*	0.75±0.02*
ROS (nM$min^{-1}mg^{-1}$ protein)	0.17±0.01	0.21±0.01*	0.38±0.03*	0.38±0.03*	0.32±0.03*	0.43±0.03*	0.33±0.03*
SOD(IU mg^{-1} protein)	23.70±0.30	18.65±0.44*	26.52±0.22*	49.62±0.92*	45.00±0.12**	62.77±0.42**	69.45±0.67***
NO (uMmL^{-1})	23.17±0.44	47.17±1.39***	33.33±1.33**	35.43±2.43**	36.03±1.80**	23.83±1.29	26.73±0.63*
AST (UL^{-1})	370.58±54.55	783.67±20.64	572.50±19.48**	567.50±31.48**	498.43±12.59**	508.00±11.43**	530.02±5.40**
ALT (UL^{-1})	75.17±2.57	96.50±1.55**	75.17±2.57	84.67±3.74*	79.50±2.26*	59.33±1.74*	60.17±2.51*
LDH (UL^{-1})	252.17±10.69	541.50±11.33*	255.33±9.48**	328.17±5.21**	247.00±7.30**	242.17±10.69*	249.33±4.96**
CK (UL^{-1})	360.67±14.5	390.33±8.58**	255.33±18.19*	265.33±18.19*	252.50±12.60**	249.17±3.07**	256.33±5.70**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs Control (DMSO). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUAE), V: (AMPUAE), Group, VI: (Pentostam + ASPUAE) Group, VII: (Pentostam + AMPUAE). Key: AST; Aspartate amino transferase, ALT; Alanine amino transferase, LDH; Lactate dehydrogenase, CK;Creatine Kinase, TBARS; Thiobarbituric acid reactive substancesNO; nitric oxide, GSH; Glutathione, GST; Glutathione-s-transferase, GSHPx;Glutathione peroxidase, SOD; Superoxide dismutase, CAT; Catalase

Table 4.14b: Effects of *A. muricata* and *A. squamosa*methanol extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice

Antioxidant	Group I	Group II	Group III	Group IV	Group V	Group V	Group VII
GSH (IUg^{-1} of Hb)	4.18±0.28	1.43±0.15***	4.02±0.55	6.27±0.15**	6.68±0.12**	9.68±0.12**	10.27±0.15**
GST (IU g^{-1} of Hb)	1.62±0.02	1.07±0.04*	2.78±0.09*	2.90±0.04*	3.90±0.05**	8.90±0.05**	9.00±0.04***
GSHPx (IU g^{-1} of Hb)	3.55±0.13	2.02±0.17*	4.22±0.74*	5.23±0.06	6.17±1.28**	17.17±0.28**	12.23±0.67***
Catalase (CAT) (µMmg^{-1} Hb)	7.58±0.06	2.15±0.03***	3.83±0.17*	6.09±0.76**	5.37±0.11**	9.37±0.11**	11.00±0.06**
TBARS/MDA (nMmL^{-1})	0.88±0.02	1.20±0.01*	0.76±0.02*	0.86±0.02	1.00±0.11*	1.00±0.01*	0.86±0.02
ROS (nM min^{-1}mg^{-1} protein)	0.17±0.01	0.21±0.01*	0.38±0.03*	0.43±0.03**	0.33±0.03*	0.63±0.03*	0.53±0.03**
SOD (IU mg^{-1} protein)	23.70±0.30	18.65±0.44*	26.52±0.22*	45.67±0.42*	41.50±0.66	74.50±0.77	87.67±0.49*
Nitric oxide NO (uMmL^{-1})	23.17±0.44	47.17±1.39***	33.33±1.33**	22.83±0.29	25.83±0.63	18.83±0.63*	20.83±0.29
AST (UL^{-1})	37.58±0.55	78.67±1.64**	57.25±0.48**	50.00±1.43**	53.00±1.40**	33.00±1.40*	30.00±1.41*
ALT (UL^{-1})	75.17±1.57	96.50±1.55**	75.17±2.57	68.33±0.74*	71.17±1.51*	44.17±0.51*	40.33±0.74*
LDH (UL^{-1})	252.17±1.69	541.50±1.33*	255.33±1.48**	205.33±11.48***	194.33±1.96**	149.33±1.46**	155.33±1.48***
CK (UL^{-1})	360.67±1.45	390.33±1.58**	355.33±1.19*	195.50±1.260*	176.33±1.30**	156.33±1.70**	152.50±1.60**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs Negative Control. Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUME), V: (AMPUME), Group, VI: (Pentostam + ASPUME) Group, VII: (Pentostam + AMPUME). Key: AST; Aspartate amino transferase, ALT; Alanine amino transferase, LDH; Lactate dehydrogenase, CK;Creatine Kinase, TBARS; Thiobarbituric acid reactive substancesNO; nitric oxide, GSH; Glutathione, GST; Glutathione-s-transferase, GSHPx;Glutathione peroxidase, SOD; Superoxide dismutase, CAT; Catalase

4.2.5.2.3 Lesion size and liver and splenic length, size and parasite burden

An increase in lesions sizes with progression of the disease was observed (Figure 4.1a-b). A decrease in leision size was observed in all the IT groups (Group II, Group IV, Group V, Group VI and Group VII) after the initiation of treatment with the extracts and the standard drug (Pentostam). However, the rate of decreasein lesion size was significantly higher ($p<0.05$) in the IT-pentostam group (Group III) and IT-pentostam+extractsgroups (Group VI and Group VII) than in the IT-extractsgroups (Group IV and Group V). In the INT (positive control) group (Group II), a significant ($p<0.05$) increase inlesion sizes was observed until the end of the experimental period.The parasite load (LDU), relative mean weight and length of liver and spleen increased with increasing time of infection(Table 4.15a-b). There was a significant ($p<0.05$)decrease in parasite load (LDU), relative hepatic and splenic length and size in allthe ITgroups of BALB/c mice (Group III, Group IV, Group V, Group VI and Group VII). In the INT (positive control) group (Group II), a significant ($p<0.05$) increase inparasite load, relative weights of liver and spleenwas observed until the end of the experimental period. A combination of the extracts and standard drug (pentosam) (Group VI and Group VII) lead to a significant ($p<0.05$) decrease in parasite load (LDU), hepatic and splenic size and length compared to the extracts (Group IV and Group V) and standard drug (Pentostam) (Group III) separately.

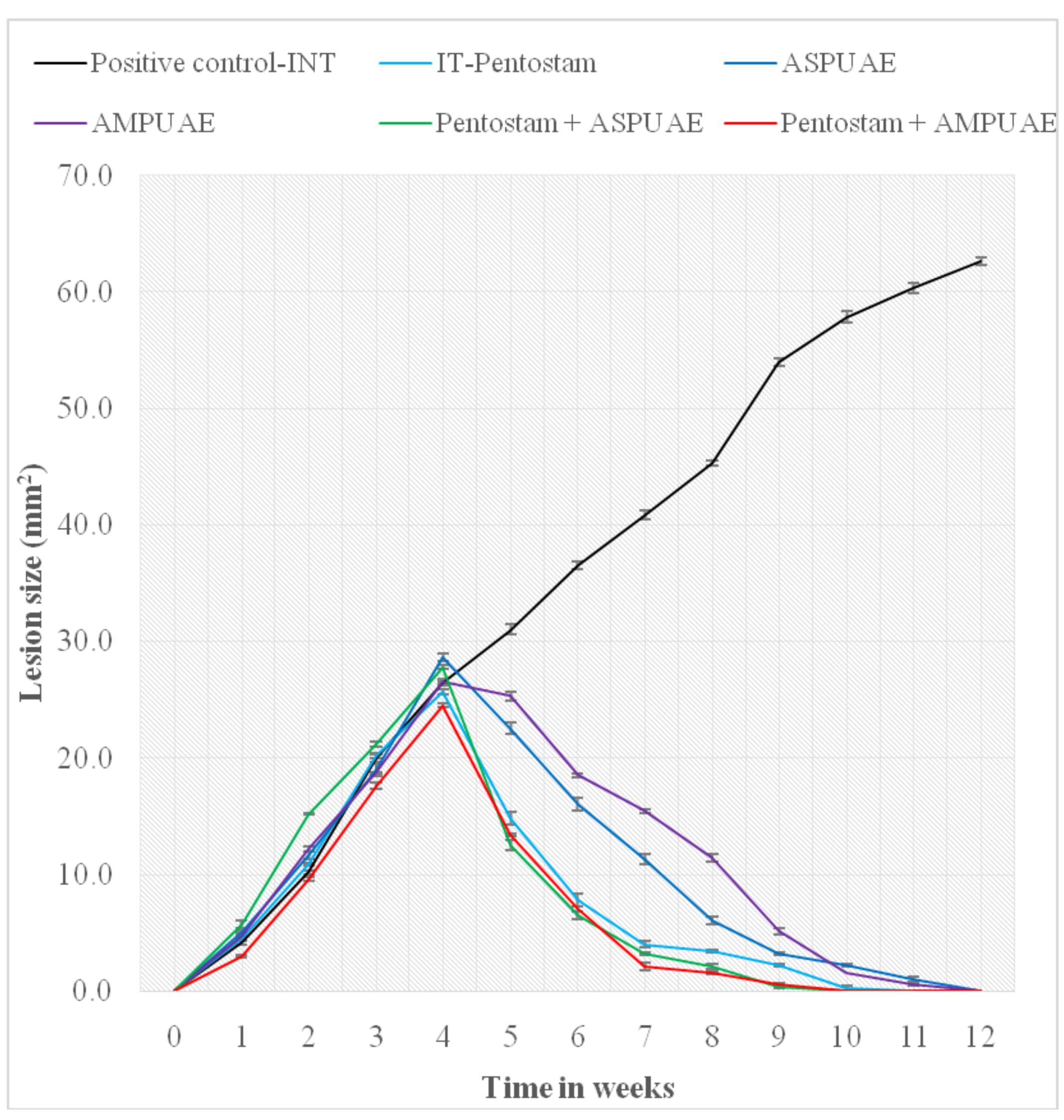


Figure 4.1a: Effects of *A. muricata* and *A. squamosa* pulp aqueous extracts on lesion sizes in *L. major* infected BALB/c mice

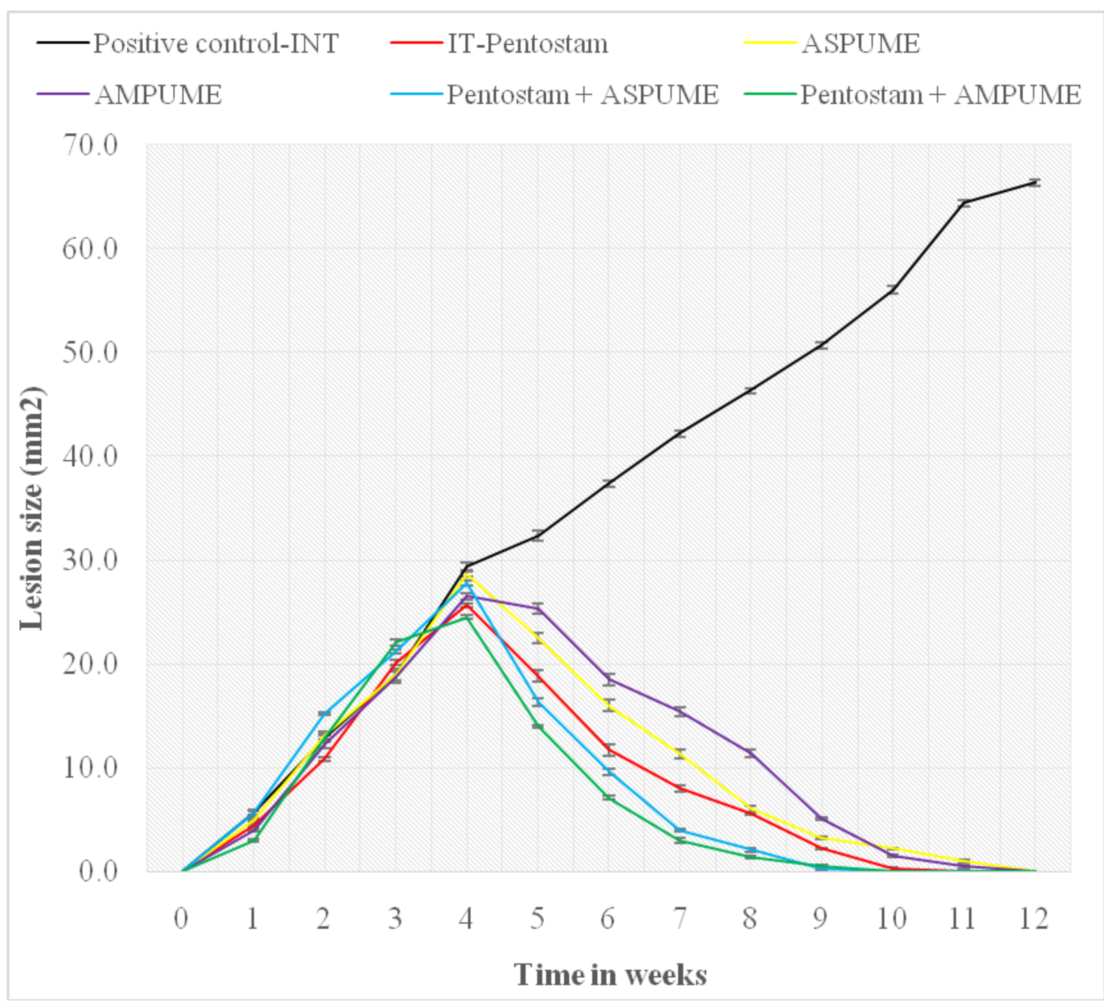


Figure 4.1b: Effects of *A. muricata* and *A. squamosa* pulp methanol extracts on lesion sizes in *L. major* infected BALB/c mice

Table 4.15a: Effects of of *A. muricata* and *A. squamosa* fruits pulp aqueous extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in *invivo* antioxidant study

Parameter	Organ	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Body Weight	Day 0	$19.99^{a}±0.19$	$20.76^{a}±0.07$	$19.93^{a}±0.15$	$19.96^{a}±0.22$	$19.93^{a}±0.13$	$19.93^{a}±0.33$	$19.93^{a}±0.11$
	Day 84	$29.74^{ab}±0.12$	$10.71^{c}±0.13$	$25.38^{e}±0.19$	$27.38^{d}±0.17$	$28.38^{b}±0.13$	$33.38^{g}±1.19$	$35.38^{f}±0.29$
Relative length (mm)	Liver	$22.08^{c}±0.11$	$51.10^{d}±0.38$	$28.15^{a}±0.13$	$25.15^{e}±0.18$	$26.15^{e}±0.27$	$22.15^{c}±0.12$	$23.15^{c}±0.08$
	Spleen	$18.83^{d}±0.32$	$43.17^{a}±0.44$	$21.17^{c}±0.11$	$24.17^{b}±0.14$	$23.17^{b}±0.17$	$19.17^{d}±0.15$	$18.17^{d}±0.19$
Relative weight (mg)	Liver	$1.44^{ab}±0.06$	$3.16^{c}±0.06$	$1.49^{b}±0.04$	$1.51^{b}±0.01$	$1.61^{d}±0.02$	$1.41^{a}±0.08$	$1.45^{a}±0.05$
	Spleen	$0.14^{a}±0.01$	$2.34^{c}±0.03$	$0.21^{b}±0.01$	$0.25^{d}±0.01$	$0.20^{b}±0.01$	$0.17^{e}±0.01$	$0.15^{a}±0.01$
LDU (10^{6})	Liver	ND	$545.44^{b}±12.58$	$2.21^{a}±0.58$	$7.15^{c}±12.58$	$12.93^{d}±4.13$	$1.03^{e}±3.13$	$0.97^{e}±0.13$
	Spleen	ND	$14.02^{a}±2.24$	$0.02^{b}±0.24$	$0.12^{c}±0.24$	$0.98^{e}±0.19$	$0.01^{d}±0.19$	$0.02^{b}±0.19$

Mean values ± SEM, (n=6). Values appended by different superscript letters within a row are significantly different (P < 0.05). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUAE), Group V: (AMPUAE), Group, VI: (Pentostam + ASPUAE) Group, VII: (Pentostam + AMPUAE), ND: Not done

Table 4.15b: Effects of of *A. muricata* and *A. squamosa* fruits pulp methanol extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in *invivo* antioxidant study

Parameter	Organ	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Body Weight	Day 0	19.99^{a}±0.19	20.76^{a}±0.07	19.93^{a}±0.15	20.08^{a}±0.11	20.10^{a}±0.11	19.98^{a}±0.11	20.01^{a}±0.41
	Day 84	29.74^{ab}±0.12	10.71^{c}±0.13	25.38^{e}±0.19	24.35^{e}±1.19	28.78^{a}±0.47	32.67^{d}±1.09	33.68^{d}±0.79
Relative length (mm)	Liver	22.08^{c}±0.11	51.10^{d}±0.38	28.15^{a}±0.13	29.55^{b}±1.12	25.55^{e}±0.01	21.00^{c}±0.27	22.30^{c}±0.97
	Spleen	18.83^{d}±0.32	43.17^{a}±0.44	21.17^{c}±0.11	23.11^{b}±1.01	26.11^{f}±0.01	19.88^{e}±0.13	18.78^{e}±0.66
Relative weight (mg)	Liver	1.44^{ab}±0.06	3.16^{c}±0.06	1.49^{b}±0.04	1.55^{b}±0.03	1.77^{b}±0.19	1.45^{b}±0.17	1.56^{b}±0.33
	Spleen	0.14^{a}±0.01	2.34^{c}±0.03	0.21^{b}±0.01	1.11^{d}±0.01	1.61^{g}±0.15	0.61^{f}±0.14	0.77^{e}±0.18
LDU (10^{6})	Liver	ND	545.44^{b}±12.58	2.21^{a}±0.58	15.55^{c}±2.01	23.55^{d}±2.01	12.55^{e}±2.04	9.55^{f}±1.11
	Spleen	ND	14.02^{a}±2.24	0.02^{b}±0.24	1.11^{c}±0.01	0.51^{f}±0.01	0.31^{e}±0.02	0.21^{d}±0.03

Mean values ± SEM, (n=6). Values appended by different superscript letters within a row are significantly different ($P < 0.05$). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUME), V: (AMPUME), Group, VI: (Pentostam + ASPUME) Group, VII: (Pentostam + AMPUME), ND: Not done

4.3 Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet

4.3.1 Proximate composition analysis of the experimental feeds

The proximate composition of RP and AFPP is presented in Table 4.16. The proximate composition parameter of RP and AFPP differed significantly ($P < 0.05$). The RP had a higher value of crude protein and crude lipids compared to AFPP. However, the AFPP had higher values of carbohydrates, moisture content, ash content, dry matter and fibre content compared to RP. Good palatability, acceptability and normal behaviors were observed during the experimental period in all groups except in the infected groups. In the uninfected group, minimal residual feed was observed.

Table 4.16: Proximate composition analysis of experimental diets used in feeding BALB/c mice (mg/100g)

Parameter	Rat Pellets (RP)	Annonaceae Fruit Palp Pellets (AFPP)
Crude Proteins	$25.28^{a}\pm0.04$	$3.81^{b}\pm0.14$
Crude Lipids	$18.60^{a}\pm0.06$	$2.95^{b}\pm0.18$
Carbohydrate	$27.24^{a}\pm2.05$	$40.12^{b}\pm0.38$
Moisture content	$2.17^{a}\pm0.98$	$6.20^{b}\pm0.12$
Ash content	$3.51^{a}\pm0.04$	$5.93^{b}\pm0.50$
Dry matter	$1.42^{a}\pm0.24$	$93.80^{b}\pm0.12$
Fibre content	$25.67^{a}\pm1.28$	$59.56^{b}\pm2.64$

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.3.2 Proximate Composition of Muscles of BALB/c mice

The proximate composition parameters such as moisture content, cruder protein, crude lipid and crude ash differ significantly ($P<0.05$) among the three groups of BALB/c mice (Table 4.17). However, no significant difference ($P>0.05$) was observed between NI groups where the same treatment was instituted on the BALB/c mice.

Table 4.17: Proximate composition of muscles of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks (mg/100g)

Parameter	Initial	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP
Moisture Content	$71.27^{a}\pm1.04$	$73.29^{a}\pm1.74$	$43.81^{b}\pm1.12$	$65.28^{c}\pm1.64$	$72.78^{a}\pm1.44$	$39.77^{b}\pm0.47$	$64.18^{c}\pm1.04$
Crude Proteins	$19.23^{a}\pm0.54$	$16.60^{a}\pm0.46$	$9.95^{b}\pm0.18$	$12.28^{a}\pm0.04$	$16.48^{a}\pm0.56$	$11.78^{b}\pm0.74$	$13.44^{a}\pm1.33$
Crude Lipids	$5.68^{a}\pm1.24$	$6.79^{a}\pm0.55$	$2.12^{b}\pm0.38$	$4.28^{a}\pm0.04$	$6.82^{a}\pm0.94$	$5.08^{a}\pm0.87$	$3.98^{a}\pm0.44$
Ash Content	$2.98^{a}\pm1.14$	$3.57^{a}\pm0.18$	$1.20^{b}\pm0.12$	$2.27^{a}\pm0.04$	$3.98^{a}\pm0.34$	$1.98^{a}\pm0.94$	$2.18^{a}\pm0.77$

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.3.3 Body mass developments, growth performance and nutrient utilization

Weekly body mass developments of different groups of BALB/c mice are presented in Figure 4.2 while the growth performance and nutrient utilization parameters are shown in Table 4.18. Greater BMG was observed in NI (NI-RP and NI-AFPP) groups with NI-RP being higher compared to NI-AFPP. A significant decrease in BMG was observed in IT (IT-RP and IT-AFPP) groups before treatment, which reversed after treatment. Further, a significant decrease ($P<0.05$) in BMG in INT (INT-RP and INT-AFPP) groups with the INT-AFPP group having greater decrease than the INT-RP group.Slightly high value of SGR, FCR, PER, PPV and ALC was observed in NI-RP group, which was statistically not different ($p>0.05$) to that in NI-AFPP. These values were significantly lower (p<0.05) in the IT (IT-RP and IT-AFPP) and INT (INT-RP and INT-AFPP) groups than in the NI (NI-RP and NI-AFPP) groups (Table 4.21). The BMG, SGR, MGR, PER and ALC differed significantly ($P<0.05$) between the IT (IT-RP and IT-AFPP), NI (NI-RP and NI-AFPP) and INT (INT-RP and INT-AFPP) groups.

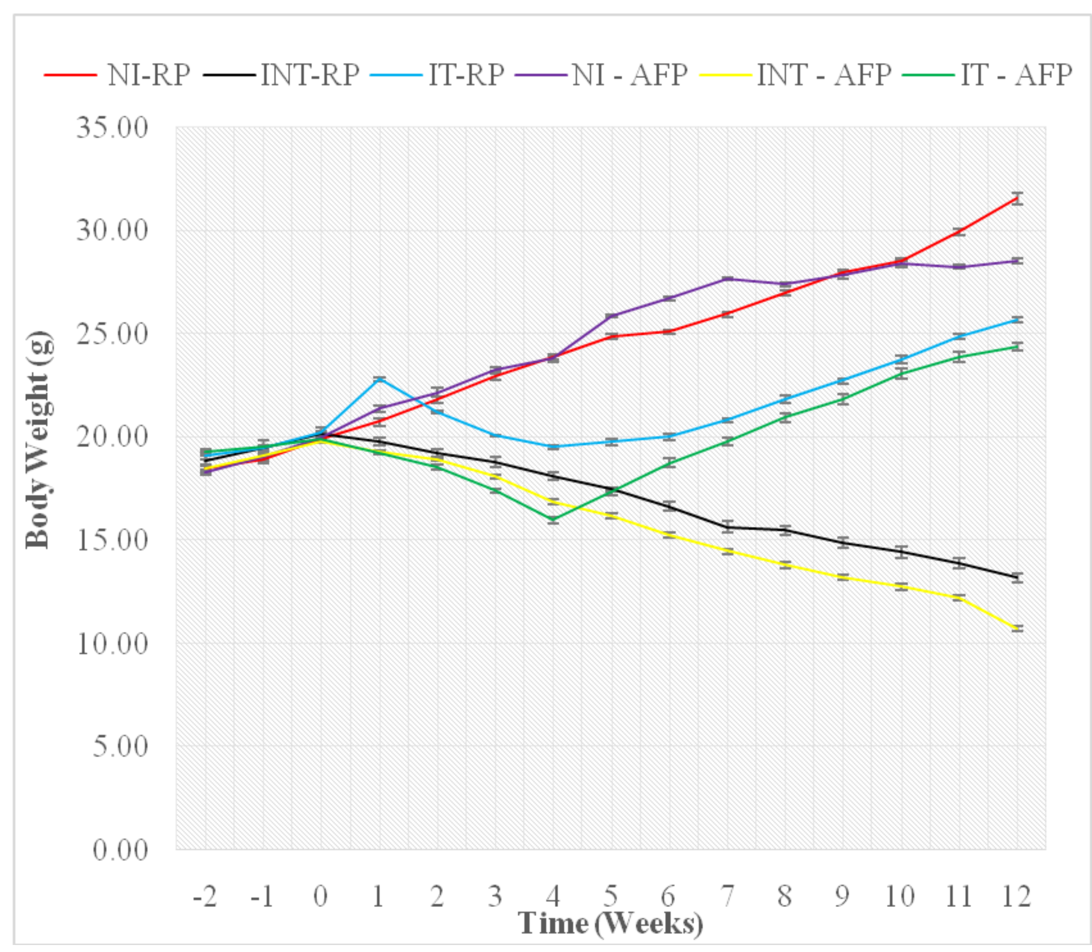


Figure 4.2: Body weight changes of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Table 4.18: Growth performance and feed nutrient utilization in in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Parameter	Rat Pellets (RP)			Annonaceae Fruit Pulp Pellets (AFPP)		
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP
IBM (g)	19.89^{a}±0.11	20.18^{b}±0.11	20.18^{b}±0.11	19.99^{a}±0.19	19.76^{a}±0.07	19.93^{a}±0.13
FBM (g)	31.02^{b}±0.21	13.18^{d}±0.13	25.68^{a}±0.10	29.74^{b}±0.12	11.71^{c}±0.13	25.38^{a}±0.19
BMG (g)	56.10^{c}±1.49	-34.62^{a}±0.79	27.31^{b}±0.66	49.11^{e}±1.39	-40.71^{d}±0.68	27.56^{b}±1.37
SGR (%)	13.24^{c}±0.30	-8.34^{a}±0.22	6.54^{b}±0.14	11.60^{d}±0.23	-9.58^{a}±0.17	6.49^{b}±0.29
MGR (g $kg^{0.8}$/day)	9.37±0.19	3.01±0.39	5.01±0.19	9.17±0.21	2.97±0.29	5.3±1.19
IBP (g)	19.23^{a}±0.54	19.23^{a}±0.54	19.23^{a}±0.54	19.23^{a}±0.54	19.23^{a}±0.54	19.23^{a}±0.54
FBP (g)	16.60^{a}±0.46	9.95^{b}±0.18	12.28^{a}±0.04	16.48^{a}±0.56	11.78^{b}±0.74	13.44^{a}±1.33
FCR	3.05^{b}±0.07	-4.93^{c}±0.11	6.28^{a}±0.18	3.51^{b}±0.10	-4.16^{c}±0.07	6.56^{a}±0.32
PER	0.33^{a}±0.01	-0.21^{b}±0.01	0.16^{c}±0.04	0.29^{a}±0.01	-0.24^{b}±0.004	0.16^{c}±0.01
PPV (%)	0.66^{a}±0.06	0.02^{b}±0.01	0.46^{a}±0.16	0.46^{a}±0.01	0.01^{b}±0.04	0.46^{a}±0.01
IBL (g)	5.68^{a}±1.24	5.68^{a}±1.24	5.68^{a}±1.24	5.68^{a}±1.24	5.68^{a}±1.24	5.68^{a}±1.24
FBL (g)	6.79^{a}±0.55	2.12^{b}±0.38	4.28^{a}±0.04	6.82^{a}±0.94	5.08^{a}±0.87	3.98^{a}±0.44
ALC (%)	52.67^{a}±3.31	22.17^{b}±2.60	40.17^{c}±6.60	49.36^{a}±4.49	18.7^{d}±6.60	38.70^{e}±6.60

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$). IBM, Initial body mass; FBM, Final body mass; BMG, Body mass gain; SGR, Specific growth rate; MGR, Metabolic growth rate; FCR, Feed conversion ratio; PER, Protein efficiency ratio; PPV, Protein productive value; ALC, Apparent lipid conversion; IBL, Initial body lipids; FBL, Final body lipids; IBP, Initial body protein, FBP, Final body protein; MGR, Metabolic growth rate.

4.3.4 Haematological changes

Hematological changes are presented in Table 4.19. A decrease in Hemoglobin (Hb) below the normal range was observed in all experimental groups except in NI groups. Red Blood Cells (RBC), Hematocrit (HCT) or Packed Cell Volume (PCV), Mean Capsular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) decreased in all the experimental groups. However, a significant decrease ($p<0.05$) in Hb was observed in the INT groups as compared to the NI groups. After treatment in the IT groups, Hb increased significantly ($p<0.05$) although it was still below the physiological range. The RBC, HCT, MCV, MCH and MCHC followed the same trend as Hb. Total white blood cells (WBC) and total leucocytes count (TLC) increased significantly ($p<0.05$) in infected groups compared to NI groups. Differentially, Leukocytes, platelets (PLT) and leukocyte populations (neutrophils, lymphocytes, eosinophils, monocytes and basophils) increased significantly in the INT groups compared to the NI groups. The increase in these parameters was within normal range in all the experimental groups. However, after treatment in the IT groups, these parameters decreased returning almost to the values in the NI groups. On comparison, the groups showed at least one significant difference among the averages of Hb ($p=0.001$), HCT ($p=0.05$), MCV ($p=0.001$), MCH ($p=0.001$), RBC ($p=0.001$), leukocytes (p=0.008), lymphocytes ($p=0.013$), neutrophils ($p=0.029$), monocytes ($p=0.002$) and basophils ($p=0.005$).

Table 4.19: Hematological changes of *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Parameter	Rat Pellets (RP)			Annonaceae Fruit Pulp Pellets (AFPP)			Ref. Range
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
HB (gdL^{-1})	$12.76^{a}\pm0.67$	$8.07^{b}\pm0.10$	$10.40^{a}\pm0.10$	$12.09^{a}\pm0.14$	$8.58^{b}\pm0.08$	$9.89^{ab}\pm0.10$	12.60 - 20.50
WBC x $10^{9}L^{-1}$	$6.11^{a}\pm0.12$	$9.08^{b}\pm0.09$	$6.58^{a}\pm0.16$	$5.91^{a}\pm0.05$	$11.97^{c}\pm0.12$	$9.91^{b}\pm0.05$	3.48 - 14.03
RBC x $10^{12}L^{-1}$	$8.39^{b}\pm0.06$	$5.96^{a}\pm0.12$	$8.05^{b}\pm0.12$	$7.04^{c}\pm0.09$	$6.10^{ac}\pm0.11$	$8.08^{b}\pm0.05$	6.93 – 12.24
HCT/PCV (%)	$48.30^{a}\pm0.88$	$43.13^{c}\pm0.79$	$46.80^{b}\pm0.08$	$46.97^{b}\pm0.88$	$44.60^{c}\pm0.63$	$44.80^{c}\pm0.47$	42.10 - 68.30
MCV (FL)	$54.28^{c}\pm0.97$	$46.28^{d}\pm0.53$	$51.44^{a}\pm0.62$	$51.28^{a}\pm0.66$	$42.30^{e}\pm0.53$	$49.61^{b}\pm0.38$	50.70 – 64.40
MCH (FL)	$30.98^{b}\pm0.44$	$23.22^{a}\pm0.19$	$26.38^{c}\pm0.38$	$26.82^{c}\pm0.54$	$22.03^{a}\pm0.19$	$26.72^{c}\pm0.42$	13.20 – 17.60
MCHC (pg)	$31.04^{c}\pm0.58$	$23.20^{b}\pm0.31$	$28.37^{ad}\pm0.19$	$27.54^{a}\pm0.16$	$25.30^{c}\pm0.12$	$27.70^{a}\pm0.97$	23.30 – 32.70
PLT x 10^{12}/L	$511.98^{a}\pm19.54$	$695.32^{b}\pm7.39$	$611.98^{c}\pm19.54$	$449.58^{d}\pm23.47$	$727.40^{e}\pm19.54$	$357.66^{f}\pm12.23$	420.00 – 1698.00
Neutrophils (%)	$24.89^{b}\pm0.51$	$30.06^{a}\pm1.15$	$23.39^{ac}\pm0.62$	$23.73^{c}\pm0.37$	$36.50^{d}\pm0.79$	$26.06^{e}\pm0.40$	9.86 – 39.11
Lymphocytes (%)	$74.62^{a}\pm0.35$	$83.95^{b}\pm0.61$	$73.57^{a}\pm0.39$	$61.95^{c}\pm0.65$	$78.20^{d}\pm0.95$	$65.62^{e}\pm1.41$	50.00 – 96.00
Monocytes (%)	$3.72^{c}\pm0.13$	$4.05^{b}\pm0.13$	$3.72^{c}\pm0.06$	$3.97^{c}\pm0.08$	$4.00^{b}\pm0.17$	$5.38^{a}\pm0.33$	3.29 – 12.48
Eosinophils (%)	$0.72^{a}\pm0.06$	$0.85^{a}\pm0.08$	$0.63^{c}\pm0.05$	$0.58^{d}\pm0.05$	$0.60^{b}\pm0.02$	$0.61^{b}\pm0.07$	0.11 – 4.91
Basophils (%)	$0.10^{b}\pm0.00$	$0.07^{a}\pm0.00$	$0.08^{ab}\pm0.00$	$0.09^{b}\pm0.01$	$0.05^{c}\pm0.01$	$0.09^{b}\pm0.00$	0.00 – 1.84
Abs Neutrophils	$3.15^{c}\pm0.24$	$4.77^{b}\pm0.19$	$4.75^{b}\pm0.17$	$3.85^{d}\pm0.17$	$7.05^{a}\pm0.29$	$4.07^{d}\pm0.19$	0.00 – 3.83
Abs Lymphocytes	$3.13^{a}\pm0.11$	$5.09^{c}\pm0.17$	$3.79^{b}\pm0.07$	$4.10^{e}\pm0.07$	$6.14^{d}\pm0.05$	$4.84^{f}\pm0.09$	2.22 – 9.83
Abs Monocytes	$0.90^{b}\pm0.03$	$1.27^{c}\pm0.06$	$0.63^{a}\pm0.04$	$0.79^{ab}\pm0.04$	$1.78^{d}\pm0.04$	$0.68^{a}\pm0.07$	0.21 – 1.25
Abs Eosinophils	$0.24^{d}\pm0.04$	$0.43^{b}\pm0.02$	$0.13^{a}\pm0.02$	$0.17^{e}\pm0.02$	$0.38^{c}\pm0.15$	$0.28^{e}\pm0.05$	0.01 – 0.49
Abs Basophiles	$0.01^{c}\pm0.0$	$0.07^{d}\pm0.00$	$0.13^{b}\pm0.00$	$0.02^{c}\pm0.00$	$0.15^{a}\pm0.02$	$0.05^{d}\pm0.01$	0.00 - 0.18

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

***4.3*.5 Liver function tests**

The function test changes are presented in Table 4.20. The activitiesALT,AST, LDH, CK, SDH and bilirubin increased significantly in the INT groups compared to the NI groups. After treatment, the activity ALT, AST, LDH, SDH, CK and bilirubin decreased almost returning to that of NI groups. The total protein, albumin, globulin and ALkP decreased significantly in INT animals compare to the NI groups. Most of the liver function tests were found to be within normal range in all IT and NI groups. A significant increase in ALT beyond the physiological range (183.49±3.18 IUL^{-1}) was observed in INT-AFPP group. Also a higher activity of AST beyond the physiological range beyond the physiological range (218.20±3.56 IUL^{-1} and 184.87±5.99 IUL^{-1}) was observed in INT-RP and INT-AFPP groups respectively. The activity of amylase increased beyond the physiological range whereas that of LDH decreases below the range in NI-RP (24.33±0.67 IUL^{-1}), NI-AFPP (17.90±0.69 IUL^{-1}) groups but increased significantly beyond the physiological range (40.17±0.29 IUL^{-1}) in the INT-AFFP group. After comparison, there was at least one significant difference among the averages of all biochemical parameters used to ascertain liver function of the mice.

Table 4.20: Liver functions in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Parameter	Rat Pellets (RP)			Annonaceae Fruit Pulp Pellets (AFPP)			Ref. Range
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
Glucose (mgL^{-1})	122.71^{b}±2.12	123.87^{c}±1.95	124.37^{a}±1.91	126.17^{d}±0.45	124.27^{a}±0.65	125.20e±0.41	106.00 – 278.00
T-Bilirubin (gdL^{-1})	9.02^{a}±0.17	11.18^{d}±0.10	9.52^{b}±0.29	10.20^{e}±0.06	12.68^{c}±0.35	9.68b±0.07	7.12 -12.05
C-Bilirubin (gdL^{-1})	2.57^{c}±0.11	3.43^{b}±0.05	2.57^{c}±0.11	3.07^{d}±0.26	4.23^{e}±0.35	2.80a±0.08	0.00 – 2.00
U-Bilirubin (gdL^{-1})	3.67^{d}±0.17	4.83^{b}±0.13	3.17^{a}±0.13	3.83^{ab}±0.16	4.33^{c}±0.20	3.00^{a}±0.11	0.00 – 3.00
ALT (IUL^{-1})	77.49^{e}±2.40	115.99^{a}±1.02	98.66^{c}±1.31	83.49^{f}±3.18	183.49^{b}±3.18	81.82^{d}±2.86	41.00 – 131.00
AST (IUL^{-1})	119.87^{b}±2.16	218.20^{a}±3.56	111.53^{c}±0.69	134.87^{d}±5.86	184.87^{e}±5.99	129.87^{f}±5.66	28.00 – 191.00
GGT (IUL^{-1})	25.43^{a}±0.55	28.77^{b}±0.48	24.60^{a}±0.46	23.77^{c}±0.60	31.43^{d}±0.56	23.77^{c}±0.60	20.00 – 40.00
ALKP (IUL^{-1})	132.36^{a}±1.53	127.36^{b}±1.33	134.03^{c}±1.21	157.36^{d}±4.40	140.70^{e}±1.70	155.70^{d}±3.03	118.00 – 187.00
T-Protein (gdL^{-1})	51.77^{b}±0.90	45.94^{a}±0.04	47.12^{c}±0.33	42.95^{d}±0.51	35.02^{e}±0.51	45.27^{a}±0.51	43.00 – 70.00
Albumin (gdL^{-1})	26.46^{b}±0.49	23.34^{c}d±0.58	35.27^{e}±0.51	24.50^{a}±0.32	22.27^{d}±0.40	26.35^{b}±0.14	27.00 – 46.00
Globulin (gdL$^{-1)}$	34.56^{e}±0.64	25.22^{d}±0.47	35.34^{c}±0.35	26.34^{b}±0.38	23.18^{a}±0.42	26.68^{b}±0.10	27.00 – 42.00
Alb/Glob ratio	0.77^{d}±0.02	0.93^{d}±0.03	1.98^{bc}±0.05	1.10^{a}±0.02	1.06^{a}±0.02	2.12^{b}±0.04	0.00 – 1.09
CPK (IUL^{-1})	3.48^{b}±0.22	4.02^{c}±0.08	1.79^{a}±6.86	1.79^{a}±0.07	2.46^{d}±0.05	1.60^{a}±0.04	2.50 – 3.70
Amylase (UL^{-1})	796.97^{b}±17.47	1580.31^{a}±54.65	896.97^{d}±7.90	813.64^{e}±23.68	1413.64^{e}±36.61	896.97^{f}±10.69	210.43 -323.57
LDH (IUL^{-1})	24.33a±0.67	34.66b±0.39	27.17c±0.68	17.90d±0.69	40.17e±0.29	24.83a±0.52	26.8 – 34.00
SDH (IUL^{-1})	29.15^{a}±0.90	34.50^{c}±0.53	31.16^{ab}±0.54	23.83^{d}±0.39	43.67^{e}±0.44	29.17^{a}±0.25	27.00 - 37.00

Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.5.6 Kidney function tests and lipid profiles

The kidney function tests and lipid profile changes are presented in Table 4.21. Creatinine, urea and blood urea nitrogen (BUN) increased significantly ($p<0.05$) in the INT group compared to NI groups. In all the IT groups, creatinine, blood Urea and BUN levels decreased significantly ($p<0.05$) compared to the INT groups. Increased creatinine levels beyond the physiological range were observed in all BALB/c mice groups while BUN decreased in NI-AFPP (25.16±0.11) and IT-AFPP (23.82±0.10) groups. However, the increased urea levels in all the BALB/c mice groups were found to be within normal range. Serum sodium, chloride, potassium, iron and phosphorous increased significantly in the INT groups compared to the NI groups although values remained within the physiological ranges. After treatment, a decrease in these ions was observed in the IT groups. The cholesterol, triacylglycerides (TAGs), high density lipoproteins (HDL), low density lipoprotein (LDL) and cholesterol/HDL ratio increased significantly ($p<0.05$) in the INT groups in NI groups indicative of liver damage and development of cardiovascular diseases. All lipid profile values were within the physiological range except HDL in INT-RP (2.73±0.11), IT-RP (1.23±0.07), INT-AFPP (3.03±0.06) and IT-AFPP (1.90±0.13) groups suggesting a relationship between leishmaniasis and cardiovascular diseases. Statistically, no significant difference between lipid profiles of NI-RP and NI-AFPP ($P=0.09$) and INT-RP and INT-AFPP ($p=0.08$) was observed but a significantly differences between NI and INT ($p=0.003$) and INT and IT ($p=0.001$) groups were observed.

Table 4.21: Kidney functions and lipid profile in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Parameter	Rat pellets (RP)			Annonaceae Fruit Pulp Pellets (AFPP)			Ref. Range
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
Creatinine (mgL^{-1})	2.57^{a}±0.16	3.32^{c}±0.15	2.49^{a}±0.19	2.12^{d}±0.11	2.87^{b}±0.25	2.48^{a}±0.10	0.50 - 0.80
BUN (mgL^{-1})	26.66^{a}±0.16	31.32^{b}±0.15	27.49^{c}±0.19	25.16^{e}±0.11	27.66^{c}±0.25	23.82^{d}±0.10	7.00 – 26.00
Urea (mgL^{-1})	7.88^{c}±0.11	9.11^{e}±0.11	7.38^{d}±0.18	6.88^{a}±0.22	10.03^{b}±0.06	6.88^{a}±0.15	7.00 – 26.00
Sodium (meqL^{-1})	143.48^{a}±0.74	147.13^{b}±1.04	142.96^{a}±0.46	135.63^{c}±1.33	143.63^{a}±0.93	137.29^{d}±0.78	125.30 – 187.40
Chloride (meqL^{-1})	116.23^{b}±0.82	121.90^{c}±1.49	117.40^{b}±0.64	121.23^{c}±1.10	131.23^{a}±1.56	122.90^{c}±1.91	109.6 – 138.80
Potassium (meqL^{-1})	6.16^{f}±0.13	6.49^{e}±0.17	5.49^{a}±0.19	7.14^{b}±0.06	6.82^{d}±0.17	6.32^{c}±0.21	7.30 – 12.07
K/Na Ratio	0.04^{a}±0.00	0.04^{a}±0.00	0.04^{a}±0.00	0.05^{b}±0.00	0.05^{b}±0.00	0.05^{b}±0.00	0.01 – 0.08
Phosphorus (meqL^{-1})	3.77^{c}±0.09	3.72^{c}±0.09	3.65^{c}±0.08	4.22^{a}±0.16	5.22^{b}±0.19	4.22^{a}±0.02	8.20 – 14.70
Calcium (mgdL^{-1})	2.59^{a}±0.06	2.30^{b}±0.03	2.74^{c}±0.04	2.76^{c}±0.11	2.68^{c}±0.09	3.43^{d}±0.06	9.40 – 12.70
Iron (mgdL-1)	230.00^{f}±4.67	405.00^{a}±6.98	241.00^{e}±3.74	340.00^{d}±3.57	418.00^{b}±2.78	317.14^{c}±5.87	210.00 – 474.00
Cholesterol (gdL^{-1})	3.75^{b}±0.18	7.13^{a}±0.16	4.81^{c}±0.16	4.22^{e}±0.14	7.13^{a}±0.16	5.31^{d}±0.26	11.10 – 24.60
Triacylglycerides (gdL^{-1})	1.16^{a}±0.04	2.48^{b}±0.05	1.68^{c}±0.05	1.34^{a}±0.06	3.11^{d}±0.08	1.81^{e}±0.03	1.050 – 5.35
HDL Cholesterol (gdL^{-1})	0.77^{a}±0.02	2.73^{c}±0.11	1.23^{b}±0.07	1.00^{b}±0.09	3.03^{d}±0.06	1.90^{e}±0.13	0.40 – 1.00
LDL Cholesterol (gdL^{-1})	1.34^{d}±0.11	3.10^{b}±0.09	2.12^{a}±0.15	1.51^{c}±0.11	3.10^{b}±0.09	2.12^{a}±0.15	
Cholesterol/ HDL Ratio	4.23^{c}±0.08	2.97^{b}±0.39	4.37^{c}±0.07	4.65^{a}±0.07	2.97^{b}±0.14	4.30^{c}±0.13	2.78 – 5.35

Mean values (n=6) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

*4.3.*7 Lesion size and liver and splenic length, size and parasite burden

The lesions sizes (mm^2) for the INT and IT groups were determined and are presented in Figure 4.3. An increase in lesions sizes with progression of the disease was observed. After the initiation of treatment, lesion sizes decreased in the IT groups. However, the rate of decrease was higher in the IT-RP group than in the IT-AFPP group. The lesion sizes of the INT groups increased significantly until the end of the experiment, with the size being larger INT-AFPP group than INT-RP group. The relative hepatic and splenic length (mm) and size (mg) for NI, INT and IT groups (Table 4.25) determined at the end of the experiment varied significantly ($p<0.05$). The relative mean weight and length of liver and spleen increased with increasing time of infection. A significant difference ($p<0.05$) was observed between weight and length of IT, INT) and NI groups. However, no significant difference ($P>0.05$) was observed between NI-RP and NI-AFPP groups and ITC-RP and IT-AFPP groups. The parasite density in liver and spleen for INT and IT groups determined after the experimental period (12 weeks) showed significant variations. In all IT groups, parasite load decreased significantly ($p<0.05$) as compared to the INT groups. The reduction in parasite load was significantly (p<0.05) more in the IT-RP groupas compared to the IT-AFPP group. After the experimental period (12 weeks), hepatomegaly and splenomegaly was observed on the INT groups. Impression smears of liver and spleen on the INT groups determined at the end of the experimental period (12 weeks) showed dissemination of *L.* majoramastigotes inside and outside macrophages.

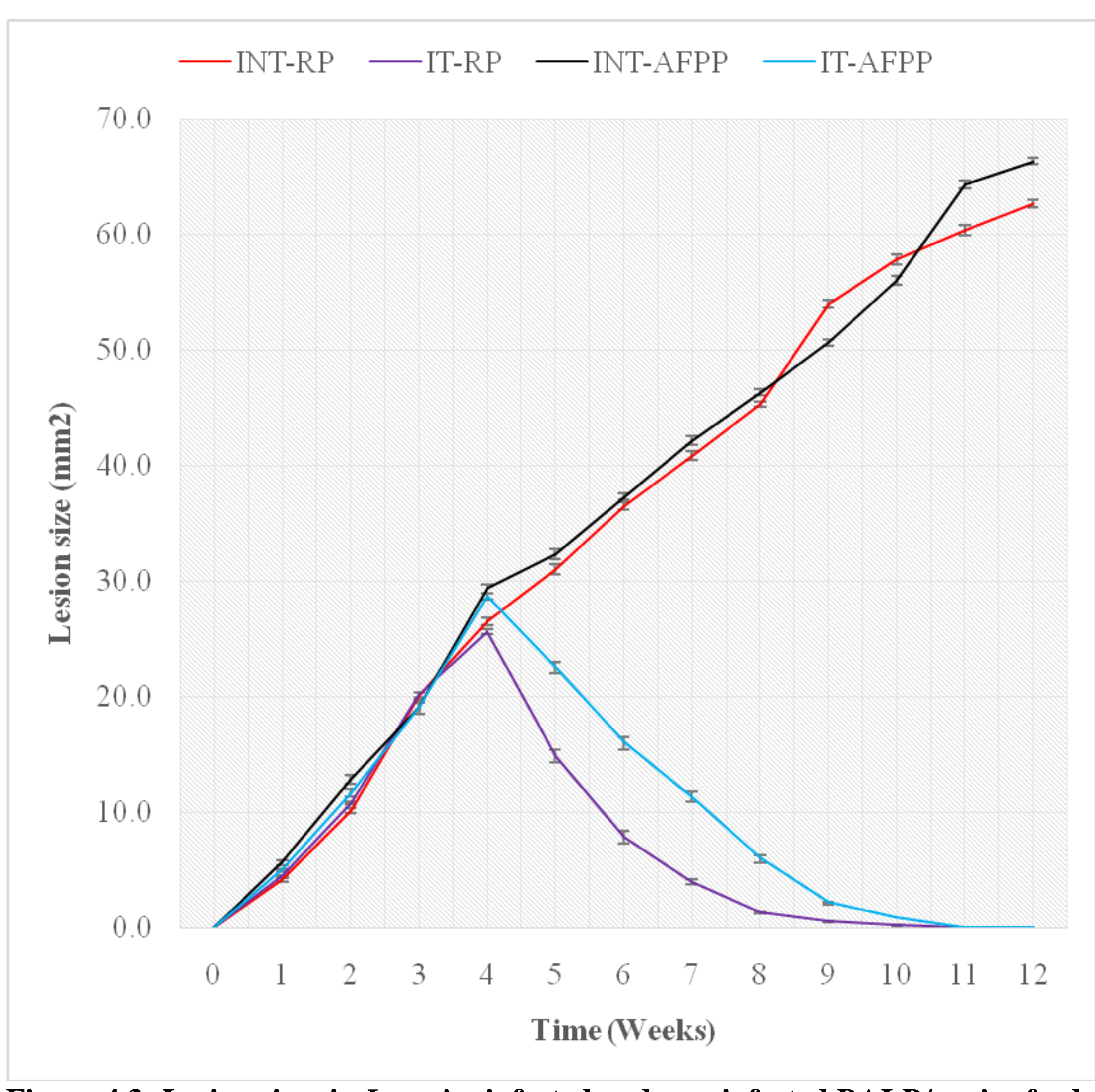


Figure 4.3: Lesion sizes in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Table 4.22: Relative weight (mg) and length (mm) of the liver and spleen in *L. major* infected and non-infected BALB/c mice feed with RP and AFPP for 12 weeks

Parameter	Organ	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP
Body Weight	Day 0	19.89^{a}±0.11	20.18^{a}±0.11	20.18^{a}±0.11	19.99^{a}±0.19	19.76^{a}±0.07	19.93^{a}±0.13
	Day 84	31.02^{b}±0.21	13.18^{c}±0.13	25.68^{d}±0.10	29.74^{ab}±0.12	11.71^{c}±0.13	25.38^{d}±0.19
Relative length (mm)	Liver	22.49^{c}±0.14	47.50^{b}±0.24	26.07^{a}±0.53	22.08^{c}±0.11	51.10^{d}±0.38	28.15^{ae}±0.28
	Spleen	15.83^{b}±0.44	40.33^{a}±0.64	19.50^{c}±0.31	18.83^{d}±0.32	43.17a±0.44	20.17^{c}±0.19
Relative weight (mg)	Liver	1.36^{b}±0.02	2.66^{d}±0.04	1.59^{a}±0.02	1.44^{ab}±0.06	3.16^{c}±0.06	1.61^{a}±0.04
	Spleen	0.14^{a}±0.00	2.48^{c}±0.07	0.15^{a}±0.00	0.14^{a}±0.00	2.34^{c}±0.03	0.21^{b}±0.01
LDU (10^{6})	Liver	ND	564.59^{a}±13.24	ND	ND	645.44^{b}±12.58	ND
	Spleen	ND	13.28^{a}±0.42	ND	ND	19.02^{a}±0.24	ND

Mean values (n=6) ± SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)

4.4 Efficacy and safety of the extracts

4.4.1 *In vitro* antileshmanial activity

4.4.1.1 Macrophage Sensitivity tests

The 24 extracts were screened preliminarily *in vitro* for antileishmanial activity against *L. major* amastigotes at a concentration of 100 µg/mL. The results of percentage infection of the host cells, mean parasite burden per host cell for 20 randomly chosen cells and percentage suppression of parasites are shown in table 4.23. Ten extracts had reduction in the percentage reduction of infected cells greater than 50%, which include ASPUAE, ASPEAE, ASPEME, ASSAE, ASSME, AMPEAE, AMPEME, AMMEEAE, AMSAE and AMSME. The percentage suppression of infected cells (chemosuppression) of the active extracts ranged between 50.84±4.16% (ASPEAE) and 65.16±6.79% (ASSAE). The summary of the result for the mean percentage of infected cells and chemo suppression after treatment with the extracts are presented in Table 4.26. Infection rate of untreated macrophages (control) was in the range of91.96±2.15% to 95.93±2.75%. The DMSO used to dissolve hexane ethyl acetate and methanolic extracts and PBS used to dissolve aqueous extracts did not show any significant effect on the percentage of infected cells as compared to the control. The effect of the extracts on the mean number of parasites per host cell showed that all the extracts suppressed the parasite number (>50%) with ASSAEgiving the greatest suppression (85.33±0.77%). Mean parasite burden per host cell in untreated macrophages was in the range of 12 to 20.

Table 4.23: Efficacies of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts (100 µg/mL) on intracellular survival of *L. major*

Treatment	Percentage of Infection of cells	Percentage Chemosuppression	No. of parasites per host cell	% Parasite Suppression
Control	95.93±2.75	ND	14.33±1.19	ND
DMSO	91.96±2.15	1.67±1.01	13.95±1.96	3.70±2.52
PBS	92.19±2.00	0.47±1.21	16.45±5.55	0.00±0.00
ASPUAE	82.18±2.17	52.66±1.78	4.47± 0.44	70.57±4.12
ASPUME	80.12±3.57	43.77±1.13	5.56±1.44	82.54±5.57
ASPUEAE	77.65±3.12	45.12±1.47	4.55±1.28	67.67±1.47
ASPUHE	84.14±1.79	41.66±1.67	5.57 ± 1.09	59.59±8.07
ASPEAE	84.44±7.00	50.84±4.16	7.00± 4.01	59.12±11.94
ASPEME	47.0 1±1.46	51.47±2.57	3.21± 0.67	74.65±4.96
ASPEEAE	53.37±1.29	44.48±1.12	6.12±1.39	59.76±3.37
ASPEHE	73.49±4.39	46.78±4.95	5.12±0.12	63.17±2.48
ASSAE	31.25± 4.47	65.16±6.79	2.38±0.47	85.33±0.77
ASSME	39.07±3.67	55.67±4.56	3.44±1.12	77.89±7.07
ASSEAE	44.54±4.47	47.86±2.47	4.67±1.29	72.23±2.29
ASSHE	74.12±8.54	48.23±7.31	5.74±0.74	61.30±2.45
AMPUAE	50.97±1.39	47.65±1.12	4.33±1.12	60.76±2.27
AMPUME	47.87±1.28	45.98±3.28	3,77±1.77	55.67±1.29
AMPUEAE	67.98±2.47	49.78±1.47	5.67±2.23	69.87±4.57
AMPUHE	43.47±2.27	47.77±2.09	4.42±3.33	63.44±4.77
AMPEAE	59.67±3.47	59.17±5.67	6.67±2.77	65.67±5.12
AMPEME	48.87±1.19	55.47±3.33	7.02±1.19	59.57±3.38
AMPEEAE	53.34±2.67	51.09±1.97	4.77±1.54	77.76±2.47
AMPEHE	47.47±2.87	49.67±2.45	3.33±2.22	66.67±1.39
AMSAE	69.67±2.34	61.22±4.47	4.54±1.12	75.56±4.12
AMSME	77.78±4.12	56.67±2.23	5.37±3.37	80.34±4.44
AMSEAE	39.67±1.33	47.35±3.45	3.21±1.29	52.47±1.11
AMSHE	44.43±2.22	49.17±2.33	4.54±1.39	60.67±3.39
Pentostam	55.61±2.39	71.35±3.77	5.61±1.12	89.56±1.77

Mean values (n=3) ± SEM.

The infected macrophages showed the presence of the parasite while the parasite were not observed in the non infected macrophages as represents in the micrograph in Plate 4.2.

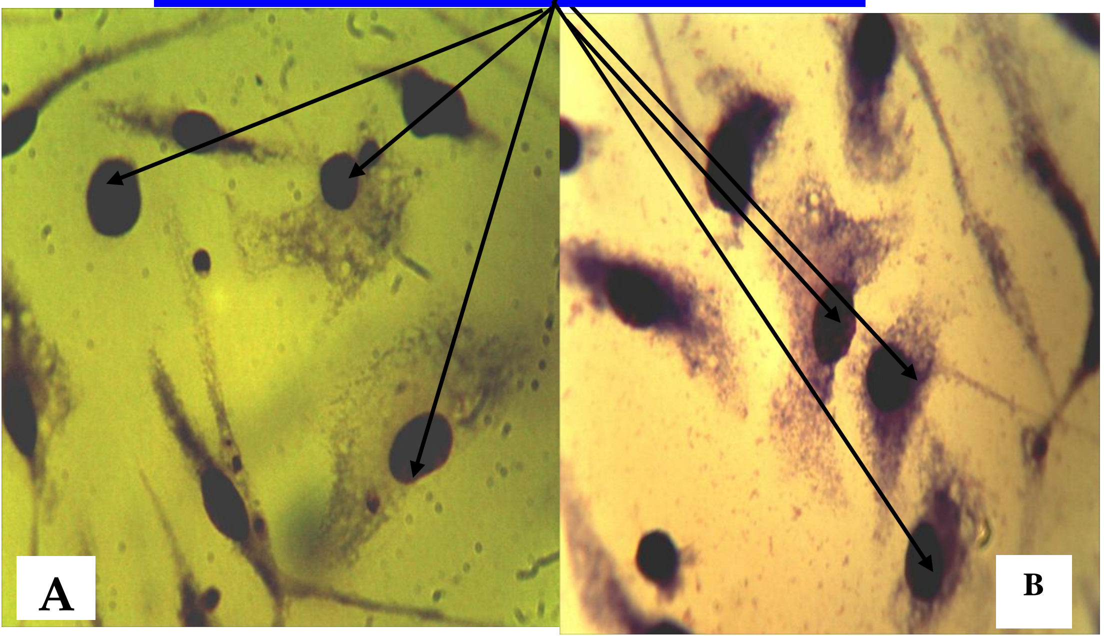


Plate 4.2: (A) Untreated macrophages with amastigotes occupying the parasitophorus vacuoles (black arrows) in the cytoplasm. Macrophages are seen with their large nuclei, which are eccentrically placed while amastigotes are small round or ovoid structures. (X1000 Giemsa stain). (B) Treated macrophages with parasitophorus vacuoles (black arrows) not occupied by amastigotes. Macrophages are viable with cytoplasm and pseudopods. (X1000 Giemsa stain).

4.4.1.2 Extracts sensitivity on infected macrophages

Freshly isolated bone marrow macrophages from BALB/c mice were infected with *L. major* promastigotes at a parasite/cell ratio of 20:1 for 24 hours and then treated with increasing concentrations of the extracts (6.25 µg/mL to 100 µg/mL) incubated for 72hours; the mean numbers of parasites per host cell was determined. Results showed a dose dependent antileishmanial effect based on the reduction in the percentage of cells infected (Table 4.24) and mean number of amastigotes per host cell (Table 4.25). Following incubation of promastigotes with various drug concentrations, a downward decrease in parasite numbers with increase in drug concentration was observed. TheASSAE (36.57±2.47) demonstrated the greatest suppression in the percentage of infected cells at the lowest concentrations of 6.25 µg/mL followed by AMSAE (38.25±0.97), AMSME (41.56±4.12) and ASSME (44.39±1.38) in that order. The other extracts showed effects at higher

concentrations.Treatment with DMSO and PBS, the solvents used to dissolve the extracts showed no significant effect on the percentage of infected cells or on the number of amastigotes per host cell in the preliminary tests therefore, they were not included in this assay.

Table 4.24: Effects of different concentrations of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts on suppression percentage rate of infected cell of bone marrow macrophages

Treatment	Suppression percentage of infected cell at different concentrations (μg/mL)					
	Control	100	50	25	12.5	6.25
ASPUAE	73.34±2.67	40.97±2.33	38.58±3.61	42.56±1.27	45.67±4.77	48.77±3.09
ASPUME	70.23±1.47	55.45±2.37	53.12±1.27	58.56±2.39	64.77±1.67	66.67±1.14
ASPUEAE	75.67±2.47	46.32±1.17	47.43±1.2	50.97±2.12	64.61±3.63	68.76±2.27
ASPUHE	75.57±3.45	44.45±2.77	48.31±4.2	54.61±3.6	58.50±2.4	59.97±3.38
ASPEAE	72.25±2.13	51.11±1.12	35.20±3.09	41.25±2.27	43.75±6.12	54.25±2.56
ASPEME	77.45±3.38	48.77±3.07	49.54±1.22	45.44±2.39	52.87±3.47	57.43±1.67
ASPEEAE	76.34±1.12	46.32±1.1	48.31±4.2	48.50±2.4	60.61±3.6	61.12±1.12
ASPEHE	73.67±1.13	48.31±4.2	47.43±1.2	47.43±1.2	66.50±2.4	67.87±3.34
ASSAE	78.67±4.67	37.32±2.23	48.47±3.34	41.12±1.47	33.75±2.29	36.57±2.47
ASSME	76.21±4.43	45.78±2.77	46.67±3.38	39.76±1.12	41.12±2.29	44.39±1.38
ASSEAE	71.12±1.17	47.72±1.18	47.43±1.28	50.32±1.14	54.61±2.16	63.37±1.78
ASSHE	73.47±2.12	46.32±1.16	46.32±1.39	48.31±4.23	64.61±3.68	66.67±2.97
AMPUAE	70.37±1.12	48.31±4.12	47.43±1.27	47.43±1.29	68.50±3.33	65.57±2.55
AMPUME	75.56±3.33	47.43±1.28	48.31±3.12	46.32±1.16	64.61±2.63	60.56±3.44
AMPUEAE	76.12±3.12	50.61±3.65	45.61±3.6	48.31±4.27	68.50±1.47	67.87±3.47
AMPUHE	71.12±2.47	48.50±2.47	48.00±1.44	64.61±3.67	68.50±2.46	62.56±1.67
AMPEAE	74.68±2.48	35.35±2.39	43.87±1.47	38.87±2.22	49.77±3.04	59.56±3.77
AMPEME	70.45±3.98	39.77±1.99	44.76±3.01	46.97±1.89	59.97±2.39	63.76±2.12
AMPEEAE	75.48±3.38	46.32±1.13	47.43±1.21	48.31±4.23	64.61±3.67	68.50±2.48
AMPEHE	77.67±4.56	47.87±3.14	46.67±1.76	48.19±1.98	65.44±3.32	67.89±2.33
AMSAE	72.25±2.38	35.25±2.11	47.35±0.79	43.45±2.77	35.75±2.29	38.25±0.97
AMSME	76.89±2.28	29.76±1.67	36.67±3.00	35.89±2.33	39.76±1.11	41.56±4.12
AMSEAE	73.34±2.38	46.32±1.11	47.43±1.23	48.31±4.2	64.61±3.6	64.45±2.29
AMSHE	72.37±3.33	47.43±1.27	46.32±1.1	48.31±4.2	68.50±2.4	67.67±3.39
Pentostam	80.87±3.76	39.87±3.46	46.45±1.88	50.22±1.16	53.35±1.47	55.42±2.67

Mean values (n=3) ± SEM.

Table 4.25: Effect ofmean number of amastigotes per host cell at different concentrationsof*A. muricata*and *A. squamosa*pulp, peel and seeds extracts on mean number of parasites per host cell

Treatment	Mean number of amastigotes per host cell at different concentrations (µg/mL)					
	Control	100	50	25	12.5	6.25
ASPUAE	5.01±0.11	2.87^{b}±0.14	3.55±0.15	3.01±0.11	3.76±0.13	4.00±011
ASPUME	4.89±0.12	3.02^{d}±0.44	3.46±0.27	4.00±0.13	3.95±0.47	4.47±0.38
ASPUEAE	4.77±0.28	2.96^{a}±0.12	3.32±0.47	4.35±0.77	4.44±0.13	4.97±1.02
ASPUHE	4.75±0.38	3.04^{d}±0.33	2.94±0.11	4.12±0.58	4.76±0.44	4.87±0.27
ASPEAE	4.55±0.17	2.86^{a}±0.27	3.35±0.12	3.01±0.76	2.71±0.21	2.98±0.33
ASPEME	5.78±0.38	2.45^{c}±0.45	3.12±0.17	2.87±0.77	3.45±0.19	4.79±0.12
ASPEEAE	4.69±0.33	3.00^{d}±0.45	3.01±0.33	3.98±0.47	4.12±0.12	4.52±0.39
ASPEHE	4.97±0.11	2.94±0.11	3.87±0.49	4.01±0.88	4.55±0.57	4.91±0.22
ASSAE	10.4±0.37	3.87^{d}±0.77	4.56±0.17	4.88±0.12	5.07±0.39	6.98±0.27
ASSME	4.37±0.49	2.35^{c}±0.28	2.51±0.37	2.82±0.38	4.42±0.52	4.81±0.23
ASSEAE	4.45±0.33	2.99^{ab}±0.22	3.45±0.17	4.25±0.67	4.51±0.12	4.61±0.17
ASSHE	4.78±0.20	3.24^{b}±0.44	3.87±0.17	4.58±0.55	4.87±0.23	5.05±1.02
AMPUAE	5.00±0.44	3.01^{d}±0.17	3.87±0.49	4.67±0.67	4.84±0.45	4.95±0.49
AMPUME	4.87±0.14	2.94^{b}±0.33	3.58±0.14	4.28±0.39	4.66±0.49	4.72±0.16
AMPUEAE	4.66±0.16	3.11^{d}±0.11	3.85±0.15	4.44±0.12	4.76±0.67	4.97±0.59
AMPUHE	4.76±0.19	2.87^{b}±0.44	3.67±0.76	4.88±0.77	5.02±0.13	6.02±1.12
AMPEAE	4.98±0.33	2.45^{c}±0.11	2.77±0.71	2.75±0.15	3.08±0.23	3.97±0.12
AMPEME	5.09±.1.21	3.02^{d}±0.25	2.84±0.81	3.76±0.09	4.00±0.78	4.57±0.47
AMPEEAE	4.56±0.13	2.88^{a}±0.22	2.45±0.14	2.76±0.11	2.99±0.12	4.88±0.38
AMPEHE	4.55±0.17	3.25^{d}±0.17	3.87±0.67	4.05±0.12	4.45±0.87	4.67±0.45
AMSAE	4.78±0.28	2.94^{ab}±0.46	2.77±0.33	2.86±0.51	2.97±0.23	4.50±0.29
AMSME	4.88±0.39	2.78^{b}±0.23	2.03±0.38	2.71±0.22	2.82±0.22	3.98±0.12
AMSEAE	4.77±0.28	2.91^{a}±0.11	2.98±0.67	2.88±0.13	3.97±0.78	4.85±0.57
AMSHE	4.99±o.13	3.12^{d}±0.12	3.87±0.33	4.13±0.47	4.58±1.02	4.94±0.52
Pentostam	2.88±0.12	2.12^{c}±0.39	2.75±0.18	2.35±0.16	2.55±0.17	2.61±0.29

Mean values (n=3) ± SEM.

4.4.1.3 The MIC and the IC_{50} values of the extracts

Minimum Inhibitory Concentration (MIC) and the IC_{50} values for the A. muricata and A. squamosa fruits pulp, peel and seeds extracts were determined against *L. major* parasites *in vitro* (Table 4.26)compared to the standard antileshmanial drug (Pentostam®). The IC_{50} of ethyl acetate extracts ranged between 0.01±0.01 µg/mL (AMPUEAE and AMPEEAE) to 0.09±0.02 µg/mL (ASPUEAE). The hexane extracts had IC_{50} values ranging between 0.01±0.01 µg/mL (AMPEHE) and 2.62±1.06 µg/mL (AMPUHE). The IC_{50} of the aqueous extracts ranged from 0.11±0.06 µg/mL (ASAE) to 2.33±0.81 µg/mL (AMPUAE). The methanolic extracts had IC_{50} ranging from 0.01±0.01 µg/mL (AMSME) to 4.79±1.17 µg/mL

(ASPUME). The most active extracts were found to be ethyl acetate followed by hexane extracts, aqueous extracts and finally methanolic extracts in that order. The MIC values ranged between 12.50±1.03 µg/mL in the most active extracts (AMPEEAE) to 55.0±2.97 µg/mL in the least active extracts (AMSHE). However, these values were very high compared to 35.37±2.47 µg/mL (Pentostam), the standard antileishmanial drug that was used in the study. Schneider's Insect Medium (SIM) was considered the negative control; the *L. major* parasites continued dividing and proliferating in the media therefore no antileishmanial activity against the growth of parasites was recorded.

Table 4.26: MIC and IC_{50} of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts

Extract	MIC (µg/mL)	IC_{50} (µg/mL)
ASPUAE	25.00±1.56	0.92±0.07
ASPUME	27.01±2.04	4.79±1.17
ASPUEAE	50.30±1.67	0.09±0.02
ASPUHE	25.00±2.33	0.02±0.01
ASPEAE	12.50±1.12	0.27±0.02
ASPEME	15.00±1.43	0.92±0.02
ASPEEAE	50.00±3,67	0.03±0.01
ASPEHE	12.50±1.06	0.58±0.04
ASSAE	12.50±1.77	0.11±0.06
ASSME	25.00±2.05	0.23±0.05
ASSEAE	25.90±1.98	0.01±0.01
ASSHE	25.05±1.47	0.87±0.08
AMPUAE	50.57±4.47	2.33±0.81
AMPUME	35.75±3.01	3.77±1.02
AMPUEAE	25.00±1.57	0.01±0.01
AMPUHE	25.77±1.67	2.62±1.06
AMPEAE	15.60±1.87	0.47±0.89
AMPEME	51.47±3.66	3.79±1.15
AMPEEAE	12.50±1.03	0.02±0.01
AMPEHE	35.55±2.33	0.01±0.01
AMSAE	25.14±1.78	0.63±0.04
AMSME	12.57±1.65	0.01±0.01
AMSEAE	12.50±1.99	0.03±0.01
AMSHE	55.12±2.97	0.23±0.08
Pentostam	15.66±1.12	1.35±0.67
Mean values (n=3) ± SEM		

4.4.2 *In vivo* antileishmanial activity

4.4.2.1 Left hand footpad sizes (LHFD)

The BALB/c mice infected with *L. major* promastigotes on the Left Hand footpad (LHFD) also developed swelling two to three weeks after inoculation thus treatment was instituted in the fourth week. All the tested extracts reduced the swelling of LHFDs, an indication of antileishmanial activities of the extracts. The effect of extracts treatments on the reduction of LHFD swelling in BALB/c mice is presented in Figure 4.4 - 4.7. Reduction in the LHFD swellings was observed in all the aqueous extracts (ASPUAE, AMPUAE, ASPEAE, AMPEAE, ASSAE and AMSAE). However, greater reduction in LHFD swelling was observed in ASPUAE group while AMPUAE group reduced LHFD swelling at the lowest rate (Figure 4.4).

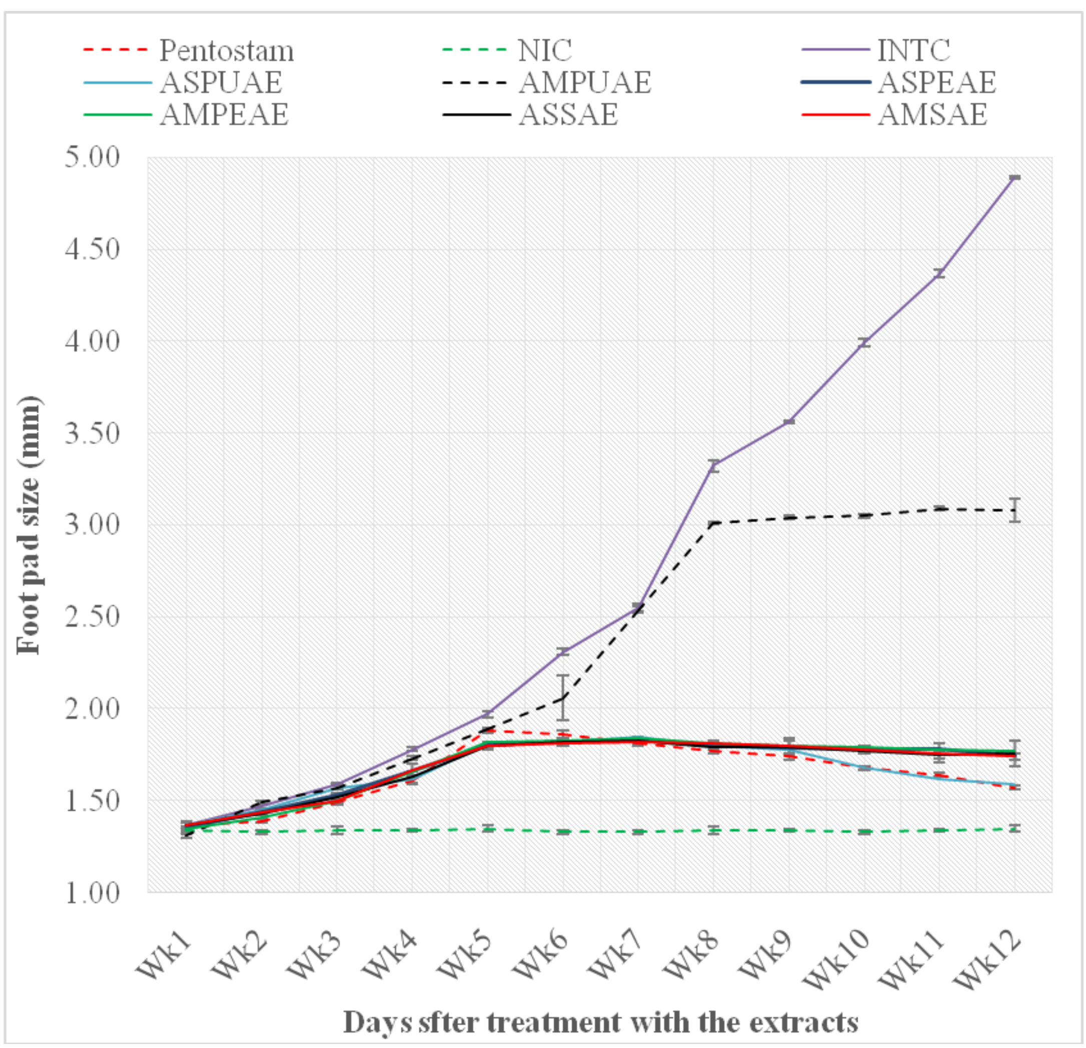


Figure 4.4: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts

Similarly, treatment with methanol extracts reduced LHFD swelling with AMPUME and ASPUME having the lowest reduction rates. The reduction in LHFD swelling in ASPEME, AMPEME, ASSME and AMSME treated groups were almost the same as Pentostam, the standard drug that was used in the study (Figure 4.5).

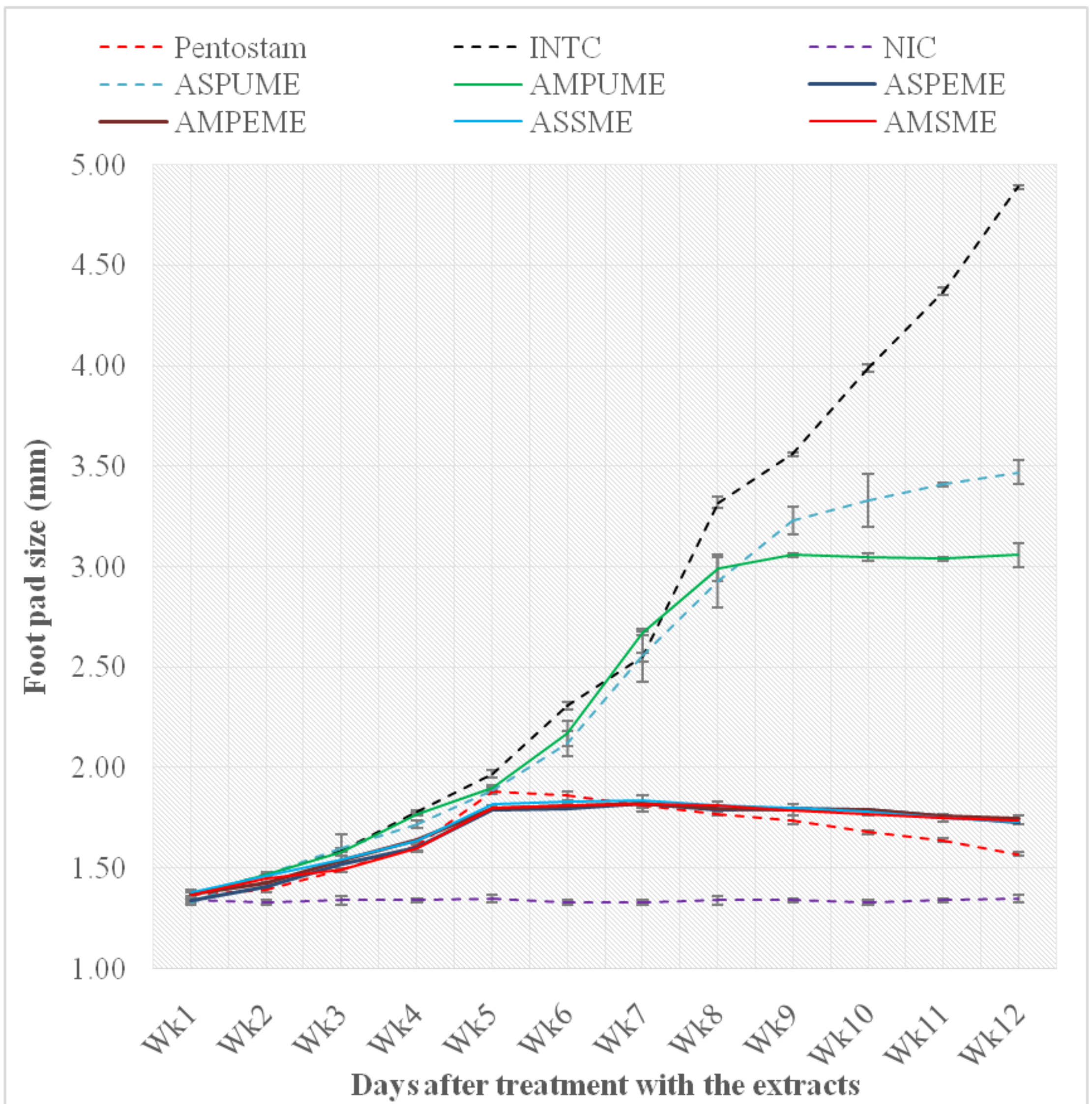


Figure 4.5: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds methanol extracts

The ethyl acetate extracts also reduced LHFD swelling with AMPEEAE having the same LHFD reduction rate as the standard drug (pentostam). Low LHFD swelling Rate was observed in AMPUEAE, AMSEAE, ASPUEAE, ASPEEAE and ASEAE (Figure 4.6).

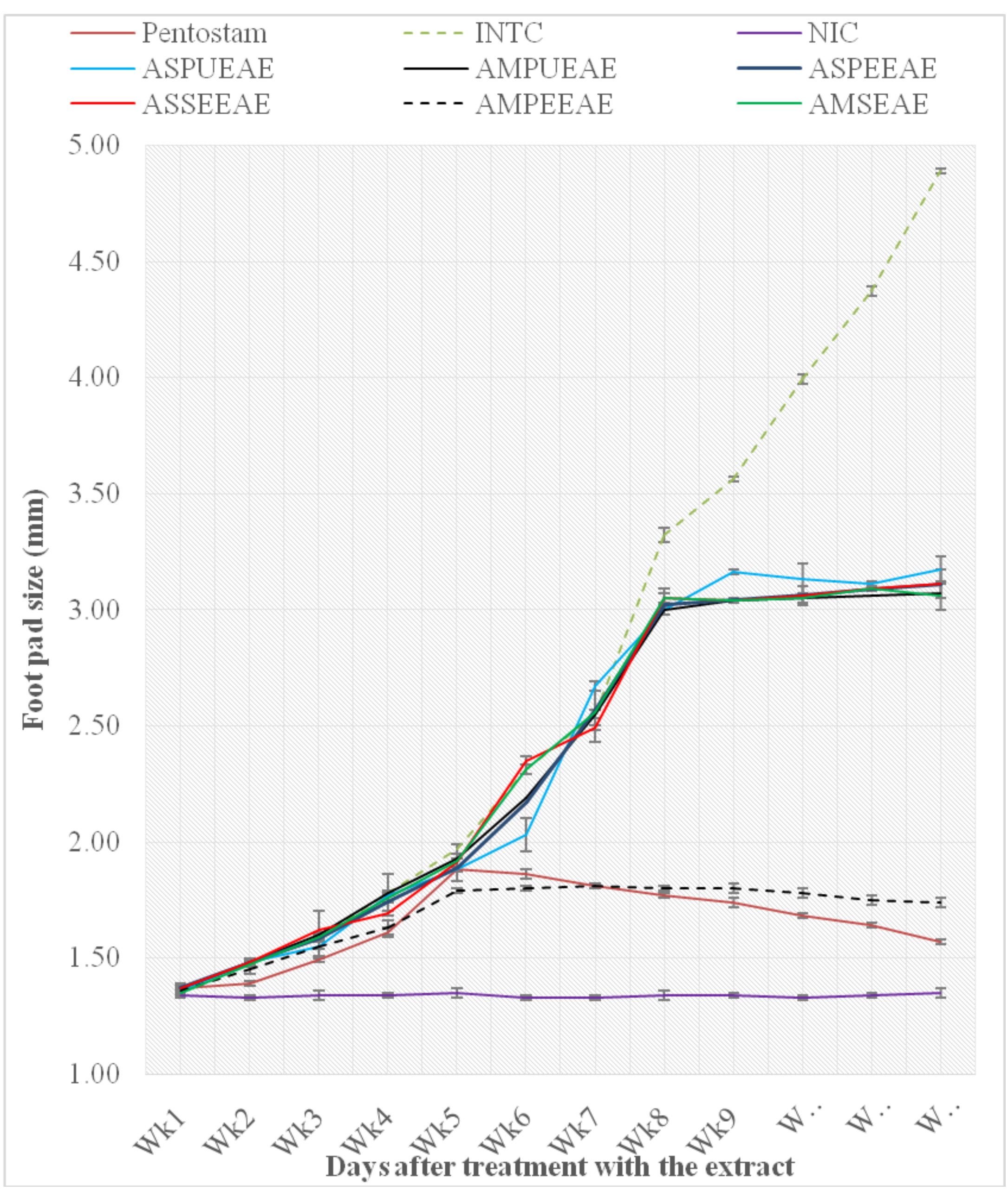


Figure 4.6: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts

The hexane extracts also had an effect of reduction of LHFD swelling after treatment (Figure 4.7). However, all the hexane extracts treated groups showed a low rate in reduction of LHFD swelling compared to the standard drug (pentostam).

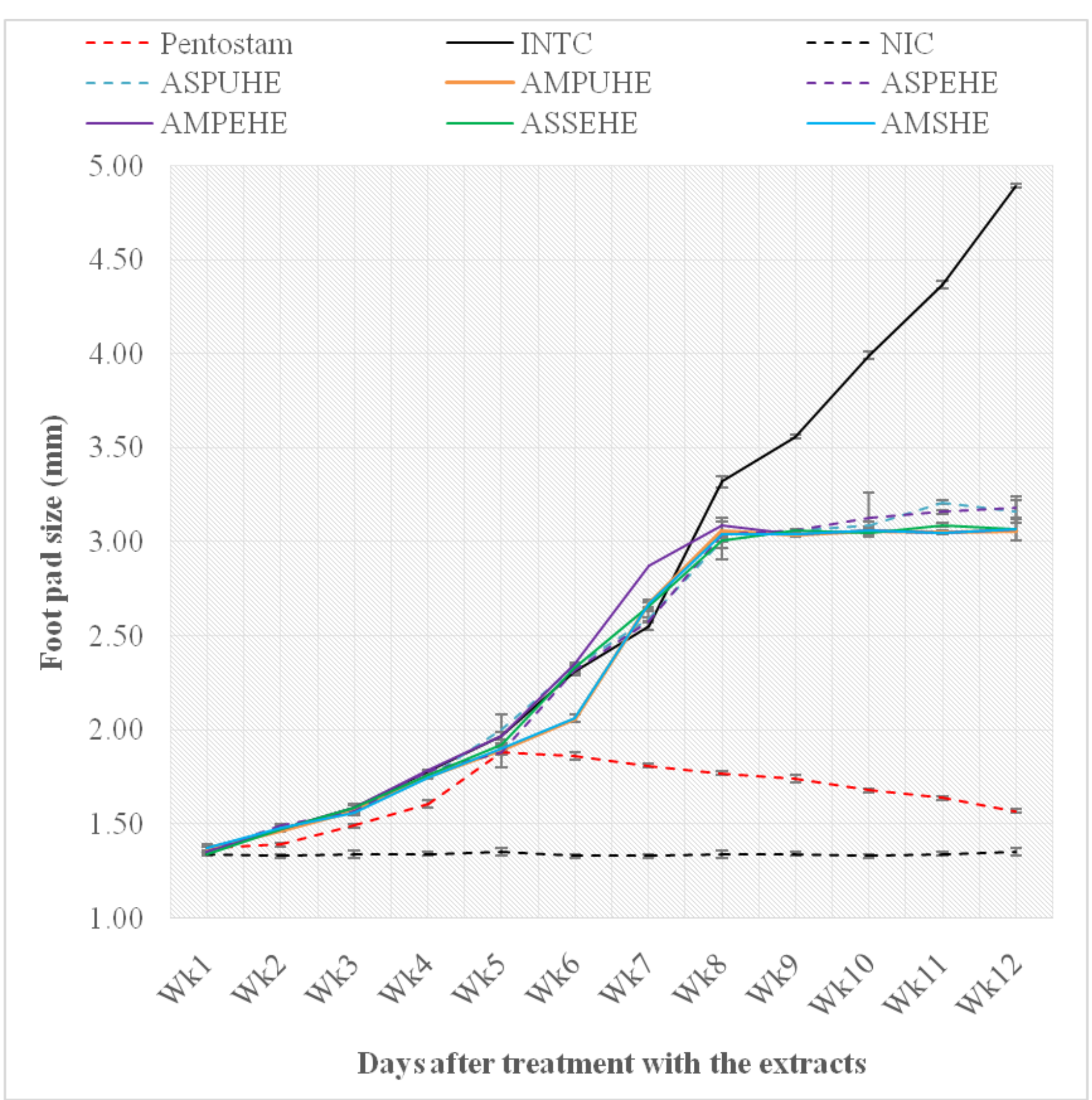


Figure 4.7: Average footpad size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts

4.4.2.2 Lesion sizes

The BALB/c mice infected with *L. major* promastigotes developed skin lesions inform of a single nodule with or without ulceration two to three weeks after inoculation thus treatment was instituted in the fourth week. Lesion sizes were almost similar in all the infected groups till the onset of treatment four weeks after infection. After two weeks (14th day of post-treatment), significant differences (*p<0.05*) in variation and reduction of lesion sizes were observed in the different experimental groups of BALB/c mice. Lesions were almost completely eliminated in the fourth month of post-treatment with the each of the extracts from pulp, peel and seeds of *A. muricata* and *A. squamosa* fruits.

The effects of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds aqueous extracts is presented in Figure 4.8. Among the aqueous extracts, AMSAE was the most active with high rate of reduction of the lesion size followed by AMPEAE while AMPUAE had the least rate of lesion size reduction (Figure 4.8)

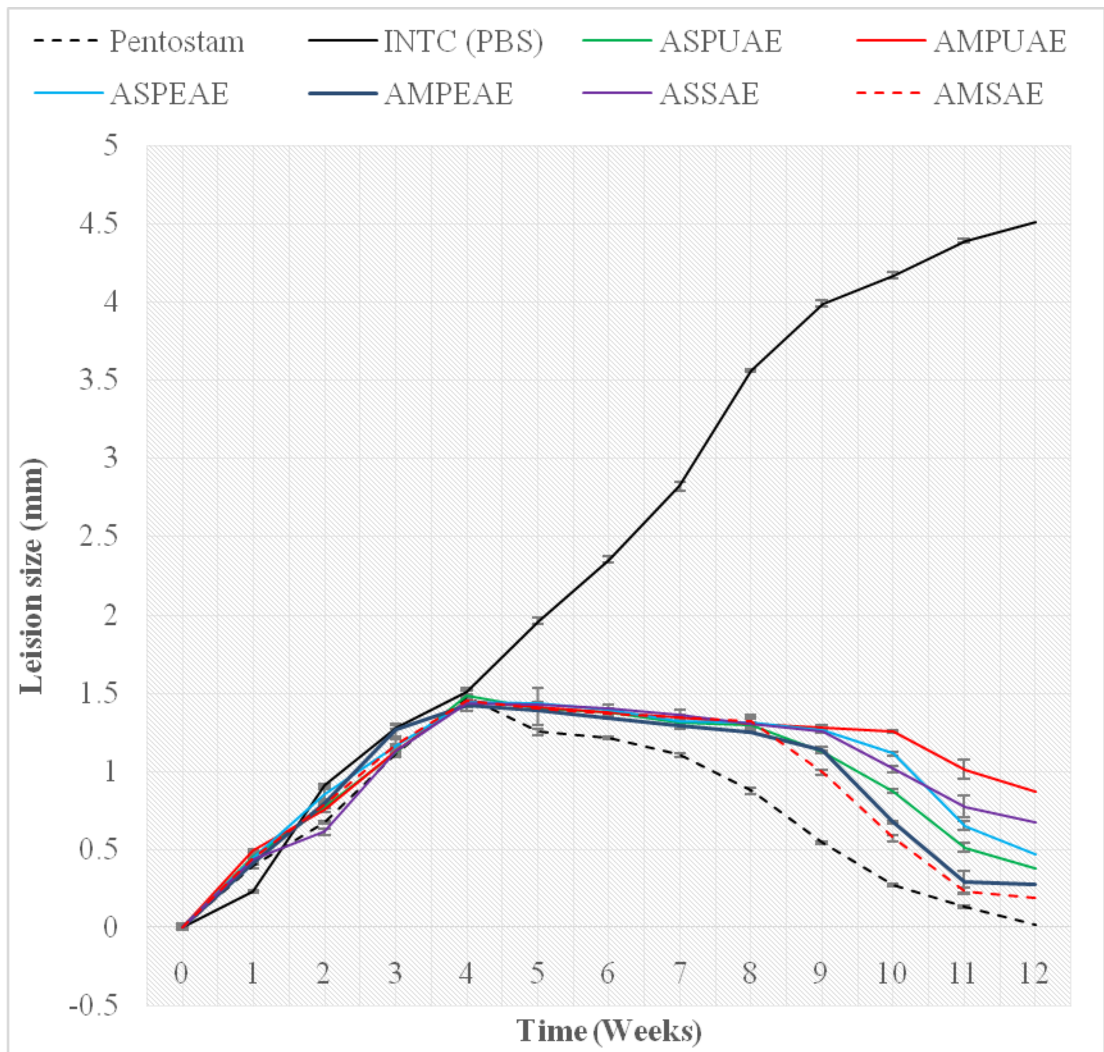


Figure 4.8: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts

The effects of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds methanol extracts on lesion size is presented in Figure 4.9. The AMSME and AMPEME had a greater effect in reduction of lesion sizes followed by ASSME and AMPUME whereas the SPEME and AMPUME had the lowest rate of reduction in leision sizes.

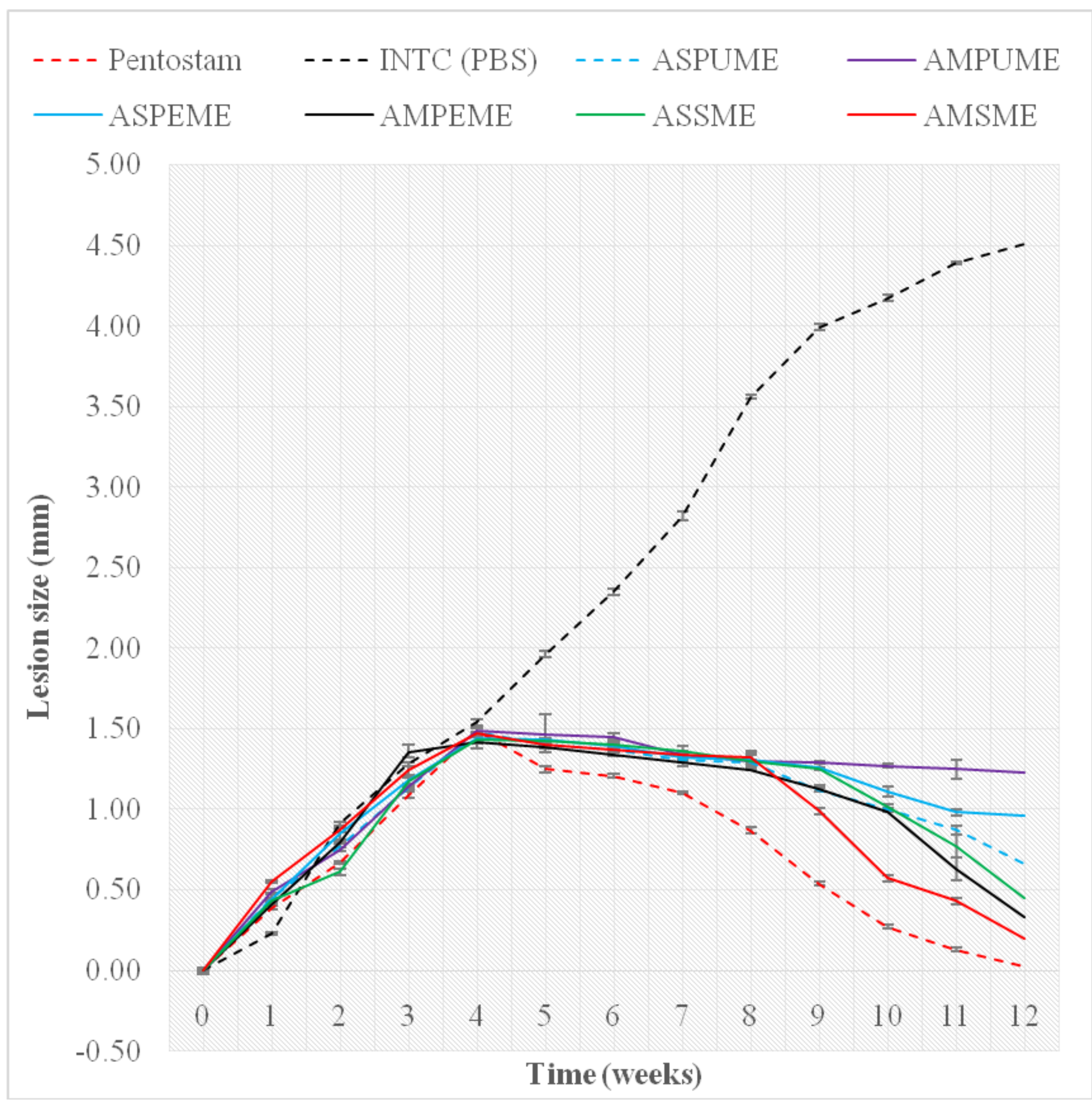


Figure 4.9: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds methanol extracts

The *A. muricata* and *A. squamosa* fruts pulp, peel and seeds ethyl cetate extracts reduced the lesion sized of the *L. major* infected BALB/c mice(Figure 4.10).However, the extract from the peel (AMPEEAE and ASPEEAE) had the higest rate of lesion size reduction. This was followed by the seeds exreact from *A. muricata* (AMSEAE) and pulp extracts (ASPUEAE and AMPUEAE) whereas the seeds extract of *A. squamosa* had the lowest rate of leision size reduction (Figure 4.10)

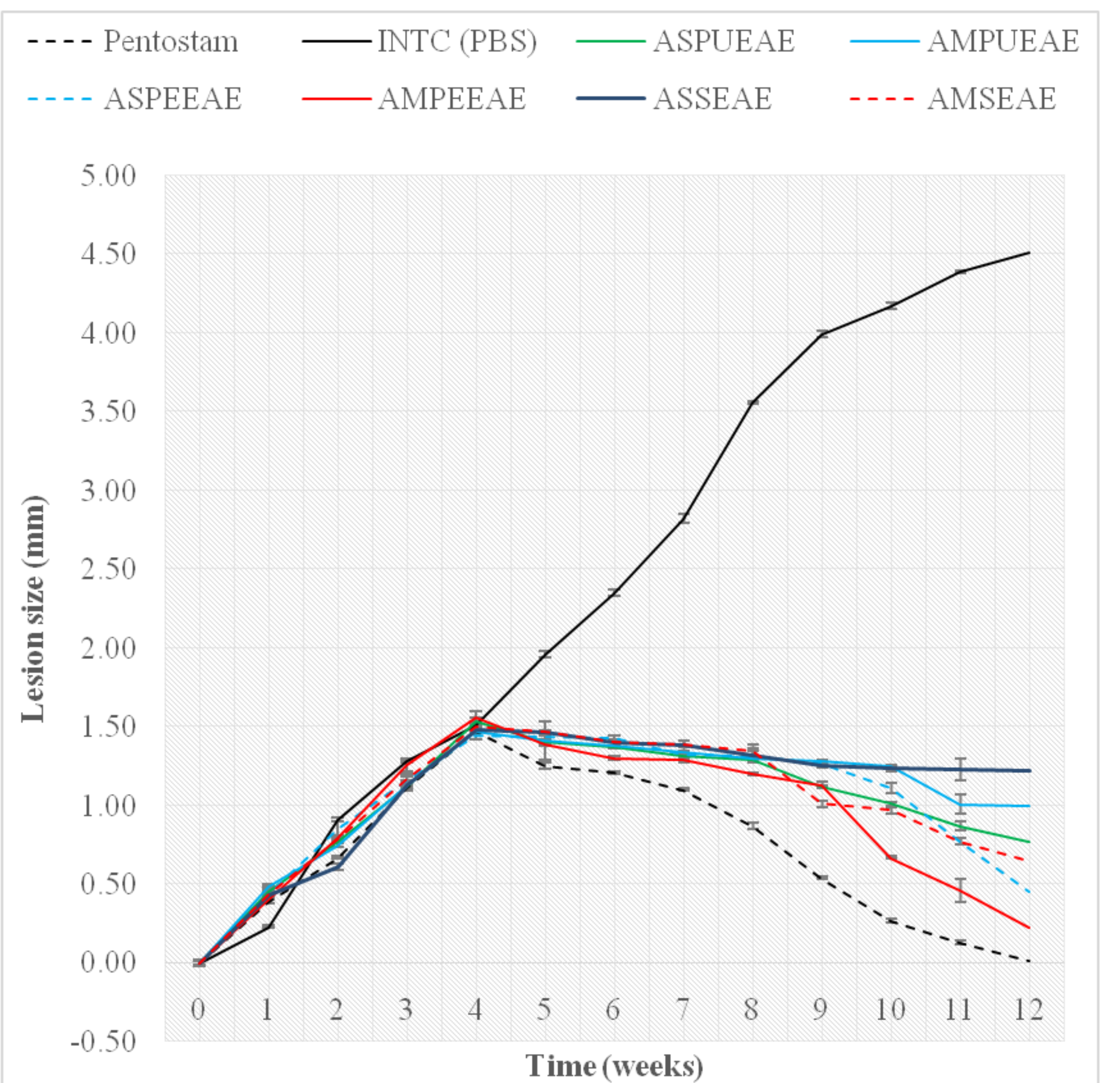


Figure 4.10: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts

Although all the *A. muricata* and *A. squamosa* fruits pulp, peel and seeds hexane extracts had an effect on lesion sizes, the rate of reduction was lowest compared to the aqueous, methanol and ethyl acetate extracts. The effects of *A. muricata* and *A. squamosa* fruits pulp, peel, and seeds hexane extracts in reduction of lesion sizes in L. major infected BALB/c mice is represented in Figure 4.11.The extracts from the peel (AMPEHE and ASPEHE) had the higest rate of lesion size reduction. The pulp extracts (ASPUHE and AMPUHE) had the lowest lesion sizes reduction rate while the seeds extracts (AMSHE and ASSHE) were in the middle between the peel and seeds extracts.

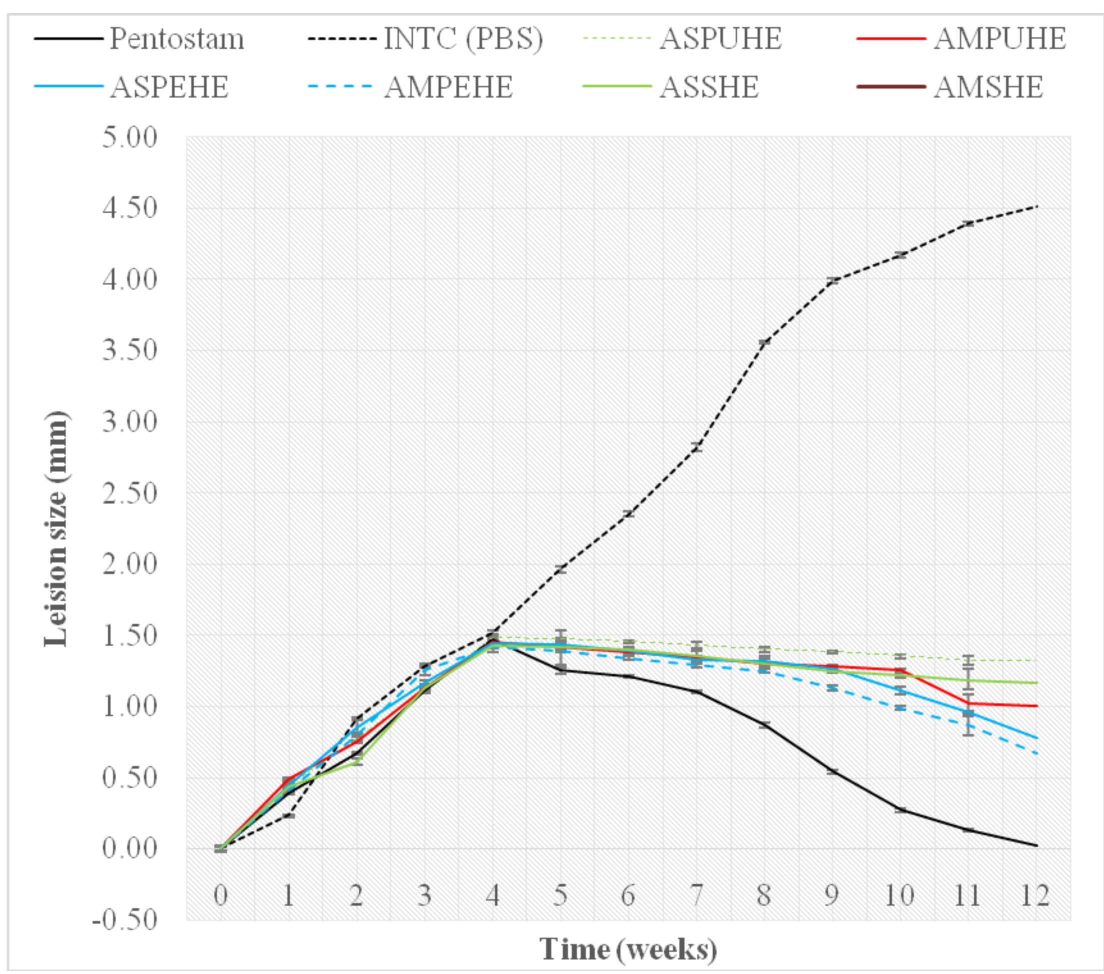


Figure 4.11: Leision size in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts

4.4.2.2 Liver and splenic lenth and size and parasitic barden

After the onset of the infection, a daily 50 mg/Kg bwt dose of each of the tested extracts within the experimental period of 3 to 4 months resulted in reductions in liver and the spleen amastigote burden (Table 4.27). In the liver, the parasite burden (LDU) ranged from 6.34±1.12 (ASPEAE) to 40.95±3.75 (ASPUHE) whereas in the spleen it was in the range of 0.13±0.01 (ASPEAE) to 0.93±0.001 (ASPUHE).

Table 4.27: Relative weight and length of liver and spleen and LDU in*L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa*pulp, peel and seeds extracts

Treatment	Relative weight (mg)		Relative length (mm)		LDU (10^6)	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
NIC	1.36±0.02	0.14±0.01	22.49±0.14	15.83±0.44	ND	ND
INTC	2.66±0.04	2.48±0.07	47.50±0.24	40.33±0.64	564.59±13.24	13.28±0.42
ASPUAE	1.47±0.01	0.71±0.01	25.47±2.66	18.87±1.12	7.15±1.09	0.13±0.02
ASPUME	1.85±0.03	1.04±0.12	29.44±3.97	23.49±1.67	33.37±2.27	1.01±0.05
ASPUEAE	1.55±0.12	1.12±0.11	28.54±2.39	19.87±1.87	25.34±2.39	1.10±0.11
ASPUHE	1.67±0.08	0.98±0.09	26.77±1.67	27.55±2.67	40.95±3.75	0.93±0.001
ASPEAE	1.42±0.06	0.82±0.02	28.56±1.47	20.33±2.27	6.34±1.12	0.13±0.01
ASPEME	1.49±0.09	0.65±0.03	24.57±3.47	21.15±3.21	8.96 ± 1.82	0.53±0.45
ASPEEAE	1.64±0.07	0.97±0.13	29.01±2,49	26.56±2.38	21.27±2.69	0.93±0.012
ASPEHE	1.57±0.16	1.09±0.11	25.38±1.12	24.45±1.12	15.67±3.44	1.12±0.13
ASSAE	1.39±0.02	0.23±0.02	26.67±1.12	19.67±1.12	10.87±1.64	0.22±0.02
ASSME	1.38±0.12	0.19±0.01	27.98±3.67	18.17±2.11	5.57±1.28	0.23±0.02
ASSEAE	1.78±0.08	1.12±0.16	33.33±1.37	20.22±2.39	55.67±6.67	0.77±0.12
ASSHE	1.82±0.18	1.45±0.18	30.67±3.67	21.12±2.12	94.58±4.54	0.85±0.17
AMPUAE	1.67±0.11	1.03±0.06	23.54±1.54	28.46±3.39	35.37±4.67	0.75±0.08
AMPUME	1.77±0.19	1.67±0.15	26.67±1.57	25.56±1.77	23.34±3.37	0.56±0.13
AMPUEAE	1.59±0.14	1.28±0.09	24.49±2.38	23.34±1.12	78.12±3.67	0.55±0.11
AMPUHE	1.91±0.17	1.85±0.12	27.67±2.27	24.43±2.27	73.46±5.12	0.45±0.19
AMPEAE	1.40±0.13	0.77±0.04	26.67±2.11	19.67±1.12	9.45±1.12	0.39±0.04
AMPEME	1.41±0.18	0.85±0.01	25.47±2.39	21.11±2.46	9.28±2.01	0.29±0.02
AMPEEAE	1.36±0.16	0.67±0.02	30.34±2.17	18.98±1.77	8.97±1.12	0.35±0.02
AMPEHE	1.75±0.15	1.00±0.11	31.12±2.12	27.27±3.12	18.76±1.77	0.67±0.42
AMSAE	1.43±0.15	0.34±0.03	28.66±3.02	20.23±2.29	9.28±1.01	0.42±0.02
AMSME	1.37±0.13	0.57±0.01	24.47±2.28	21.54±1.67	7.35±1.12	0.55±0.03
AMSEAE	1.69±0.12	1.23±0.18	24.14±1.47	27.12±3.39	37.15±1.09	0.45±0.19
AMSHE	1.75±0.16	1.11±0.12	25.45±2.23	24.54±3.13	42.33±3.33	0.10±0.003
Pentostam	1.35±0.08	0.18±0.01	24.53±2.29	18.54±2.49	2.22 ± 0.13	0.10±0.003

Mean values (n=3) ± SEM.

The infection as also characterized by presence of the parasites in the liver and spleen (Plate 4.3) and increase in relative weight and length of the liver and spleen (Plate 4.4) as a result of accummulation of the parasites in these organs. However after treatment with the extracts there was a reduction in the relative weight and lenth of the liver and spleen (Table 4.20)

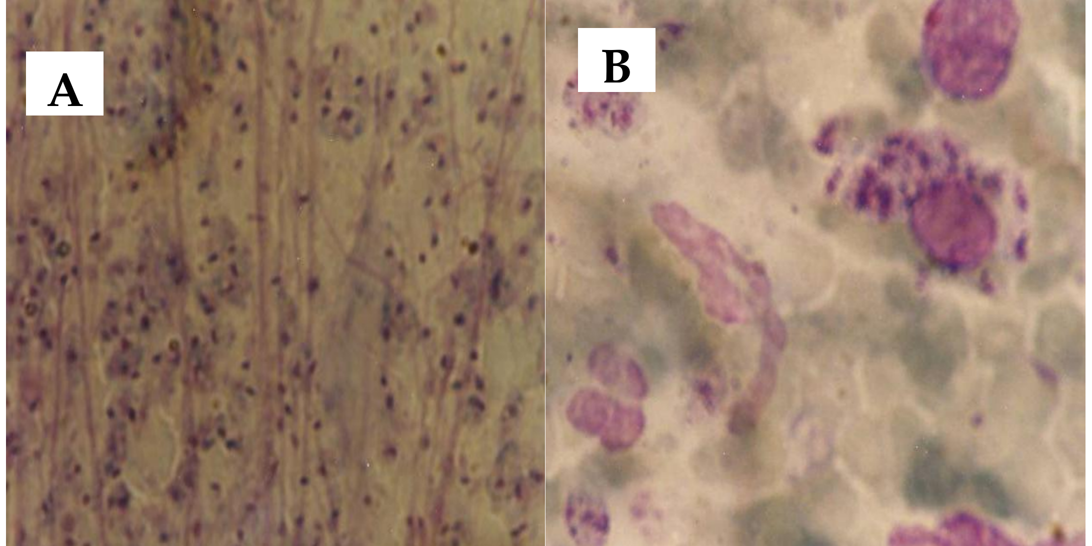


Plate 4.3: Impression smears of liver (A) and spleen (B) on 12th week showing dissemination of *L. major* amastigotes inside and outside macrophages

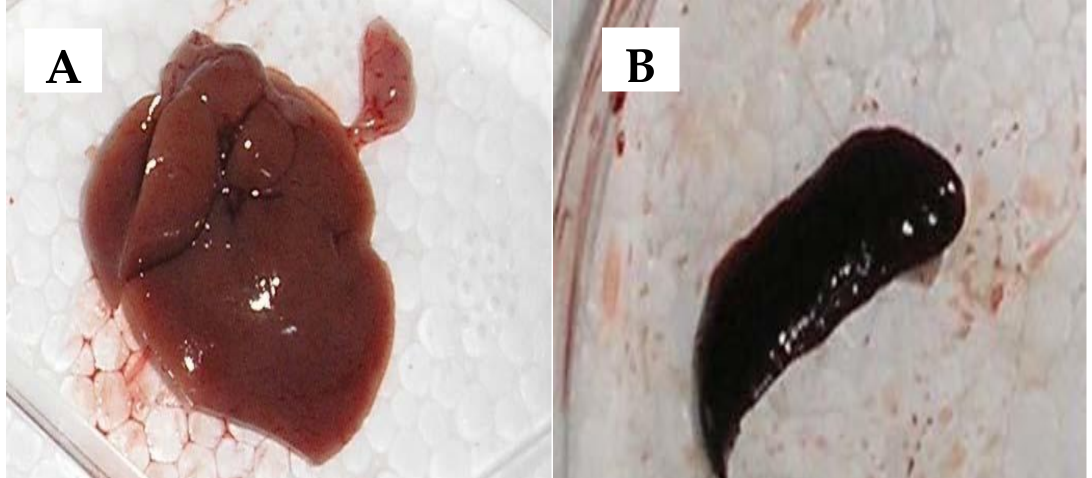


Plate 4.4: Enlarged liver (A), Enlarged spleen (B) with weight (3.16±0.06 gm) and length (51.10±0.38 mm) respectively in BALB/c mice on 12th week

4.4.2.3 Body weight changes in BALB/c in antileishmanial activity study

The severity of infection in the INTC groups increased dramaticallyas illustrated by an increase in relative weight, LDU and positive for amastigotes of the liver and spleen.Further, all the INTCBALB/c mice groups showed poor health status and great body weight loss. However, deaths did occur in all the extracts treated groups. The NIC group showed a greater body weight gain throughout the experimental period. Before treatment withaqeous extracts, the animals showed considerable weight loss (Figure 4.8). A low overall body weight gain was observedin the order: AMSAE > AMPEAE > ASPEAE > ASSAE > AMPUAE > ASPUAE.

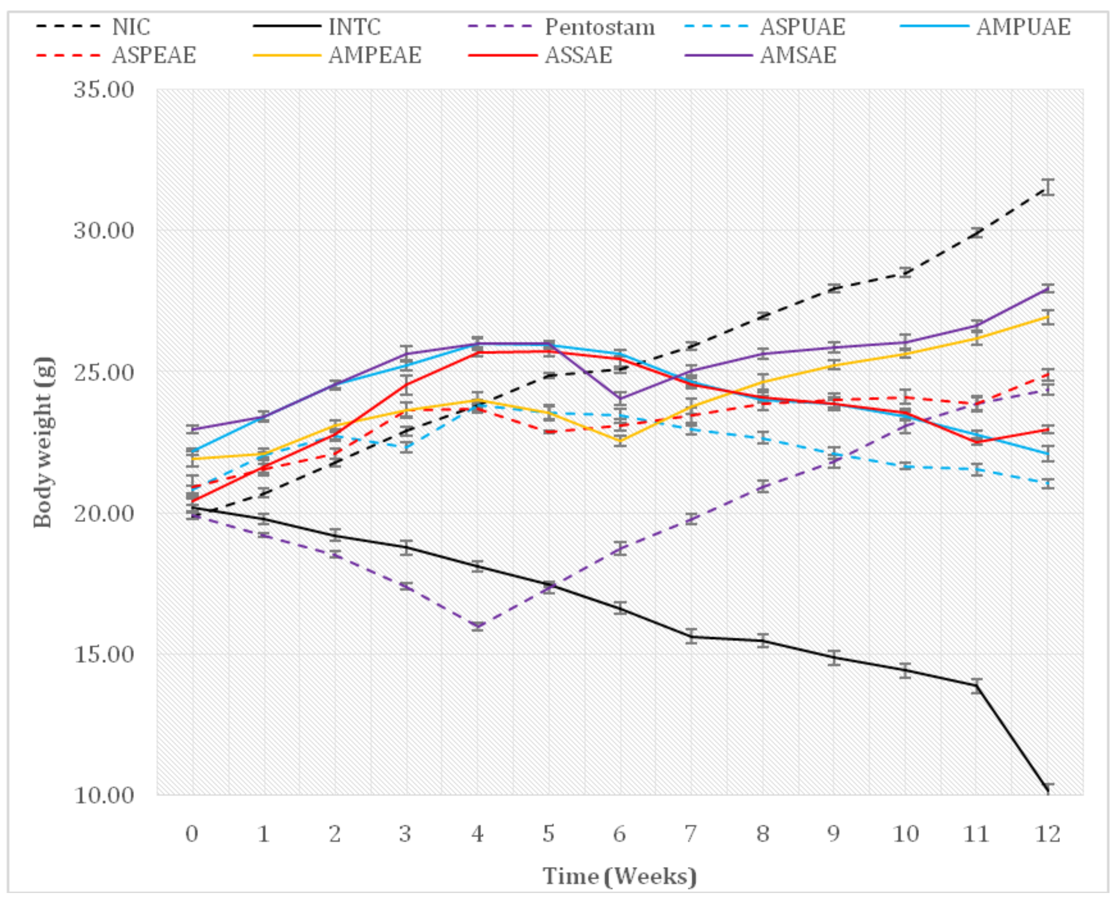


Figure 4.12: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds aqueous extracts

The effects of methanol extracts on body weigh before and after treatment are presented in Figure 4.9. The mice showed weight loss before treatment. Body weight gain was observed in ASPEME, AMPEME, ASSME and AMSME treated groups while AMPUME and ASPUME group showed a decrease in weight even after treatment. The gain in body weight in ASPEME, AMPEME, ASSME and AMSME treated groups of BALB/c mice were higher than the body weight gains in the Pentostam treated group.

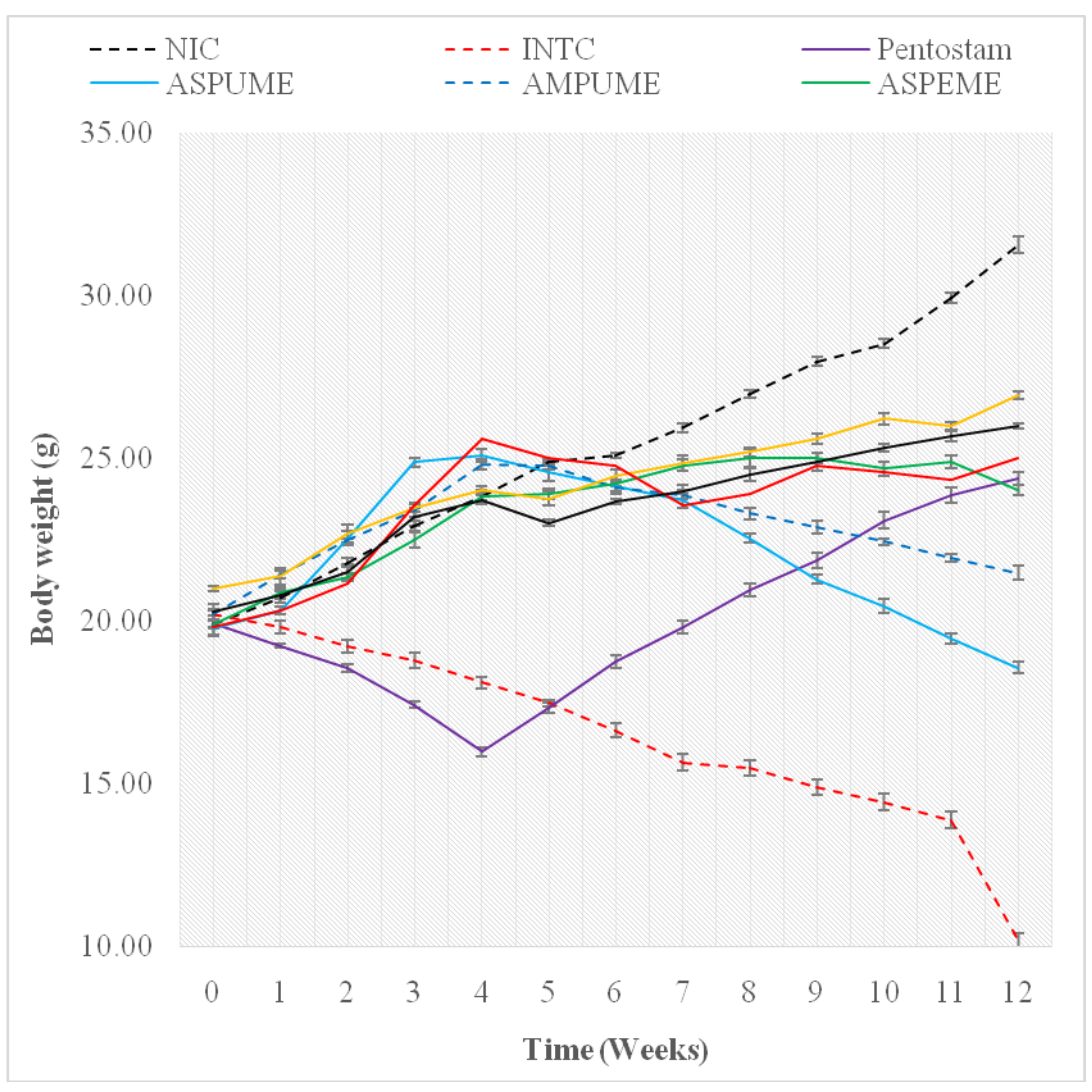


Figure 4.13: Body weight changes in*L. major* infected BALB/C mice treated with *A. muricata*and *A. squamosa*pulp, peel and seeds methanol extracts

The effects of *A. muricata* and *A. squamosa* fruits ethyl acetate extracts on body weight of BALB/c mice before and after treatment is presented in Figure 4.10. The AMPEEAE group showed a higher gain in body weight than pentostam group. This was followed by AMSEAE, AMPUEAE, ASPEEAE, ASSEAE and ASPUEAE in that order.

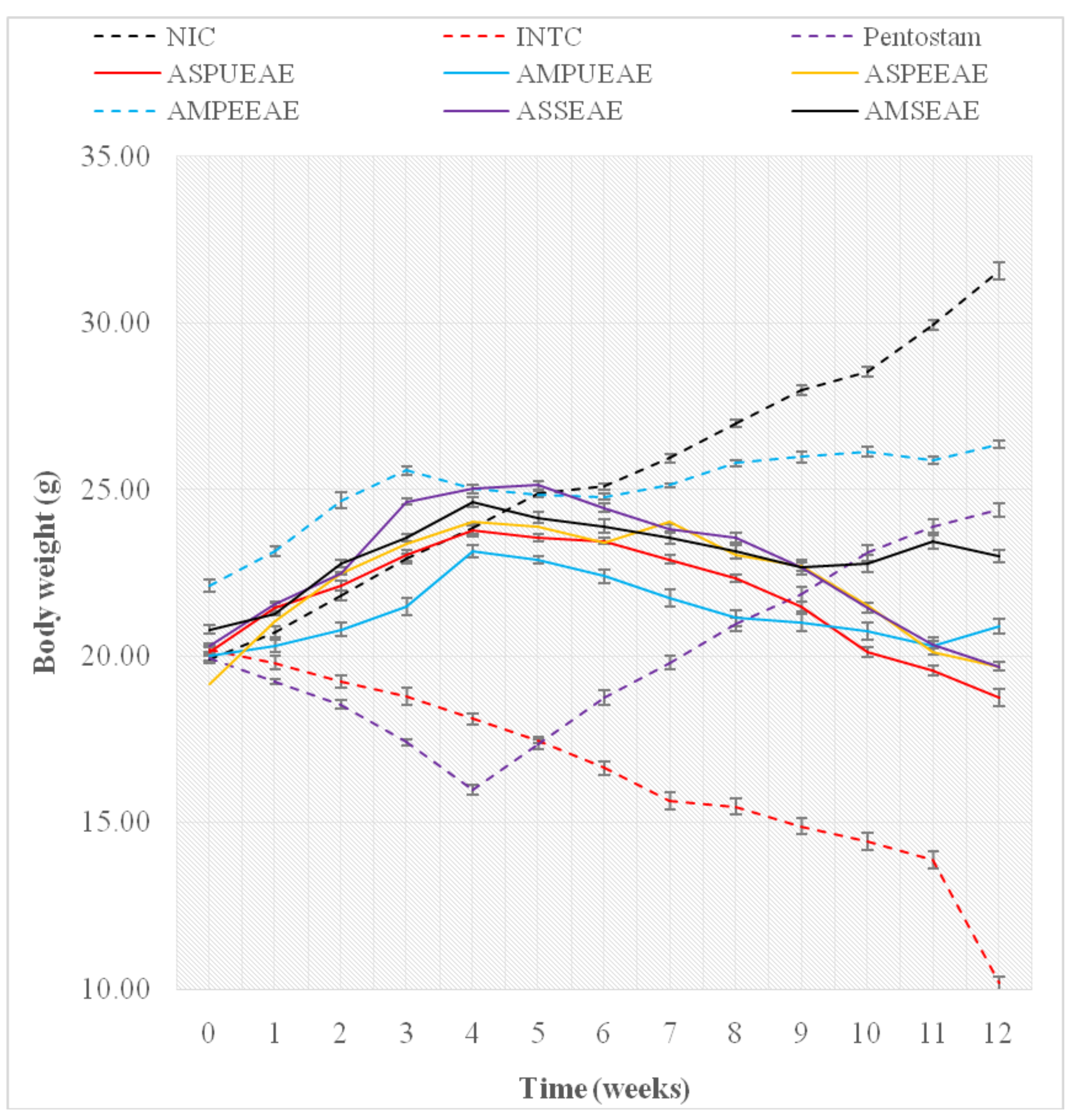


Figure 4.14: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds ethyl acetate extracts

The animals showed weight loss before treatment but a body weight gain were observed in AMPEHE, AMSHE and AMPUHE groups. However, ASPEHE, ASSHE and ASPUHE did not gain weight **e**ven after treatment (Figure 4.11). The AMPEHE, AMSHE and AMPUHE groups had a greater body weight gain as comapared to the standard drug (pentosam).

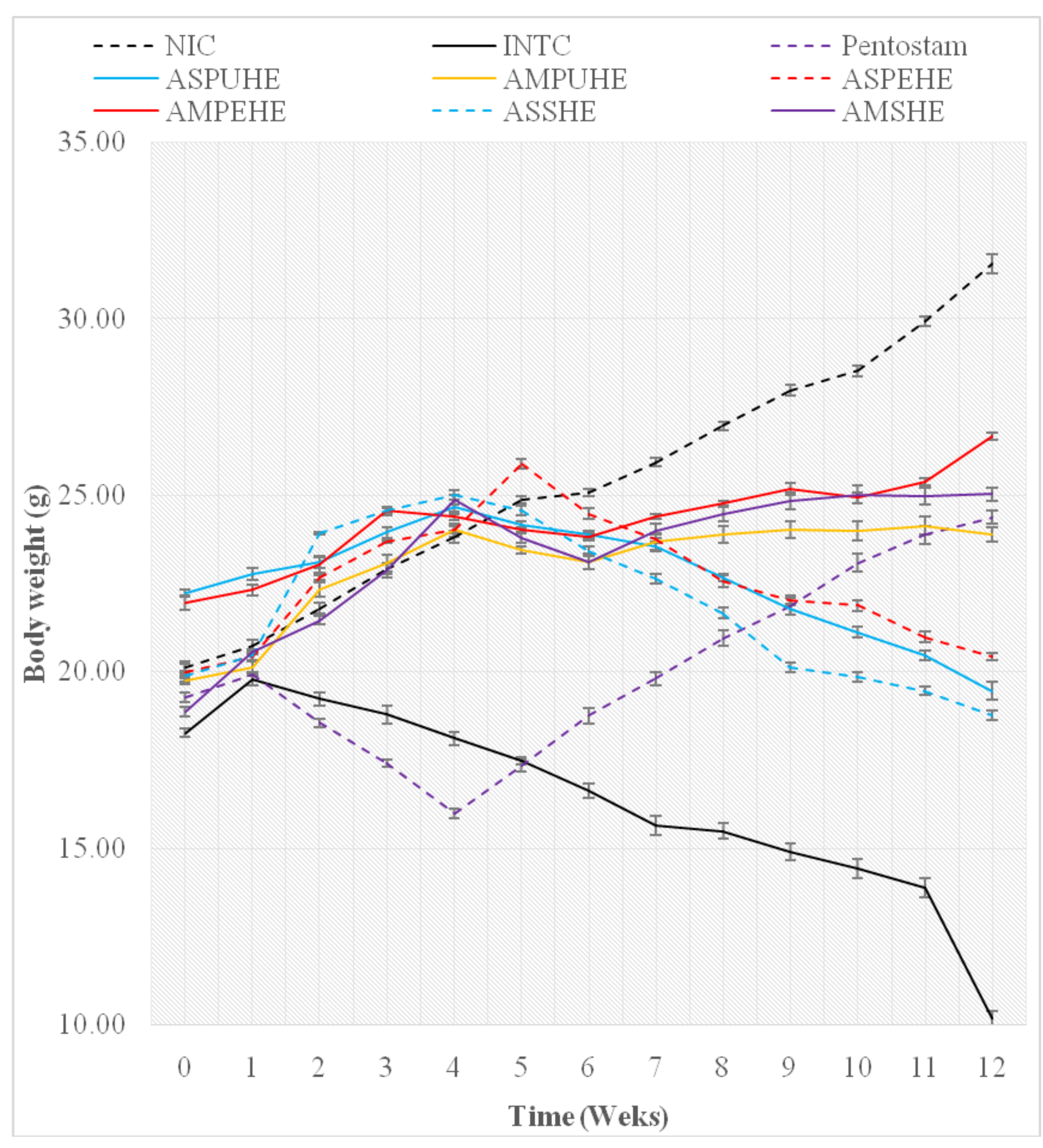


Figure 4.15: Body weight changes in *L. major* infected BALB/c mice treated with *A. muricata* and *A. squamosa* pulp, peel and seeds hexane extracts

4.4.3 Determination of safety of the extracts

4.8.3.1 *In vitro* toxicity studies

The *in vitro* cytotoxic activities as demonstrated by the IC_{50} values of the extracts against normal mammalian cells (vero cells) are shown in Table 4.28. Based of the classification scheme as outline by OEDC, (2008), the extracts ranged from highly toxic with IC_{50}; 2.54±0.44 μg/mL (AMPUHE) to 7.55±1.19 μg/mL (AMPUME), toxic with IC_{50}; 12.85±2.80 μg/mL to 94.07±5.81 μg/mL (AMPUEAE) and moderately toxic with IC_{50}; 104.81±1.16 μg/mL (AMPEME) to 292.94±10.10 μg/mL (AMPUAE). Eight of the extracts were highly toxic extracts (ASPUME, ASPEHE, ASSAE, AMPUME, AMPUME, AMPUHE, AMPEAE and AMPEHE), 9 extracts were toxic (ASPEAE, ASPEEAE, ASSME, ASSEAE, AMPUEAE, AMSAE, AMSEAE and AMSHE) while 7 extracts were moderately toxic (ASPUAE, ASPUEAE, ASPUHE, AMPUAE, AMPEME and AMSME)

Table 4.28: Cytotoxicity activities of *A. muricata* and *A. squamosa* pulp, peel and seeds extracts on normal mammalian cells (vero cells)

Extract	IC_{50} (μg/mL)
ASPUAE	250.94±6.10
ASPUME	7.55±1.19
ASPUEAE	129.41±8.89
ASPUHE	114.36±8.28
ASPEAE	74.58±3.49
ASPEME	104.88±9.80
ASPEEAE	24.07±3.72
ASPEHE	5.30±1.72
ASSAE	3.67±1.47
ASSME	19.51±3.67
ASSEAE	12.85±2.80
ASSHE	6.45±2.14
AMPUAE	292.94±10.10
AMPUME	2.75±1.07
AMPUEAE	94.07±5.81
AMPUHE	2.54±0.44
AMPEAE	4.82±1.16
AMPEME	104.81±1.16
AMPEEAE	75.74±3.55
AMPEHE	3.49±1.11
AMSAE	29.25±1.18
AMSME	116.49±7.12
AMSEAE	22.85±2.80
AMSHE	17.83±1.42
Pentostam	0.267±0.02

Mean values (n=3) ± SEM

4.4.3.2 *In vivo* acute toxicity testing

All the mice survived the treatment and appeared normal before, during and post-treatment period of 72 hours without presenting anyabnormal clinical signs(hyper- or hypo-activity, aggression, unusual locomotion, catalepsy, prostration, skin edema or redness, loss of body hair, piloerection, apnea, cyanosis, convulsions, salivation, lacrimation, corneal opacity, conjunctivitis, diarrhea, constipation, tremor, hypotonia, hypertonia).

4.4.3.2 *In vivo* subacute toxicity testing

4.4.3.2.1 Body weight changes of BALB/c mice in subacute toxicity study

In the acute oral toxicity studies, no mortality was observed even at the highest dose (2500 mg/Kgbwt) in all mice groups after the fourteenth day of post extracts administration suggesting an LD_{50}> 2500 mg/Kg bwt. The effectsof *A. muricata*and *A. squamosa*fruits pulps, peels and seeds aqueous, methanol, ethyl acetate and hexane extracts on body weight of BALB/c mice in subacute toxicity study is presented in Appendix V-X. Variable changes in body weights were recorded in all groups of mice. Overall in all extracts tested and control (DSMO) groups of BALB/c mice gained weight. However, the rate of weight gains in the 100 mg/Kg extracts treated groups was lower compared to that of the control (DMSO) groups. Weight loss was recorded in the groups of mice that received doses >500 mg/Kgbwt. Significant decreases in body weight was recorded in the dose 1000mg/Kg bwt on 12th and 14^{th}day ($p<0.05$) and 2500 mg/Kg on 10th, 12^{th}and14^{th} day ($p<0.05$) of post extracts administration. However, no reduction in water and food consumption was observed. Weight gain was recorded in all the 100 mg/Kg bwt extracts dosage groups which were not significantly different ($p>0.05$) from those of the control (DSMO) groups.Overall, treatment with*A. muricata* and *A. squamosa* fruits pulps, peels and seeds aqueous, methanol, ethyl acetate and hexane extracts lead to an insignificant increase ($P>0.05$) in body weight in all doses up to a maximum in either $6^{th}$and 8^{th}day of post extracts treatment depending of the extract (Appendix V-IX)**.** The increasing body weight with increased dosage of the extracts reversed in the 8^{th}day of post extracts treatmet in majority of the extracts. A significant decrease in body weight was observed in the 10th, 12th, and 14th day of post extracts treatment

4.4.3.2.2 Effects of the extracts on organs in subacute toxicity study

The effects of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds aqueous, methanol ethyl acetate and hexane extracts in BALB/c mice on relative weights of organs (organ weight/animal weight); heart, kidney, liver, lungs, spleen and stomach were evaluated. Macroscopic examination did not show any changes in the colour of organs of the extracts treated groups compared withcontrol (DMSO)group. The effects of *A. muricata* and *A. squamosa* fruits aqueous, methanol, ethyl acetate and hexane extracts on on relative weights of organs (organ weight/animal weight); heart, kidney, liver, lungs, spleen and stomach of BALB/c mice is represented in Appendix XIa-c and Appendix XIIa-c. There were no significant ($p>0.05$) effects in relative weights of liver, kidney and heart and spleen on the extracts treated groups compared to the control (DMSO) at low doses of the extracts (<500 mg/Kg bwt). However, a significant ($p<0.05$) effect of the extracts treated groups were observed on the relative weight of the stomachwhen compared to the control (DMSO groups) groups. An insignificant ($p<0.05$) increase in weights of heart, kidney, liver, lungs and spleen was recorded in higher doses of the extracts (500, 1000 and 2500 m/Kg bwt). Further, insignificant dose-dependent decreases in relative weight of organs were observed in extracts treated groups compared to control (DMSO).

4.4.3.2.4 Haematological profile of BALB/c mice in subacute toxicity study

The effects of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds aqueous, methanol, ethyl acetate and hexane extracts on haematological parameters of BALB/c mice is represented in Appendix XIIIa-c and Appendix XIVa-c.The BALB/c mice treated with *A. muricata* and *A. squamosa* aqueous, methanol, ethyl acetate and hexane extracts showed a significant increase ($p<0.05$) in WBCs and lymphocytes levels at high extracts doses (Appendix XIIIa-c and Appendix XIVa-c).A general decrease in haematological indices (RBCs, HGB, PCV, MCV, MCH and MCHC) was observed in all pulp and peel extracts treated groups in lower doses, which increased with dosage increase, was observed compared to theDMSO (control) group. However, this trend was observed to deviate in some extracts treated groups of BALB/c mice compared to the DMSO (control) (Appendix XIIIa-c and Appendix XIVa-c). It was observed that all the groups of BALB/c mice treated with

A. muricata fruit seeds extracts (AMSAE, AMSME, AMSEAE and AMSHE) had elevated levels of HGB, MCV, MCH and MCHC when compared to the DMSO (control) group. In contrast to extracts from the pulp and peel of both *A. muricata* and *A. squamosa* fruits, such as AMPUAE and ASPEAE, the RBC, HCT, HGB, MCV, MCH and MCHC decreased insignificantly in lower dosage and increased in higher dosage in the extracts treated groups compared to the DMSO (control) group.Further, a dose dependent increase in WBCs and lymphocytes was recorded in all the extracts treated groups when compared to the DMSO (control) group.

4.4.3.2.5 Biochemical parameter of BALB/c mice in subacute toxicity study

The effects of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds aqueous, methanol, ethyl acetate and hexane extracts on biochemical parameters of BALB/c mice is represented in Appendix XVa-c and Appendix XVIa-c. In all the extracts tested, there were significant decreases ($p<0.05$) in serum glucose levels especially in BALB/C mice treated with 1000 mg/Kg and 2500 mg/Kg compared with vehicle group. Further, there were insignificant decreases ($P>0.05$) in albumin levels coupled with significant increases ($p<0.05$) in serum creatinine levels only in BALB/c mice treated with the highest dose (2500 mg/Kg) in all the extracts tested. However, there were significant decreases ($p<0.05$) in serum cholesterol and HDL levels in the BALB/c mice treated with 100 mg/Kg extracts which reversed with increased doses of the extracts in all the experimental groups compared to the DMSO (control) group also in all the extracts tested. An insignificant decrease in the levels of serum urea ($P>0.05$) was observed at lowere doses (100 mg/Kg), which reversed upon increase in the dosage in all the extracts treated group compare to the DMSO (control) group.The creatine and LDLs increased significantly ($P<0.05$) in a dose dependent manner in all the tested extracts as compared to the DMSO (control) group. Further, in all the extracts treated groups, an insignificant ($P>0.05$) dose-independent decrease in ALT activity and a dose-dependent increase in ASTand ALkP activities were observed. Amylase levels also increased significantly ($P<0.05$) in a dose dependent monner in all extracts treated group copared to the DMSO (control). The levels of serum TAGs decreased significantly ($P<0.05$) in a dose dependent manner whereas there were no changes in albumin

4.5 DNA Binding and DNA Topo I inhibitory activity

4.5.1 DNA Binding activity

The crude plant extracts are complex matrices containing several phytochemicals. Therefore, the IC_{50} values of the studied extracts were expected to be higher than those for pure compounds. The IC_{50} values and the percentage decrease in absorbance obtained for the DNA-methyl green assay as an indicator of plant extract(s) DNA binding interaction are presented in Table 4.34. The extracts with higher IC_{50} values had low percentage inhibition than the extracts with low IC_{50} values (Table 4.47). The recorded percentage decrease in absorbance of the aqueous extracts (ASPUAE, ASPEAE, ASSAE, AMPUAE, AMPEAE and AMSAE) ranged between 18.14±2.67% (AMPUAE) and 38.06±1.47 (ASPEAE) with IC_{50} values of ranges between 67.66±2.44 µg/mL (ASPEAE) and 137.44±33.33 µg/mL (AMPUAE). The methanolic extracts (ASPUME, ASPEME, ASSME, AMPUME, AMPEME and AMSME) followed with percentage decrease in absorbance ranges between 17.14±2.67% (AMSME) and 41.01±1.09% (AMPEME), IC_{50} values of 62.97±3.37 µg/mL (AMPEME) and 159.79±9.44 µg/mL (AMPUME). The ethyl acetate extracts (ASPUEAE, ASPEEAE, ASSEAE, AMPUEAE, AMPEEAE and AMSEAE) percentage decrease in absorbance ranged between 9.05±1.67% (AMSEAEA) and 20.50±2.01% (AMPEEAE) with IC_{50} values of 115.87±7.67 µg/mL (AMPEEAE) and 171.99±19.47 µg/mL (AMSEAEA). The hexane extracts (ASPUHE, ASPEHE, ASSHE, AMPUHE, AMPEHE and AMSHE) had the lowest affinity to bind DNA with binding percentages decrease in absorbance ranging between 4.04±1.12% (AMSHE) and 10.09±1.39% (AMPUHE) with IC_{50} values of 165.97±24.65 µg/mL (AMPUHE) and 270.98±37.57 µg/mL (AMSHE).

Table 4.29: Percentage decrease inabsorbance of DNA methylene green and IC_{50} values of *A. muricata*and *A. squamosa*pulp, peel and seeds aqueous, methanol, ethyl acetae and hexane extracts

Extract	% Decrease in Absorbance	IC_{50} (μg/mL)
ASPUAE	20.50±2.01	108.42±7.67
ASPUME	18.14±1.12	133.66±11.45
ASPUEAE	11.14±1.67	156.17±5.67
ASPUHE	7.05±1.98	223.20±32.89
ASPEAE	38.06±1.47	67.66±2.44
ASPEME	22.65±2.28	104.45±4.67
ASPEEAE	12.14±1.67	158.08±15.55
ASPEHE	6.77±1.11	227.59±41.87
ASSAE	26.46±1.37	95.69±9.01
ASSME	36.76±1.47	66.80±6.07
ASSEAE	16.14±3.01	147.54±13.09
ASSHE	8.84±1.27	177.20±21.97
AMPUAE	18.14±2.67	137.44±33.33
AMPUME	11.74±2.67	159.79±9.44
AMPUEAE	19.94±2.00	130.59±6.44
AMPUHE	10.09±1.39	165.97±24.65
AMPEAE	26.76±1.47	80.99±4.48
AMPEME	41.01±1.09	62.97±3.37
AMPEEAE	20.50±2.01	115.87±7.67
AMPEHE	8.01±2.19	189.54±28.65
AMSAE	30.67±1.47	77.46±5.67
AMSME	17.14±2.67	131.73±10.77
AMSEAE	9.05±1.67	171.99±19.47
AMSHE	4.04±1.12	270.98±37.57
Positive (Cucurbitacin)	87.67±3.45	20.19±1.12
Negative (Dexamethasone)	2.33±0.98	33.76±2.02

Mean values ± SEM, (n=3)

Out of the 24 extracts studied, none of them had IC_{50} values 0 – 50 μg/mL while 6 extracts had IC_{50} values between 50 μg/mL – 100 μg/mL. The IC_{50} ranging between 100 μg/mL – 150 μg/mL had 8 extracts, the range of 150 μg/mL – 200 μg/mL had 7 extracts while only 3 extracts had IC_{50} values <200 μg/mL.

4.5.2 DNA Topoisomerase I inhibitory activity

Eight (8)of the 24extracts with percentage inhibition ranging between 17.14±2.67% (AMSME) and 40.01±1.09% (AMPEME) with IC_{50} values ranging between 62.97±3.37 (AMSME) and 131.37±10.77 (AMSME) were investigated for their DNA Topo I inhibitory activities. Screening of these eight extracts (ASPEAE, ASPEME, ASSAE, ASSME, AMPEAE, AMPEME, AMPEEA and AMSAE) indicated activity against Topo I mediated relaxation of supercoiled DNA at the concentration of 100 μM (Figure 4.19).

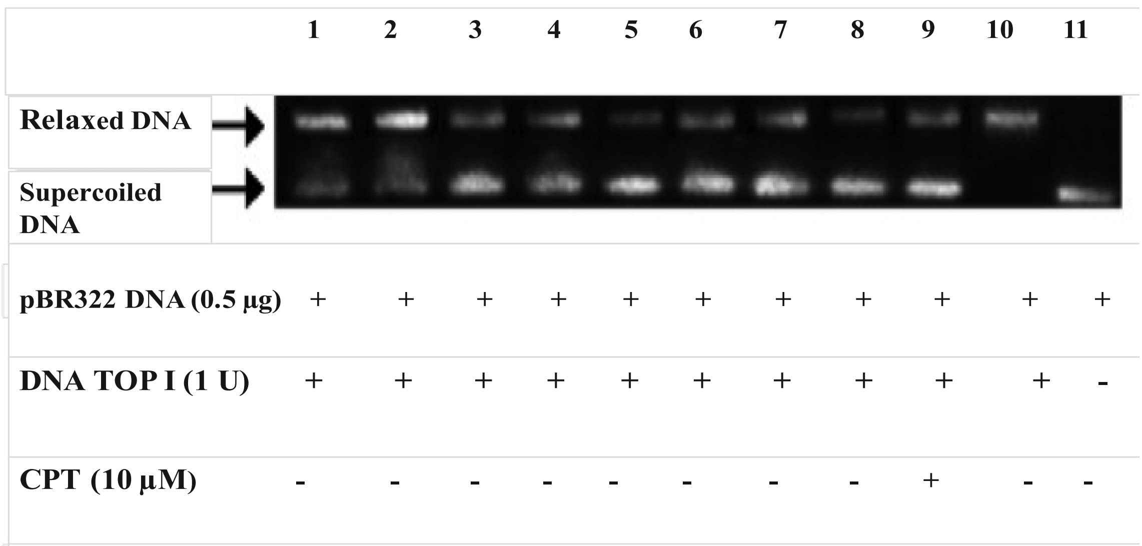


Figure 4.16: DNA Topo I inhibitory activities ofExtractsat 100 µM. Extracts (DNA + Topo I + tested compounds);ASPEAE, ASPEME, ASSAE, ASSME, AMPEAEAMPEME, AMPEEA andAMSAE in lane 1-8 respectively. Positive control (DNA + Topo I + CPT) lane 9.Negative control (DNA + Topo I) lane 10 and DNA alone lane 11

The Topo I inhibition activity of the eitht (8) hits were further tested at lower concentrationsof 5 µM and 25 µM. At a concentration of 25 µM,fiveextracts in lane 3, lane 4, lane 5, lane 6 and lne 8 showed DNA Top I inhibitory activities (Figure 4.13). Negative Topo I inhibitory acivities ware observed inlane 1, 2 and 7.

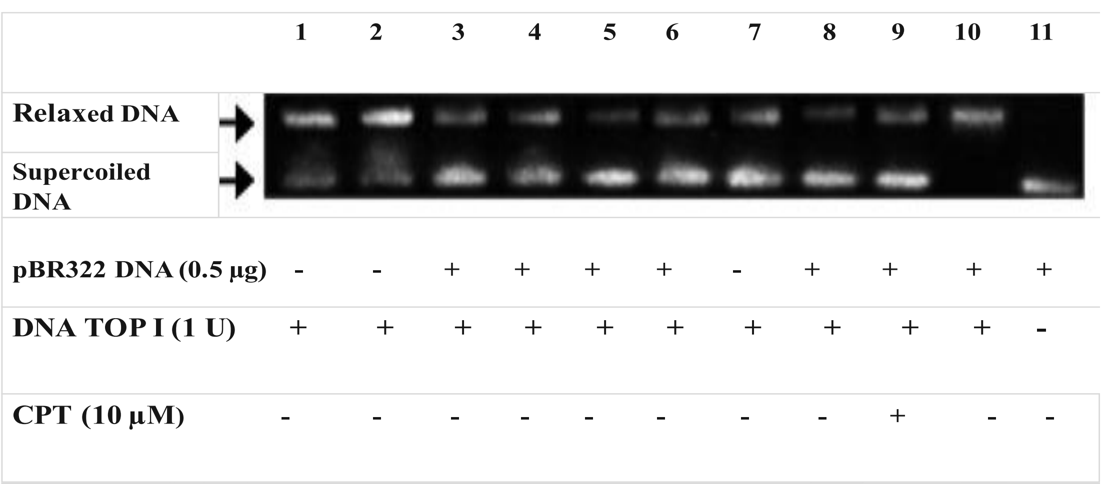


Figure 4.17: DNA Topo I inhibitory activities of Extracts at 25 µM (DNA + Topo I + tested compounds). Positive extracts; AMSAE (lane 1), AMPEME (lane 3), AMPEAE (lane 4), ASSME (lane 5), ASSAE (lane 6) and ASPEAE (lane 8). Negative extracts; AMPEEA (lane 2) and ASPEME (lane 7). Positive control; (DNA + Topo I + CPT)(lane 9, Negative control (DNA + Topo I) (lane 10) and DNA alone (lane 11)

At a concentration of 5 µM three AMPEME (lane 3), ASSME (lne 5) and ASPEAE (lane 8)exhibited DNA Topo I inhibitory activity while negative DNA Topo I inhibitory activity was observed in five *extracts;* AMSAE lane I, AMPEEA lane 2, AMPEAE lane 4, ASSAE lane 6 and ASPEME lane 7(Figure 4.14)**.**

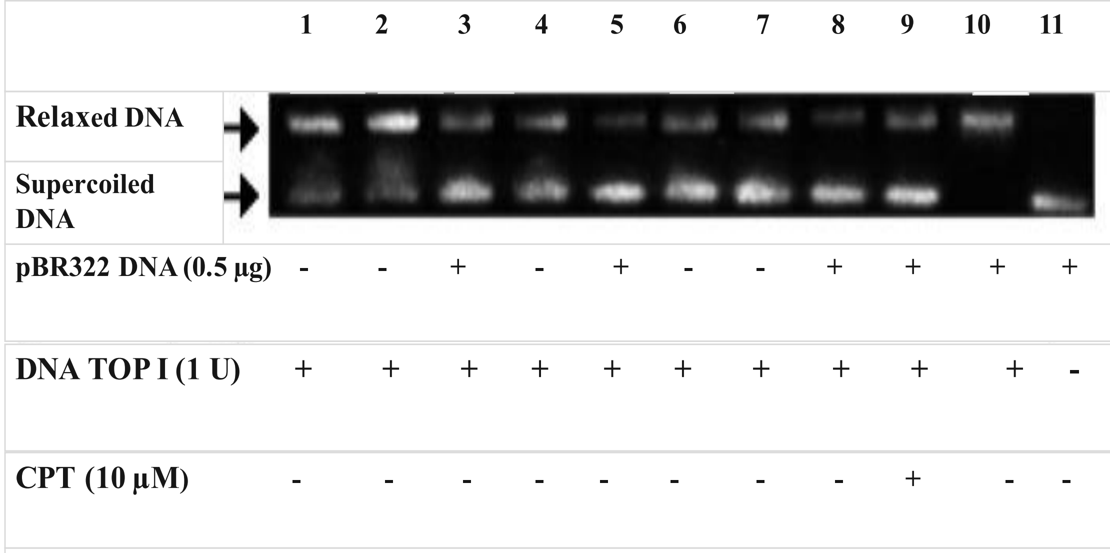


Figure 4.18: DNA Topo I inhibitory activities of Extracts at 5 µM (DNA + Topo I + tested compounds). Positive extracts; AMPEME (lane 3), ASSME (lane 5) and ASPEAE (lane 8). Negative extracts;AMSAE (lane 1), AMPEEA (lane 2), AMPEAE (lane 4), ASSAE (lane 6) and ASPEME (lane 7). Positive control; (DNA + Topo I + CPT)(lane 9). Negative control (DNA + Topo I) (lane 10) and DNA alone (lane 11)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical screening

The percentage yields in dry extracts obtained from *A. muricata* and *A. squamosa* fruits pulps, peels and seeds during extraction were dependent on the water solubility (g/100g), relative polarity and eluent strength of the solvents used. Methanol and water gave the highest extracts percentage yields compared to ethyl acetate and hexane because of their high solubility to water (g/100g), relative polarity and eluent strength (Appendix I). Further, other studies conducted on other types of fruits and foodstuffs (cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin and milk) correlates percentage yields varying depending on the species of the fruit, experimental conditions and the technique used for the extraction (Parry *et al*., 2006; Mariod *et al*., 2010). Although water has greater solubitity, relative polarity and eluent strength than methanol, the high polarity naturally sets limitations to the amount of compounds that can be extracted (Eloff, 1998). The high percentage yields of methanol as a solvent used in extraction of phytochemicals from medicinal plants is in line with previous works (Alawa, 2008). In terms of structure and forms, the methanol and ethyl acetate extracts appeared caramelized and gluey probably because of the high sugar content and other similar compounds extracted by methanol and ethyl acetate (Alawa, 2008) as compared to aqueous or water (pelleted) and hexane (oily). The colours of the extracts ranged from green, yellow to brown. The peelextracts had the deepest colours (Blackish green) followed by that of seeds (Blackish brown) while that of pulpswere light coloured (light brown) (Plate 4.1). However, the colour of the extracts obtained may largely depends on the plant part used during as describe in studies conducted on other plant species (Alawa. 2008).

Tannins, flavonoids, saponins, terpenoids, phenols, glycosides, alkaloids, anthocyanins, steroids and cholesterol, coumarins, fixed oil and fats were detected in one or more parts (pulp, peel and seeds) of *A. muricata*and*A. squamosa*, a finding that is in agreement with other studies (Folorunso & Modupe, 2007; Savithramma *et al.,* 2011; Soni *et al.,* 2011; Vadharajan *et al*., 2012; Onyechi *et al*., 2012; Florence *et al*., 2013; Biba *et al*., 2013) making them a potential source ofraw material for

antileishmanial drug discovery. Tannins and saponins have been associated with antioxidants, antimicrobial, antiviral, anti-inflammatory, and anticarcinogenic and immunostimulant properties (Akiyama *et al*., 2001; Trease & Evans, 2002; Etebu, 2012; Onyechi *et al*., 2012) an indication of the potential health benefits ofthese fruits to cure a number of diseases. Saponins and tanninsimpart astringent and bitter taste to plant-based foods making them undesirable when present in very high quantities in ready-to-eat foods such as fruit while identified types of saponins have been associated with adverse effects on fishes and other aquatic lives (Francis *et al*., 2002; Etebu, 2012). However, in the current study, the content of tannins and saponins of *A. muricata* and *A. squamosa* fruits when ripe is just adequate thereby eroding any cause for alarm in the consumption of these fruits. The intake of glycosides in edible fruits has been associated with certan toxicity (EFSA, 2009). However, detection of glycosides indicates existence of compounds with active genins and at least one reducing sugar that have relaxant and calming effects on the heart and muscles when consumed in small doses (Onike, 2010; Dembitsky *et al*., 2011; Usunomena, 2012). Therefore, their detection in this study suggest the inclusion of *A. muricata* and *A. squamosa*fruits in human diet especially leishmaniasis patient for supportive therapy.

Flavonoids are acclaimed for antioxidant and antimicrobial activities (Dembitsky *et al*., 2011) while coumarins are anti-stress compounds and postulated to have antiviral and anticoagulant activities (Trease & Evans, 2002). Positive and negative inferences for coumarins were obtained in pulp, peel and seeds for *A. muricata* and *A. squamosa* respectively, corroborating earlier findings in species from other countries (Onyechi *et al*., 2012) thus extending the potentiality of these fruits as raw materials in nutraceutical industries. However, Coumrins are not commonly assessed in phytochemical analysis of most fruits resulting into limited data (Trease & Evans, 2002). Terpenoids are responsible for wound healing properties leading to wound contraction and epithelialisation (Varadharajan *et al*., 2012). General test for terpenoids gave negative inferences for the pulp of *A. muricata*. Although terpenoids were detected in *A. squamosa* in this study, the data in its detection is scarce (Edeoga *et al*., 2005). Some samples, which tested positive to terpenoids, had a negative inference for the sterols, which confirms the hypothesis that all sterols are terpenoids,

but the vice-versa is untrue (Trease & Evans, 2002). The detection of terpenoids in the pulp further indicates the medicinal potential of the edible portions of *A. muricata* and *A. squamosa* fruits.

An inverse relationship exists between intake of plant sterol and risk of some cancers (Valko *et al.*, 2007). The detection of this plant chemical therefore, suggests the potential health benefits associated with the consumption of *A. muricata* and *A. squamosa* fruits. Phenolic compounds are plant secondary metabolites, which constitute one of the most common and widespread groups of compounds in plants. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to diseases and many other functions (Zimmerman& Snow, 2018). Studies on phenol content of fruits of different genotypes of *Annona* species from different countries under different geographical and climatic regions have been conducted (Onyechi *et al.*, 2012; Mariod *et al.*, 2012; Biba *et al.*, 2013; Bhardwaj *et al.*, 2014).This study of the Kenyan variety supplements the result indicating that *A. muricata* which possessed significantly higher phenol content could be a potential source of antileishmanial agentsreplacing the existing leishmania chemotherapywhich are resistant to the parasite besides being having serious adverse effects.

5.2 Proximate, nutritional composition analysis and antioxidant activities

5.2.1 Proximate and nutritional composition analysis

The increasing demand and scientific awareness on nutritional and functional properties of edible fruits, a current focus of international research neccisated the study. The dry matter of food material is a function of the rate of nutrient uptake and dry mass accumulation (Hocking, 1994) whereas moisture content gives an indication of available dry matter and plays a major role in determining the propensity of the food to spoilage (Appiah *et al.*, 2011). The high moisture content of fresh pulp, peel and seeds was contributed by the bulk tissue weight of fleshy and succulent of the fruits. Higher moisture content in fruits reduce their stability and shelf life making them perishable by providing a suitable medium for many reactions to occur (Hussain *et al.*, 2009; Appiah, *et al.*, 2011). However, the succulency nature of fruits makes them a good source of raw materials for juice manufacturing as a supplement to whole fruits. However, it has been revealed that high moisture content

in fruits tends to promote microbial contamination and chemical degradation (Hussain *et al*., 2009). Analogous moisture content values have been obtained in other studies for all the samples (Folorunso & Modupe, 2007; Lim, 2012) and heterologous results (Onimawo, 2002; Pareek *et al*., 2011) where the moisture content of *A. muricata* pulp was found to be significantly high.The relatively high moisture content can be beneficial as moisture content makes the fresh fruit juicier and more palatable to the consumer (Barrett *et al*., 2010). The high moisture content as observed in the *Annona* speciespulp is responsible for theperishability and short shelf life of the fruits (Worrell *et al*., 1994).

The organic matter content of a plant material is the measure of the total lipids, proteins and carbohydrates (FAO/WHO, 2004). The results from this study indicate that the pulp and seeds of *A. muricata* and *A. squamosa*can be described as having high nutritional value. The protein contents in the pulp, peel, and seeds of *A. muricata* and *A. squamosa* fruits obtained from this study are in agreement with other studies (Onimawo, 2002; Pareek *et al.,* 2011; Dembitsky *et al*., 2011; Bhardwaj *et al*., 2014; Boake *et al*., 2014). Generally, the fruits had protein contentscomparatively higher than literature values for some other common fruits (Lozano, 2006). However, Mariod *et al*., (2012) reported lower values in *A. squamosa* pulp, which could be due to environmental factors. Proteins are required in our daily diet since they are the building block of the body and are important components of various enzymes and nucleic acid (Wardlaw & Smith, 2009). The results of this study suggest that the fruits of *A. squamosa* and *A. muricata* can be good source of proteins in consumer's diet.

Carbohydrates serve organisms as energy sources and as essential structural component of nucleic acid, which stores genetic information (Wardlaw & Smith, 2009). The pulp of *A. squamosa* recorded significantly higher amount of total carbohydrates (38.24±2.18 mg/100g) compared to significantly lower amount for *A. muricata* pulp (2.42±0.88 mg/100g). These values are surfficient and may be considered to be of nutritional significance hence the potential of *A. squamosa* as a source of energy hence its inclusion in the diet. Maximum reducing sugars were recorded in *A. squamosa* pulp recorded the higest amount of reducing sugars than *A. muricata* pulp. Therefore, it can be concluded that *A. squamosa* pulp contain

significant amount of reducing sugar that can beused as a source of resistance against biotic stresses to the plant (Morgan &Connolly, 2013)besides being utilized by humans (Wardlaw & Smith, 2009).

Titratable acidity (TBA) in fruits plays a role in taste, color, and indicator of the quality and microbial stability of the fruit juice and determines maturity (Oniwamo *et al*., 2002). The TBA in *A. muricata* and *A. squamosa* fruits pulp, peel and seeds was found to be in the range between 0.18±0.01 mg/100g to 0.78±0.05 mg/100g.In contrast to other studies, Onimawo *et al.,* (2002) obtained slightly higher titratable acidity values of 0.79% mg/100g (seeds) and 3.43 mg/100g (pulp) and Othman *et al*., (2014) who also revealed titratable acidity ranging from 0.10-1.25 mg/100g in freshly matured fruit of *A. muricata*. Due to moderate levels of titratable acidity, *A. squamosa* and *A. muricata* fruits can have better acceptability for the consumers.

Crude fibre content of foods gives an indication of its dietary fibres (Anderson *et al*., 2010) with a number of beneficial effects related to its digestibility and food retention in the gastrointestinal tract (GIT) (Champ *et al*., 2003; Anderson *et al*., 2010). As an important component of the diet, it enhances faecal bulk, prevents constipation, stimulating peristalsis and decreasing the risk of many disorders such as constipation, diabetes, cardiovascular diseases and obesity (Champ *et al*., 2003).Fibre content measured in different parts of *A. squamosa* and *A. muricata* fruits were found to be in the range of 38.69±1383 mg/100g *(A. squamosa* seeds) to 72.95±0.86 mg/100g (*A. muricata* pulp). These results were comparable with most other fruits seeds and pulps of the same and other families (Champ *et al*., 2003). Therefore, their inclusion in the diet can provide good roughage, which can be helpful for maintaining the health of the gastro-intestinal tract, weight regulation and thus decreasing the risk of many disorders. Considering the health benefits of dietary fibre as elaborated by Champ *et al*., (2003), the relatively high crude fibre content observed in the studied fruits is much desired.

Fats are important part of the diet because they are vital to the development of various organs and systems (Zimmerman & Snow, 2012). As a concentrated source of calories, especially in infants, fat helps to resolve the potential problems of high calorie needs and small stomach capacity (Wardlaw & Smith, 2009; Wardlaw *et al*., 2009). However, the pulp of the *A. muricata* and *A. squamosa* fruits had low content

of crude fat than the seeds. The values obtained support studies on essential oils extraction from fresh fruits of *A. muricata*in the seeds with potential for human health (Dembitsky *et al*., 2011).

Significantly, higher fat content was obtained for *A. muricata* compared to *A. squamosa*. The result was not comparable with 26.8 mg/100g fat content in the seeds of *A. squamosa* (Mariod *et al*., 2010) and 22.2 mg/100g in the seed kernels of *A. squamosa* (Rana, 2015). The values of fat content 26.13±1.05 mg/100g (*A. squamosa*) and 33.67±1.97 mg/100g (*A. muricata*) in the pulp are sufficient to cater for the needs in the diet. Therefore, the seeds of *A. muricata* and *A. squamosa* could be very useful as a source of fats and oils for both domestic and industrial uses. Instead of discarding them as waste, they can be utilized in oil industries and cooking oil if it is deodorized.

The fatty acid profile shows both short and medium carbon chain SFA; Caprylic acid (C8:0), Capric acid (C10:0), Lauric acid (C12:0), Myristic acid (C14:0), Palmitic acid (C16:0) and Stearic acid (C18:0), MUFA; Oleic acid (C18:1) and PUFA; Linoleic acid (C18:2) and Linolenic acid (C18:3). Amongst the SFA, Palmitic acid was predominant, followed by Myristic acid, Stearic acid, Capric acid, Lauric acid and finally Caprylic acid in that order. This result is consistent with studies conducted in other fruits such as kiwi (*Actinidia chinensis*), passion fruit (*Passiflora edulis*) and guava (*Psidium guajava*) (Piombo *et al*., 2006) and safflower and poppy fruit (Bozan & Temelli, 2008) where palmitic acid was the most abundant SFA; Stearic acid being the least common while the others (Caprylic acid, Capric acid, Lauric acid, Myristic acid) exists as intermediates during fatty acids biosynthesis. Further, in these studies conducted by Piombo *et al*., (2006) and Bozan & Temelli, (2008), linoleic acid was the abundant PUFA compared to linolenic acid whereas oleic acid was the abundant MUFA.

The pulp and seeds of *A. muricata* and *A. squamosa*fruits showed significant amounts of essential fatty acid,linoleic acid; 372.45±24.34 mg/100g (*A. muricata* pulp), 500.16±17.12mg/100g (*A. muricata* seeds), 80.13±3.16 mg/100g (*A. squamosa* pulp), 173.22±8.97 mg/100g (*A. squamosa* seeds) and linolenic acid; 100.12±2.38 mg/100g (*A. muricata* pulp), 196.67±17.82 mg/100g (*A. muricata*

seeds), 67.33±10.03 mg/100g (*A. squamosa* pulp), 129.99±2.86 mg/100g (*A. squamosa* seeds) in different proportions. Greater amount of linoleic acid compared to linolenic acid may not be desirable since the high polyunsaturation nature of linolenic acid (C18:3) compared to linoleic acid (C18:2) makes it more susceptible to oxidation (Piombo *et al*., 2006; Bozan & Temelli, 2008), making biological membranes to be susceptible to lipid peroxidation. In this study, linoleic acid was found to be higher than linolenic acid making the fruit safe for inclussion in the human diet.

The ash content present in any food is a measure of quality for assessment of functionalproperties of foods (Hofman*et al*., 2002). The levels of total ash in *A.squamosa* and *A. muricata* were up to 8.93±0.69 mg/100g (*A. muricata* pulp) which was higher than some of the common fruits such as avocado, papaya and banana (Daramola *et al*., 2000; Mandle *et al*., 2012). More over, the ash content gives an approximate measure of total mineral elements composition of foods not withstanding contaminations, which may lead to higher than factual values (USDA, 2009). Iron, copper, zinc, calcium, magnesium, phosphorous, sodium and potassium were detected in the pulp and seeds of the fruits. The study observed high content of calcium (857.16±6.39 mg/100g in *A. muricata* pulp), sodium (843.38±16.25 mg/100g in *A. muricata* pulp) and potassium (322.25±13.11 mg/100g in *A. muricata* pulp), magnesium (395.54±4.58 mg/100g in *A. squamosa* pulp) and phosphorous (146.30±4.02 mg/100g in *A muricata* seeds) whereas iron, copper, zinc and selenium were detected in trace amounts. While sodium and potassium are important in the body fluid, calcium plays an essential role in bone formation and magnesium is involved in enhancement of activities of metabolic enzymes, zinc is critical to the normal functioning of the immune system (Choi *et al*., 2011). Low Na/K ratio is evident due to high content of potassium compared to sodium. Diets with low Na/Kratio is of nutritional importance since it is associated with lower incidence of hypertension (Choi *et al*., 2011). Since calcium was found to be among the mostabundant mineral element present, *A. muricata* and *A. squamosa*fruits can be considered an appropriate dietary source of calcium to maintain the biological role of nerve transmission, musclecontraction, glandular secretion as well as mediating vascular contraction and vasodilation (Straub, 2007). It is significant that due to

notable mineral elements content, the pulps and seeds of *A. squamosa* and *A. muricata* fruits can satisfy a substantial portion of the mineral elements requirement. The values of mineral elements obtained in this study were different from earlier studies performed in other regions of the world(Onimawo, 2002; Onyechi *et al*., 2012; Mariod *et al*., 2012; Othman *et al*., 2014; Lugwisha *et al*., 2016). The difference could be due to varietal differences, impact of type and composition of soil of fruit origin and time of the experiment (Marinova*et al*., 2005; Mariod *et al*., 2012).The study indicates that *A. muricata* and *A. squamosa* fruits are source of tocopherol and ascorbic acid. The recommended daily intake (RDI) of ascorbic acid is about 30 mg/day for adults and 17 mg/day for children (NRC, 1989; USDA, 2009). Ascorbic acid content was found in the range of 19.60 mg/100g to 39.24 mg/100g. Studies conducted by Pareek *et al.,*(2011), Othman *et al*. (2014), Boake *et al.,*(2014) and Lugisha *et al*., (2016) on *A. muricata* and *A. squamosa* fruits claimed almost the same value of ascorbic acid with the current study. This signifies the potential use of the fruits as sources of ascorbic acid.

5.2.2 Carotenoids analysis and quantification

Dietary carotenoids provide health benefits in decreasing the risk of disease, particularly certain cancers and eye disease (Seddon *et al*., 1994; Jacob, 1995; Giovannucci *et al*., 1995; Johnson, 2002; Giovannucci, 2002a; Johnson *et al*., 2008). Although fruits have provided a delicate balance interms of nutrition and health, a shift from using fruits to nutritional supplement has always been experienced (Van Duyn & Pivanka, 2000). Consumer interest in the relationship between diet and health has increased the demand for information on functional foods. Moreover, the rapid advances in science and technology, increasing health-care costs, changes in food laws affecting label and product claims, an aging population, and a rising interest in attaining wellness through diet among othe factors fuel interest in functional foods (Van Het Hoff *et al*., 2000; Wang *et al*., 2002; Giskes *et al*., 2002; Wardlaw & Smith, 2009; Obuntibejua *et al*., 2013;).

The carotenoids that have been most studied in this regard are β-carotene, lycopene, lutein, and zeaxanthin. In part, the beneficial effects of carotenoids are thought to be due to their role as antioxidants (Johnson *et al*., 2008). The β-carotene may have

added benefits due its ability to be converted to vitamin A (Johnson *et al*., 2008). Furthermore, lutein and zeaxanthin may be protective in eye disease because they absorb damaging blue light that enters the eye (Johnson *et al*., 2008). Food sources of these compounds include a variety of fruits and vegetables, although the primary sources of lycopene are tomato and tomato products (Giovannucci, 2002b). Additionally, egg yolk is a highly bioavailable source of lutein and zeaxanthin (Van Het Hoff *et al*., 2000; Van Duyn & Pivonka, 2000). These carotenoids are available in supplement form. However, intervention trials with large doses of β-carotene found an adverse effect on the incidence of lung cancer in smokers and workers exposed to asbestos (WHO, 2008). Until the efficacy and safety of taking supplements containing these nutrients can be determined, current dietary recommendations of fruits and vegetables are advised (Wardlaw & Smith, 2009; Obuntibejua *et al*., 2013)

Credible scientific research indicates many potential nutritional and health benefits from naturally occurring food components and manufucnutritional supplement (Rodriguez-Amaya *et al*., 2006). However, toxicity arising from these manufactured nutritional supplements is an issue. Further, the retention of carotenoids and other functional foods is a major concern. Alteration or loss of carotenoids during processing and storage of foods through physical removal such as peeling, geometric isomerization, and enzymatic or non-enzymatic oxidation has been reported (Rodriguez-Amaya *et al*., 2006). Since good eating habits and healthy lifestyle is crucial, supplements, natural and chemically produced products are used in provision of nutrition (Wardlaw& Smith, 2009). This has led to ignorance on the importance of dietary fruits, which has been exacerbated by the presence of a suitable substitute for these essential components in the diet (van Rooyen *et al*., 2008). Further, carotenoids cannot be synthesized in animals or humans are found in fruits and vegetables therefore need to be part of the dietary intake.

The *A. muricata* and *A. squamosa* fruits are underutilized exotic fruits not only in Kenya but also in other countries such as India (Bhardwaj *et al*., 2014) and Brasil (Pinto et al., 2005; Badrie & Schauss, 2009). Preliminary screening has revealed the nutraceutical potential of these fruits and their uses as valuable source in functional foods (Pinto *et al*., 2005; Onyechi *et al*., 2012; Mariod *et al*., 2012; Boake *et al*.,

2014; Bhardwaj *et al*., 2014; Othman *et al*., 2014; Lugwisha *et al*., 2016)which has been confirmed by the results of this study. The *A. muricata* and *A. squamosa*fruits grown in Kenya have been ignored interms of research in the nutritional and health benefits accrued from their consumption probably leading to their underutilization. The results from the current study indicate the presence ofneoxanthin, violaxanthin and zeaxanthin, α-carotene, β-carotene γ-carotene, chlorophyll a, and chlorophyll b in *A. muricata* and *A. squamosa* fruits. Further, the results of this study showed that under the conditions used, the fruits contained several unidentified components, eluted at 11.8, 15.9, and 16.3 minutes retention time (Appendix II). Based on their retention times and spectral characteristics, these are most likely to be xanthophyll esters, exhibiting identical UV-vis spectra, but are less polar and elute differently. Similar results under isocratic elution in mango fruits reported by Burns *et al*., (2003) suggest that they could be xanthophyll based on their spectral characteristics.

Adequate intake of fruits forms an important part of a healthy diet and can help to prevent diet-related chronic diseases (Van Duyn & Pivonka, 2000; During &Harrison, 2004; WHO, 2008). However, gender, age and income, family origin and socioeconomic status and education and nutritional knowledgeinfluences fruit intake (Wang *et al*., 2002Giskes *et al*., 2002; Wardlaw *et al*., 2000; Wardle, 2008). Family origins affect the purchasing power of food, food choice, food preparation and food availability, which in turn affects consumption (Van Het Hoff *et al*., 2000; Obuntibejua *et al*., 2013). In Kenya, studies have not been undertaken to generate data on the low popularity of *A. muricata* and *A. squamosa* fruits in diets. The study therefore, reveals the importance of Kenyan *A. muricata* and *A. squamosa* fruits in supplementing dietary nutrition due to the high levels of carotenoids presence. In this way, *A. muricata* and *A. squamosa* fruits can provide a source of bioactive compounds with nutritional and functional properties beneficial to health, which should stimulate the pharmaceutical and food industries to develop new products. This is the first report on carotenoid composition of Kenyan *A. muricata* and *A. squamosa* fruit species. The data generated in the present study could be exploited for nutritional purpose to suggest sources of carotenoids as a part of daily meal to consumers, to overcome health disorders such as age-related diseases, including eye

diseases such as cataract, diabetic retinopathy, glaucoma, and age-related macular degeneration (Johnson *et al*., 2008).

5.2.3 *In vitro* and *in vivo* antioxidant activity in relation to leishmaniasis

It has been reported that ROS and RNS production are highly elevated during CL (Faria *et al*., 2007; Alkathiri *et al*., 2017), an indication that free radicals and oxidative damage play a vital role in pathogenesis. Natural antioxidantsof plant origins have been studied extensively for their effectiveness in the treatment of toxic injury (Abdel-Moneim, 2014a; Othman *et al*., 2014b). Further, they have been shown to support the skin's underlying structure and lower the synthesis of collagen-degrading enzymes, resulting in younger-looking skin (Aslam *et al*., 2006; Alkathiri *et a*l., 2017). The *L. major* causes inflammation via mast cell stimulation and enhancingsecretionof pro-inflammatory mediators (Cragg and Newman, 2013; Alkathiri *et a*l., 2017). Morevover, ROS and RNS produced during an inflammatory response leads to oxidative injure in non-infected cells (Alkathiri *et al*., 2017). During oxidative damage, free radicals that play a role in collagen damage are released (Sakthianandeswaren *et al*., 2005; Sheikholeslami *et al*., 2014). Natural productsfrom plants have animportant contribution in the search for new leishmanicidal drugs (Sakthianandeswaren *et al*., 2005; Tiuman *et al*., 2011; Al-Olayan *et al*., 2014; Alkathiri *et al*., 2017). Thus, convenctional therapy combined with natural products has been the trend in management of leishmaniasis (Metwally *et al*., 2016; Alkathiri *et al*., 2017).

In this study, *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts revealed marked antioxidative activities accompanied with DNA protective effects against H_2O_2-induced toxicity both *invitro* and *in vivo*. This indicates that the *A. muricata* and *A. squamosa* fruit pulp can be included in the diet of leishmaniasis patients to remove ROS and RNS produced by the interaction between the parasite and the host macrophages. The evaluated antioxidant activity of *A. muricata* and *A. squamosa* fruits through *in vitro* models are higher compared to earlier conductedstudies (Nawwar *et al*., 2012; Roy *et al*., 2011; Nandhakumar & Indumathi, 2013; Samuagam *et al*., 2014; Samuagam *et al*., 2014). This further expands the therapeutic pontenial of *A. muricata* and *A. squamosa* fruits. Higher total

phenol and flavonoid contents in methanol extracts in this study might be the cause of antioxidant acivities as reported in other studies (Nadhakumar & Indumathi, 2013; Gulçin *et al*., 2010).

Methanoland aqueous extracts the fruits pulp of *A. muricata*(AMPUAE and AMPUME) and *A. squamosa*(ASPUAE and ASPUME) were evaluated for*in vivo*antioxidant acivities using both non-enzymatic oxidants (GSH, NO, TBRS, MDA and ROS) and enzymatic antioxidants (GST, GSHPx, CAT and SOD) and organ protection ability using serum AST, ALT, LDH and CK activity. The findings of this study suggested that these extracts possess antioxidant potential in BALB/c mice. This study shows that the extracts strengthened both enzymatic and non-enzymatic antioxidant to prevent the extent of lipid peroxidation and normalized level of both cardiac and hepatic markers in serum. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of ellular membrane in organs producing these enzymes such as the liver (Drotman *et al*., 1978). An increased level of ALTand AST assesses the extent of hepatic damage with ALTbeing more specific than AST (Arthur *et al*., 2012a; Arthur *et al*., 2012b, Arthur *et al*., 2011). The results of this study revealed significance difference seen among negative control groups (Group I) and positive control (Group II) with IT groups (Group III, Group IV, Group V, Group VI and Group VII) where the levelsof serum enzymes were lowered.

The principle of antioxidant activity is based on the availability of electrons to neutralize any free radicals (Nadhakumar & Indumathi, 2013). During extracts therapy, BALB/c mice showed normalized level of both the cardiac markers LDH and CK suggesting the cardiac potential capacity of phenolic compounds that may be present in the extracts. Further, the present study also showed the antioxidant and radical scavenging mechanism extracts by antioxidant assays and analysis of lipid peroxidation.In free radical elimination, cellular antioxidants play an important role in the existence of equilibrium between enzymes under normal conditions (Binu & Harikumaran, 2015). Excess production of free radicals makes biological system to lose this equilibrium resulting in the establishment of an oxidative assult. In this study, the extracts IT groups (Group IV and Group V)showed significant ($p<0.05$) change in the activity of antioxidant like GSH, GST and GSHPx. Endogenous

antioxidants (GSH, GSHPx and GST) play a major role in the maintenance of redox potential and GSH act as a substrate for GST and GSHPx (Yoshida *et al*., 1997; Ilavenil, 2012). The GSHPx reduces free H_2O_2 to water and lipid hydroperoxides to their corresponding alcohols as described by other studies (Binu & Harikumaran, 2015). Several isoenzymes of GSHPx exist and GSHPx1 is the most abundant version found in the cytoplasm of many mammalian tissues (Gulçin *et al*., 2010).

The GSH level is an important factor in leishmania parasite induced toxicity and elevated GSH level increases GSHPx1 activity. In CL,a decreased GSH levels have been observed (Jafari *et al*., 2014). The GSH, the main nonenzymatic antioxidant molecules in cells plays a protective role in the metabolism of several toxic agents (Alkathiri *et al*., 2017) by acting as a free radical-trapping agent to preserve cytochrome P450 via blocking LPO (Moskaug *et al*., 2005). The results from previous studies on mice infected with *L. major* and treated with plants extracts (Aguiar *et al*., 2010; Jafari *et al*., 2014) showed remarkable elevated levels of GSH and GSHPx. In this study,*A. muricata* and *A. squamosa* fruits aqueous and methanol extracts markedly increased and maintained GSH and GSHPx activities in *L. major*infected BALB/c mice.

In biological systems, LPO is thought to be a toxicological phenomenon and low concentration of LPO products and byproducts are seen in biological system (Binu & Harikumaran, 2015). The byproduct lipid peroxides such as malondialdehyde (MDA) always alter the function of membrane proteins and lead to the production of peroxynitrite ($ONOO^-$) radicals (Binu & Harikumaran, 2015). The LPO occurs as a result of oxidative stress resulting from ROS and RNS over-production due to host defense against leishmania parasite (Dkhil *et al*., 2013) and are responsible for cellular damage (Abdel-Moneim & El-Khadragy, 2013). Moreover, the production of free oxygen radicals ($O_2^{\bullet-}$) in excess depletes protective enzymatic antioxidants, resulting in cellular injury observed in *Leishmania* infection (Ozbilge *et al*., 2005). Increased LPO has been described in VL inhamsters (Sen *et al*., 2001), humans (Neupane *et al*., 2008) and dogs (Heidarpour *et al*., 2012). Further LPO in patients with active CLhas been reported (Vural *et al*., 2004; Abdel-Moneim, 2014a). Inhibitory effects of compounds present in extracts on the LPO process may be

closely related to the metal reducing activity (Ito *et al*., 2005; Alkathiri *et al*., 2017). In human CL, serum antioxidant activities, MDA and NO levels are elevated (Vural *et al*., 2004; Serarslan *et al*., 2005). In this study, the extracts IT groups of BALB/c mice (Group IV and Group V) showed low level of MDAcompared to positive control group of BALB/c mice (Group II). Prevention of LPO after *A. muricata* and *A. squamosa* fruit pulp aqueous and methanol extracts treatment could be attributed to the radical-scavenging effect of the antioxidant constituents of the extracts.

The NO play a role in inducing inflammatory response and its toxicity is propagated only when they react with $O_2^{\bullet -}$to form peroxynitrite ($ONOO^-$), which damages biomolecules such as proteins, lipids, and nucleic acids (Abdel-Moneim, 2014b). In the present study, NO generation was restrained by the extracts, suggesting its applicability as a potent and novel therapeutic agent for scavenging NO. Further, the extracts may affect regulation of pathological conditions caused by excessive generation of NO and its oxidation product peroxynitrite ($ONOO^-$). Enhanced levels of NO and peroxynitrite ($ONOO^-$) have been reported in the blood and lesions of mice infected with *L. amazonensis* (Shirbazou & Jafari, 2012). The ROS and RNS in the form of peroxynitrite ($ONOO^-$) produced by the reaction of superoxide radical with NO has been indicated in the production of cytotoxic effects (Ou, *et al*., 2006; YukIlhami, 2011) thus related in the development of oxidative damage. In this *in vivo* model of the study, the level of NO declined in in the extracts IT groups (Group IV and Group V) compared to thepositive control (Group II). Therefore, the extracts could have potential natural compounds that are reactive nitrogen scavenger.

A further decrease in the activities of CAT and SOD has been reported in Canine VL (Heidarpour *et al*., 2012) and *L. major* infected ALB/c mice (Jafari *et al*., 2015). In thisstudy, treatment with the extracts (Group IV and Group V) caused a significant increase in CAT and SOD activities. The ability of the extracts treatment to prevent reduction inenzymaticantioxidantsmay be attributed to host defense for protection against toxic oxygen metabolites (Alkathiri *et al*., 2017). Results of other studies on other plant species have shown a positive effect of polyphenols and flavonoids on enzymaticantioxidant activities *in vivo* (Abdel-Moneim & El-Khadragy, 2013;

Abdel-Moneim, 2014a; Othman *et al*., 2014b). Furher, there was a higher effect that was observed when the extract from *A. muricata* and *A. squamosa* fruits pulp were combined with the standard drug (Pentostam) (Group VI and Group VII). The finding of this study revealed that *A. muricata* and *A. squamosa* fruits aqueousand methanol extracts caused a significant increase in GSH GSHPx, GST, SOD and CAT and decrease in NO, MDA, ALT AST and ALkP in *L. major* infected BALB/c mice. Thus, the extracts may be included in the formulation of the existing leishmania chemotherapy for better management of the disease.

5.3Assesment of growth performance of BALB/c mice on Annonaceae fruit pulp pellets diet

This study investigated the rate of utilization AFPP on NI, INT and IT groups of BALB/c mice. Lack of significance difference in BALB/c mice exposed to the same conditions (IT-RP vs IT-AFPP, INT-RP vs INT-AFPP and NI-RP vs NI-AFPP) on growth performance (BMG, SGR and MGR) and feed utilization parameters (FCR, PER and ALC) suggests that AFPP may be a good dietary source protein, lipids and carbohydrates. Further, this indicates that RP and AFPP had equal rate of digestion, absorption and utilization of nutrients in BALB/c mice. In the present study, SGR of 13.24±0.30 (NI-RP), 11.60±0.23 (NI-AFPP), 6.54±0.14 (IT-RP) and 6.49±0.29 (IT-AFPP) is an indicative of good growth performance. However, the SGR of all INT groups; INT-RP (-8.34±.022) and INT-AFPP (-9.58±0.17) were negative. This is an indication that *A. muricata* and *A. squamosa* fruit can be included in the diet for supportive therapy of leishmaniasis.

The *L. major* is an aetiological agent of CL, which is a parasite of the skin on humans (Makwali *et al*., 2012). However, in BALB/c mice and other animal models, it attacks visceral organs in addition to the local lesion at the point of inoculation (Shirbazou & Jafari, 2012; Makwali *et al*., 2012; Jarallah, 2015; Jarallah, 2016) thus a good model to study haematological changes, lesion size, organ length and parasite burden and other pathophysiological aspects of leishmaniasis. The parasites can be deposited in major visceral organs involved in the synthesis of major macromolecule after the digestion, absorption and transport (Anstead *et al*., 2001; Malafaia, 2009; Malafaia *et al*., 2009). Therefore, BALB/c micewere chosen as a suitable model for the investigation of interaction between malnutrition and leishmaniasis in this study.

In earlier studies, malnourishment contributed to higher parasite loads in the blood, skin, bone marrow, lymph node, liver and spleen (Carrillo *et al*., 2014) favouring the development of leishmaniasis. Based on these observations, this model provides an excellent opportunity to elucidate the factors implicated in severe malnutrition in leishmaniasis. Moreover, as it has been shown in this study, the model can be used for further investigation on the relationship between severe malnutrition and leishmaniasis Malnutritional decreases is weight-for-age in proportion to the level of dietary protein deficiency (Anstead *et al*., 2001). Cure of the disease depends upon the development of an effective immune response that activates macrophages (Kaur *et al*., 2013), which can be promoted and enhanced by good nutrition.

During leishmaniasis, there is a profound immunosuppression in the host that promotes the survival of parasites (Serafim *et al*., 2010; Khademvatan *et al*., 2011). Studies on supportive therapy on leishmaniasis have been conducted (Anstead *et al*., 2001; Aslam *et al*., 2006; Faria *et al*., 2007; Malafaia, 2009; Malafaia *et al*., 2009; Metwally *et al*., 2016; Alkathiri *et al*., 2017). In this study, it is revealed that treatment of infected animals coupled with good nutrition brought the levels of most biological parameters such as Hb and TLC to normal range as compared to the abnormal levels that do not receive such nourishment in infected animals (Serafim *et al*., 2010).

Kidney function tests include estimation of urea, BUN and creatinine. Renal abnormalities caused by leishmaniasis have been well documented in experimental animal studies and are comprised of interstitial and glomerular abnormalities (Salgado *et al*., 2003). The Na^+, K^+, Cl^- and K^+/Na^+ ratio values give an indication of electrolyte/water balance, whereas high levels of Ca^{+2} implies thyroid or parathyroid, intestine, pancreas, kidney and borne metastasis (Jarallah, 2015; Jarallah, 2016). Although in this study there were elevated levels of Na^+, K^+, Cl^- and K^+/Na^+ ratio, the role of infection caused by *L. major* on damage of these organs causing the elevated levels of these parameters is yet to be established. The reason may be that these parameters can vary with mouse strain/stock, age, sex, blood sampling method, environmental conditions pathogen status and the laboratory as well as nutrition (NAS, 1995; Anstead *et al*., 2001; Suckow *et al*., 2005; Malafaia, 2009; Malafaia *et al*., 2009; Santos *et al*., 2016). Variables that must be considered in establishing a

range of reference values in mice include sex, age, genetic variation, diet and environmental conditions (NAS, 1995; Suckow *et al*., 2005; Santos *et al*., 2016). In this study, mice of the same sex and age infected with the same strain of *L. major* were used. Therefore, they may provide a useful starting point to investigate the effect of leishmaniasis and nutrition on hematological parameters. Since significant variation of biological parameters may occur between individual mice strain, stock, laboratories and method of sampling, individual laboratories should establish normal reference values for their facility (Suckow *et al*., 2005; Santos *et al*., 2016).

The most important organ concerned with majority of biochemical activities in the human body is the liver. Since it has a great capacity to detoxify toxic substances and synthesize useful body metabolites, damage inflicted by hepatotoxic agents is of grave consequence metastasis (Jarallah, 2015; Jarallah, 2016). To assess the damage caused to the liver, activities of enzymes; ALT, AST, ALkP and SDH and the concentration of bilirubin, albumin, globulin, albumin/globulin ratio and total serum protein were measured. The increased level of ALT, AST, ALkP, and bilirubin is conventional indicator of liver injury. In leishmaniasis, liver damage due to high parasitic load in INT groups resulting in elevated levels of ALT, AST, ALkP and SDH an increased levels of bilirubin which were observed in this study. Albumin is used to detect liver damages whereas globulin and total protein content are used to detect immunoglobulin status, a key indicator in fighting of infections in organisms that is affected by nutrition (NAS, 1995; Crook, 2006; Anstead, *et al*., 2001; Malafaia, 2009; Malafaia *et al*., 2009). The stabilization of serum bilirubin, ALT, AST, and ALP levels in the IT-RP and IT-AFPP groups is a clear indication of the improvement in the functional status of the liver cells as described in other studies (Achlyia *et al*., 2004). The increase in AST and ALT levels has been reported in VL in different animal models (Mathur *et al*., 2008; Jarallah, 2015; Jarallah, 2016) depicted in this study for CL using BALB/c mice model. In this study, there was no significant elevation in the levels of liver function parameters in all the IT (IT-RP and IT-AFPP) groups compared to INT (INT-RP and INT-AFPP) groups.

The mononuclear phagocyte system (spleen, liver, bone marrow, intestinal mucosa and mesenteric lymph nodes) is the normal habitat of leishmania parasite (Makwal *et al*., 2012; Jarallah, 2015; Jarallah, 2016). However, the parasite may be found in

endothelial cells of the kidneys, suprarenal capsules, lungs, meninges and in cerebrospinal fluid (Beaver *et al*., 1984; Makwali *et al*., 2012; Shirbazou & Jafari, 2012). In leishmaniasis there can be possibilities of multiple organ damage as indicated by elevated levels of non-specific markers such as LDH indicating possible damage of liver, heart, skeletal muscles and lungs and CK commonly elevated in heart and skeletal muscle damage and muscular dystrophies.

These effects are consequences of the stimulation of the immune system by *L. major*, which promotes the inflammatory components of atherosclerosis, which are primarily the parasite-activated macrophages (Fernandes *et al*., 2013). Studies suggest that other pathogens contribute to the atherogenesis and pathogenesis process in leishmaniasis (Murray *et al*., 1995; Deonhoff *et al*., 2002; Burnett *et al*., 2004; Portugal *et al*., 2004; Mussa *et al*., 2006; Ameen, 2010; Eissa *et al*., 2012). The increased inflammation caused by these pathogens promotes macrophage activation and migration to the atheroprone sites (Fernandes *et al*., 2013). Alternatively, proatherogenic status may be attributed to the systemic oxidative stress induced by infection, which enhances lipoprotein or endothelium oxidation (Fernandes *et al*., 2013). Pathogens involved in atherosclerosis development usually induce a systemic infection instead of a localized infection such as an *L. major* infection (Fernandes *et al*., 2013). However, even localized infections, such as odontologic ones, may be associated with the development of atherosclerosis (Meurman *et al*., 2004; Fernandes *et al*., 2013).

In this study, microscopic examination of stained impression smear of liver and spleen showed the density of amastigotes demonstrating the pathological effect of parasite. The results of this study have demonstrated that the infected BALB/c mice show the hepatosplenomegaly sign of pathological effect of *L. major*promastigote in the infected BALB/c mice. The weight of liver was increased with increasing days of infection in the INT group compared to NI group after 12 weeks. For both the liver and spleen, INT group had the highest LDU. Significantly ($p<0.05$) higher parasite load in the liver and spleen occurred in INT group and the lowest in IT group. There were no significant differences between the following groups, NI-RP and NI-AFPP ($p>0.05$), IT-RP BALB/c mice and IT-AFPP BALB/c mice ($p>0.05$), INT-RPand INT-AFPP ($p>0.05$), indicating that there is no difference between the two feeds.

5.4 Efficacy and Safety of the extracts

5.4.1 Antileishmanial activity of the extracts

Ongoing search for better leishmanicidal compounds has gained increasing scientific attention and shifted to plant-derived products (Mishra *et al*., 2011a; Mishra *et al*., 2011b; Wink, 2012; Roy *et al*., 2012; Adebayo *et al*., 2013; Alkathiri *et al*., 2017; Et-Touys*et al*., 2017). Researchers have focused on microorganisms and plants as a source of new alternatives for the treatment of leishmaniasis (Mishra *et al.,* 2011a; Mishra *et al*., 2011b;Wink, 2012; Roy *et al*., 2012; Adebayo *et al*., 2013). This study was carried out to investigate the leishmanicidal effects of pulp, peel and seeds of *A. muricata* and *A. squamosa*fruitsaqueous, methanol, ethyl acetate and hexane extracts growing in the coast region of Kenya. A preliminary study of the 24 extracts that were tested at 100 µg/mL, 10 extracts markedly inhibited the infection rate of the *in vitro* forms of *L. major* amastigotes. However, 14 of the 24 extracts also showed direct effect on the amastigotes as they significantly reduced the number of amastigotes per host cell when compared to the control. Th eASSAE exhibited the highest leishmanicidal activity as it suppressed the infection of amastigotes significantly with an inhibition percentage of 65.16±6.79% reducing the parasite levels up to 85.33±0.77% compared to the INTC group. The observed leishmanicidal activities of the extracts suggest that these extracts possess selective activities against *L. major*. Thus, the *in vitro* promising antileishmanial activities at 100 mg/mL of the tested extracts serve as advancement in the search for new safe and efficacious antileishmanial drugs.

The results of this study are in agreement with other studies which investigated potent aantileishmanialactivity *in vitro* (Shapaz *et al*., 1994; Shapaz *et al*., 1996; Jaramillo *et al*., 2000; Osorio *et al*., 2007; Villa-Nova *et al*, 2011; de Lima *et al*., 2012) some of them being higher than the commercial compound (pentamidine) used to treat diseases caused by different strains of leishmania (Jaramillo *et al*., 2000; Osorio *et al*., 2007). Further, Isoquinoline, alkaloids isolated from Annonaceae species (Tempone *et al*., 2005; Tempone *et al*., 2011), inhibits trypanothione reductase an essential antioxidant enzyme that protects the parasites of

*Leishmania*and *Trypanosoma* from ROS generated by the host defense cells. The mammalian forms of *L. major* responsible for the clinical manifestation of leishmaniasis in humans is the amastigotes differing in cellular, molecular and biochemical characteristics from the insectal forms, the promastigotes (Gupta *et al.*, 2001). The fact that the tested extracts had direct effect on the multiplication on amastigotes explains their preferred use in the study. This is paramount for theestablish predictive value for antileishmanial activity in plant-based drugs.

Besides, validation of results of any study on chemotherapeutic potentials of leishmaniasis drugs must be performed using the parasites forms mimic the disease response in humans (Gupta *et al.*, 2001). In this study, the antileishmanial activities of the extracts were assessed by determining the proportion of macrophages free from the parasites (rate of infectivity andchemosuppression) and mean amastigote number per macrophage or host cell (number of parasite) as described by Alawa, (2008). These two methods used in the study are in line with the concept adopted for*in vitro* assessment of the susceptibility of leishmania parasites to potential and therapeutic experimental agents (Neal and Croft, 1984; Alawa, 2008). However, due to its simplicity, microscopic counting in leishmania parasite susceptibility assays has become the gold standard (Habtemariam, 2001; Alawa, 2008).

Further, variation in different leishmania species in drug sensitivity has been observed due to leishmania *spp* residing different types macrophages and intracellular survival adaptations (Croft & Coombs, 2003; de Lima *et al.*, 2012). Purification of the active compounds fromcomplex mixture of the crude extracts from plants might be considered to increase their antileishmanial activity. In this study, the methanolic and aqueous extracts of seeds and peel proved to be more active than the hexane and ethyl acetate extracts in contrast with other studies (de Lima *et al.*, 2012). These results can only be explained after complete investigation of the chemical constituents and biological evaluation of the less active hexane and ethyl acetate extracts.

The *A. muricata* and *A. squamosa* extracts have been previously evaluated against other protozoan infections including leishmaniasis (Igwe& Onabanjo, 1989; Iwu *et al.*, 1992; Iwu *et al.*, 1994; Atawodi *et al.*, 2003; Ajaiyeoba *et al.*, 2006). They have also been used against different agents of CL (*L. major, L. aethiopica* and *L.*

mexicana)in African countries including but not limited to Sudan, Ethiopia, Cameroon and Nigeria(Iwu *et al*., 1992; Iwu *et al*., 1994) besides agents of VL (*L. amazonensis*, *L. braziliensis* and *L. donovani*) in different parts of the world (de Lima *et al*., 2012). However, these conducted studies have concentrated on the leaves, bark and seeds but not the pulp and the peel of the fruits. In Kenya, there are no published scientific data available on the antileishmanial use of *A. muricata* and *A. squamosa*. In this study, the extracts of *A. muricata* and *A. squamosa* had both *in vitro* and *in vivo* effects on *L. major*. This is the first study in Kenya, undertaken to evaluate the *in vitro* and *in vivo* effects of the crude extracts of the pulp, peel and seeds of *A. muricata* and *A. squamosa* fruits extacts on *L. major*, the etiologic specie for CL. This may serve as a pioneer study in *A. muricata* and *A. squamosa* screening exercise for the discovery of cheaper, less toxic and readily accessible leads to new antileishmanial drugs in Kenya.

5.4.2 *In vitro* toxicityof the extracts

In this study, the *in vitro*cytotoxicity effect of pulp, peel and seeds aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata*and *A. squamosa*fruits were evaluated on normal mammalian cell line (vero cells). The increasingly popularity and use of *A. squamosa* and *A. muricata* in treatment of various diseases is due to ethnobotanical report on its selective cytotoxic activity (George *et al*., 2006; George *et al*., 2012). Although the extracts were not tested against cancer cell lines, *in vitro* studies have shown more selective toxicity to cancer cell lines than to normal cells (George *et al*., 2006; George *et al*., 2012; Nawwar *et al*., 2012). The *A. muricata* and *A. squamosa*fruitspulp, peel and seeds aqueous, methanol, ethyl acetate and hexane extracts were classified as highly toxic (IC_{50}< 10 μg/mL), toxic (10 < IC_{50}< 100 μg/mL), moderately toxic (100 <IC_{50}< 1000 μg/mL) and potentially non-toxic (IC_{50}>1000 μg/mL) (OEDCD, 2008). The results showed that 8 extracts were highly toxic to vero cells with IC_{50} ranging between 2.54±0.44 μg/mL and 7.55±1.19 μg/mL, 9 extracts were toxic with IC_{50} ranging between 12.85±2.80 μg/mL and 94.07±5.81 μg/mL and 7 extracts were moderately toxic with IC_{50} ranging between 104.81±1.16 μg/mL and 292.94±10.10 μg/mL. Cytotoxic activity (IC_{50}) is related to

antileishmanial activity by determining their corresponding selectivity index if performed on peritoneal macrophages (de Lima *et al*., 2012).

5.4.3 *In vivo*toxicity of the extracts

Medicinal plants preparations, commonly known as herbal medicines orCAM therapiesareused for primary health care (FAO/WHO, 2004). Greater attentionof herbal medicine as alternatives to orthodox therapy in treatment of various illnesses is increasing in awareness, interest and demand (Crook, 2006; Arthur *et al*., 2011).Further, studies have revealed that saponins, tannins, glycosides, flavonoids among other phytochemicals present in Annonaceae (Onimawo, 2002; Lozano, 2006; Folorunso & Modupe, 2007; Onyechi *et al*., 2012; Biba *et al*., 2013; Florence *et al*., 2014; Othman *et al*., 2014; Bhardwaj *et al*., 2014) contribute immensely to their bioactivity and usage in treating various diseases including antioxidant activity and hepatoprotective effects (Igwe & Onabanjo, 1989; Pinto *et al*., 2005; Adewole & Ojewole, 2006; Ajaiyeoba *et al*., 2006; George *et al*., 2006; Owuor & Kisangau, 2006; Adewole & Ojewole, 2009; Ugwu *et al*., 2011; Chukukwa *et al*. 2011; Nawwar *et al*., 2012; Mariod *et al*., 2012; Arthur *et al*., 2012a; Arthur *et al*., 2012b; George *et al*., 2012; Nandhakumar & Indumathi, 2013). However, toxicity of the various herbal preparations has been an issue of concern (Ghani, 2003; Nwaka & Hudson, 2006). Thus, the need to evaluate toxicity profiles of different extracts preparations.

Phytochemical analysis and detection of chemical constituents of plants extract using different solvents and extraction methods in search of bioactive agents forms the basis for drug synthesis (Ogbonnia *et al*., 2009). Aqueous extracts have been used for centuries and have received acceptability and ethnomedicinal usage in countries with well-established traditional medicine (Athur *et al*., 2012a; Arthur *et al*., 2012b). However, they are thought to contain only limited compounds (Eloff, 1998), thus the use of other solvents in extraction of bioactive constituents from plants for therapeutic purposes. The study was dedicated to evaluate the toxicological effects of extracts obtained from various solvents from the three parts (pulp, peel and seeds) of *A. squamosa* and *A. muricata* fruits. In the acute oral toxicity study of all the extracts studied, no mortality and changes in the behaviour of BALB/c miceadministered

with up to 2500mg/Kg bwt were observed. Further, the abnormal clinical signs such as hyper- or hypo-activity, aggression, unusual locomotion, catalepsy, prostration, skin edema, loss of body hair, piloerection, apnea, cyanosis, convulsions, salivation, lacrimation, corneal opacity, conjunctivitis, diarrhea, constipation, tremor, Hypotonia, hypertoniaas repoted in others studies(Arthur *et al*., 2011; Arthur *et al*., 2012a; Arthur *et al*., 2012b) were not observed.

An estimated LD_{50} of ≤ 5 g/Kg bwt usually indicates safety (Ghosh, 1984; OECD, 2008). Other studies conducted established that if the median lethal dose of a test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies (Salawu *et al*., 2009). The chemical labeling and classification of acute systemic toxicity based on oral LD_{50} valueshas been described as very toxic ≤ 5 mg/Kg; toxic > 5 ≤ 50 mg/Kg; harmful > 50 ≤ 500 mg/Kg; and no label, >500 ≤ 2000mgK/g (OECD, 2008). In this study, LD_{50} extracts studied was estimated to be ≤ 5g/Kg bwt, hence the extracts may be considered safe for oral use for the management ofleishmaniasis.

Body weight changes serve as a sensitive indication of the general health status (Arthur *et al*., 2011). A varied response of BALB/c mice to the extracts at high dose levelswas observed. The higher doses ofaqueous, ethyl acetate and hexane extracts from peel, pulp and seeds of *A.muricata* and *A. squamosa* fruits resulted in varied decline in body weight of BALB/c mice throughout the duration of experiment with a decline starting early from day 6 in some of the extracts. At the same dose, increase in body weights was observed in BALB/c mice for the aqueous and methanol extracts from pulp. However, weight gains were observed in all BALB/c mice administered 100 mg/Kg/dayin all the extracts.Therefore, it may be stated that at 100 mg/Kg bwtnot all the extracts interfered with the normal metabolism of BALB/c mice as corroborated with non-significant difference from BALB/c in control group.

It may be assumed that at higher dose, the crude extractscontained high amount of toxic substance, which could interfere with gastric function and decreased food conversion efficiency as described in other studies (Chokshi, 2007; Arthur *et al*., 2011). Further, lack of changes in thefeeding habits and well-accepted diets by the BALB/c mice treated with 100 mg/Kg/daybwt suggests that theextracts did not possibly cause any alterations in carbohydrate, protein or fat metabolism in the

experimental animals used. It may also be assumed that 100 mg/Kg/day treatments with the extracts did not adversely interfere with the nutritional benefits such as weight gain and stability of appetite of the BALB/c mice that were continually supplied with food and water *ad libitum*. However, it may not be concluded that the opposite is true for the BALB/c mice administered 1000 mg/Kg/dayand 2500 mg/Kg/day, but a general observation can be made that overdose of the extracts could have resulted in loss of appetite and decrease in body weight as confirmed by other studies (Arthur *et al*., 2011).

The absence ofchanges in colour of organs of BALB/c mice treated with various doses of the extracts compared with control group on macroscopic examinations may not be sufficient to exclude adverse effects of the extracts studied. Thus, hypertrophies of organs, the first hand indication of toxicity of chemical or biological substance were evaluated. The absence of insignificant hypertrophicliver, kidney, heart and spleen at all doses gives no indications of the susceptibility of these systems to toxicity of substance that may be present in these extracts. However, increased levels of amylase activity ($p<0.05$) and relative weights of stomach ($p<0.05$) on BALB/c mice administered 1000 mg/Kg bwt and 2500 mg/Kg bwt is a sign of possible mild adverse or toxic gastrointestinal (GIT) effects of the extractsas also described in other studies (Arthur *et al*., 2011). The average significant increase in relative weight of the stomach ($p<0.05$) may be due to inclusion of female BALB/c mice in the experimental groups as reported by earlier reports demonstrating uterine stimulant activity due to overdose of *A. muricata* in rats (Taylor, 2002). Therefore, overdose of extracts of both *A. muricata* and *A. squamosa*in females should be avoided due to the observed effect on the uterus (Arthur *et al*., 2011)

Generally, the insignificant changes ($p>0.05$) in haematologicalindices (RBC, HGB, HCT (PCV), MCV, MCH and MCHC) except WBCs and leucocytes which increased significantly ($p<0.05$) in the extracts treated groups of BALB/c mice compared to the DMSO (control) group is an indication of safety ofthe extracts studied. The observed significant increase ($p<0.05$) in WBCs and lymphocytes emphasize beneficial effect of extracts in improving immunity and general well being ofextracts treated groups of BALB/c miceas reported by other studies (Ogbonnia *et al*., 2009; Arthur *et al*., 2011). However, the observed non-significant

dose independent decrease in HBG, RBCs, PCV, MCV, MCH and MCHC in lower dose followed by an increase at higher doses could be used to justify that the studied extracts at all doses do not induce anaemia, making them safe. Contrary to documented use of *A. muricata* in floristic studies as a tonic (Mshana *et al*., 2000)this was not observed in the current study and in other studies (Arthur *et al*., 2011; Arthur *et al*., 2012a).

The liver and heart release AST and ALT while ALkP has both hepatic and bone sources. Therefore, elevations in the levels of these enzymes are indicators of liver, heart and bone damages (Crook, 2006; Arthur *et al*., 2011). The general lack of significant changes relative weights of the liver and heart is an indication of safety and lack of deleterious effect on the liver and the heart. However, the significant dose-dependent decrease in ALT, increase in ASTand increase ALkP ($p<0.05$) in BALBC mice extracts treated groups at 1000 mg/Kg and 2500 mg/Kg compared to control groupsuggestpossible occurrences of deleterious effect of the extracts on liver, heart and bone functions at higher dasage.

Plasma albumin, BUN, urea and creatinine levels can be used in the assessment of renal functions (Kachmar & Grant, 1984; Wasan *et al*., 2001; Crook, 2006; Arthur *et al*., 2011). The observed combined insignificant decrease in plasma albumin and a significant increase creatinine and urea levels ($p<0.05$) in BALB/c mice administered with extracts at higher doses may be a sign of impaired renal function. Thus, the extract at 1000 mg/Kg and beyond may likely cause kidney damage. Further, the extracts showed a remarkable decrease in plasma glucose, total cholesterol and TAGs levels at higher doses indicating the presence of hypoglycaemic and hypolipidaemic agents in the extract and thus giving the credence to the use of *A. muricata* and *A. squamosa*as hypoglycaemic agent as reported in other studies (Adewole & Ojewole, 2006; Adewole & Ojewole, 2009; Arthur *et al*., 2011). Generally, changes in HDL-cholesterol and LDL-cholesterol levels can be used to indicate faulty lipid metabolism (Barnett and Gara, 2003). The general lack of significant difference in lipid profile in all groups of BALB/c mice indicates that the extracts at all doses had no effect on lipid metabolism. The significant increase in HDL-cholesterol levels ($p<0.05$) and a non-insignificant increase in LDL-cholesterol levels ($p>0.05$) observed in BALB/c mice administered 100 mg/Kg bwt extracts is

an indication that low dose of the extract can reduce the cardiovascular risk factors which contribute to death of diabetic subjects (Barnett & Gara, 2003). The reduction of the cardiovascular risk factors can further give support to the traditional use of the herbal formulation of *A. muricata* as a hypoglycaemic agent as reported in earlier studies (Adewole & Ojewole, 2006; Adewole & Ojewole, 2009; Arthur *et al*., 2011).

5.5 DNA Binding interactions and DNA Topo I inhibitory activity

5.5.1 DNA Binding interactions of the extracts

The DNA methyl green bioassay is one of the simplest and comprehensive techniques used to study DNA binding interactions with other compounds with a high throughput (Attard & Pacioni, 1996). During the assay, the methyl green binds quantitatively to DNA forming a DNA-methyl green complex, hence identifying the agents with a high affinity for the DNA (Attard & Pacioni, 1996). The affinity determines the displacement of methyl green, leading to a colourless carbinol (Kurnick, 1950; Kurnick & Foster, 1950; Krey & Hahn, 1975). In this study, DNA methyl green assay was used to determine the affinity of *A. squamosa* and *A. muricata* fruitspulp, peel and seeds aqueous, methanol, ethyl acetate and hexaneextracts.

Studies have shown that pure compounds of plant origin may be associated with low IC_{50} values (Burres *et al*., 1992; Goda & Badra, 2005). Further, due to crude extracts being complex matrices with several phytochemicals, their IC_{50} values are expected to be higher than those of pure compounds (Attard &Pacioni, 1996) may due to presence of inhibitors. Therefore, it is reasonable that in the case of extracts investigated in this study, higher IC_{50} values were acceptable. The studies involving DNA binding interaction of different plant species extracts with IC_{50} values >70 μg/mL may be considered as active (Attard & Pacioni, 1996). Therefore, only 3 extractsout of the 24 extracts involved in this study (AMPEME, ASPEAE and ASSME) translating to 12.5 % had IC_{50} values <70 μg/mLthus had the ability to displace methyl green from the methyl green DNA complex. It is likely that these extracts contain compounds that may act as intercalating agents at the DNA level.All the active extracts (AMPEME, ASPEAE and ASSME) were extracted using water and methanol, which are polar solvents.

There is relationship between the affinity of the extracts to bind DNA and phytochemicals present (Attard &Pacioni, 1996). In this study, alkaloids were not the predominant phytochemical in AMPUAE (IC_{50}; 137.44±33.33 µg/mL) and ASPUAE (IC_{50}; 108.42±7.67 µg/mL) and had low affinity to interact with the DNA in the methyl green complex. Terpenoids, flavonoids, phenols and saponins predominated mainly in active polar aqueous extract (ASPEAE; IC_{50}, 67.66±2.44), polar methanolic extracts (AMPEME; IC_{50}; 62.97±3.37 µg/mL and ASSME; IC_{50}; 66.80±6.09 µg/mL). In other conducted studies, a correlation between the phytochemical class and DNA-methyl green displacement activityandability to inhibit DNA topoisomerase II was observed (Peebles *et al*., 2001). The percentage decrease in abasorbance of DNA methyl green in this studies were even superior compared to studies conducted by Attard and Pacioni, (1996) on other plant species. Majority of the extracts with DNA binding interaction in the study were from polar solvents which confirms studies by Attard & Pacioni, (1996) and Correa *et al*., (2007) on other plant species. It has been reported that naturally occurring products intercalating with DNAto be alkaloids (Berger, 2001; Frei *et al*., 2002; Cao *et al*., 2005; Qin *et al*., 2006) while others do not intercalate with DNA (Ishida and Asao, 2002; Kluza *et al*., 2003). Variation in [DNA]/[Etract] molar ratio (Tayeb *et al*., 2003), changes in ionic strength (Kluza *et al*., 2003), size and structure of the ligands may affect the interaction (Ni *et al*., 2006) are among the factors that may affect ability of the extracts to interact with the DNA. Thus, some of these factors may be responsible for the low affinity DNA interaction found in this study.

Since 37.5% of the extracts studied showed a DNA interaction with decrease in percentage absorbance of DNA methyl green >20.00%, it can thus be inferred that the *A. muricata* and *A. squamosa* fruits pulp, peel and seeds aqueous, methanol, ethyl acetate and hexane extracts have the potential as a source of phyto-compounds with possible pharmacological applications. Studies have been conducted to investgate the effects of reducing the molecular weight of antileishmanial compounds on DNA binding affinity, antileishmanial and cytotoxicity activity (Banerjee*et al*., 2012). Although the DNA used was not isolated from the leishmania parasite, the study provides insights on the potential of the extracts used to interact with DNA from the parasite. It is important to mention that few literature reports exist on the evaluation

of ethnopharmacological bioactivity of these *A. muricata* and *A. squamosa* fruits pulp, peel and seeds extracts through DNA binding interaction. It is therefore necessary to continue with the isolation, identification, and evaluation of the secondary metabolites responsible for this DNA interaction among the more active extracts obtained from this study. Thisprovide base line data for *A. muricata* and *A. squamosa* fruits pulp, peel and seed extracts to develop antileishmanial agents.

5.5.2 DNA Topo I inhibitory activity of the extracts

Most of the topoisomerase inhibitors in use are sourced from natural products (Xin *et al*., 2017) and thus there is considerable interest in identifying novel inhibitors from plant products. In continuation of the search for new topoisomerase inhibitors, the study investigated aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* and *A. muricata* from pulp, peel and seeds. Although studies have demonstrated potential DNA binding interactions ofplant extracts(Pommier *et al*., 2010; Sarkar & Mandal, 2011; Ghate *et al*., 2014a; Ghate *et al*., 2014b; Najuddin*et al*., 2016; Kar & Chattopadhyaya, 2016), their effect on DNA topoisomerases was not explored previously. The observed inhibition of DNA Topo I at very low concentration (0.75 µM) with mixtures of different plant extracts (Kar *et al*., 2017), suggests synergistic association between compounds present in the different extracts in inhibition of DNA Topo I (Xin *et al*., 2017). Adverse toxic side effects limit the evaluation and used use ofDNA Topo I inhibitors for the treatment of various illnessesincluding leishmaniasis (Banerjee*et al*., 2012; Kar *et al*., 2017).

However, isolation of pure compounds from the crude extracts may increase, enhance or improve the DNA Topo Iinhibitory activities (Xin *et al*., 2017).Thus, the need for natural non-toxic Topo I inhibitors, which may have possible leishmanial chemotherapeutic potentials. The study demonstrates inhibitory effect of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds extracts on synthetic DNA Topo I at 5 µM. However, the effect of mixture of different extracts and concentration on DNA Topo I inhibition was not investigated. Studies carried on DNA Topo I inhibitory activity revealed that majority of the active extracts are from polar solvents and contain flavonoids (Xin *et al*., 2017). Although synthetic DNA Topo I was used, the study demonstrate that plant extracts are potent inhibitors of DNA Topo I, a property

that may be helpful in development of antileishmanicidal agents. Thus *in vitro* and *in vivo* studies of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds extracts using purified DNA and DNA Topo I from the parasite and host should be conducted.

CHAPTER SIX

6.0 CONCLUSSIONS ANDRECOMENDATIONS

6.1 Conclussions

The results of present study indicate that *A. muricata* and *A. squamosa* pulps, peels and seeds exhibited significant amounts mineral composition, proximate composition, antioxidant activity and phytoconstituents. Proximate composition provides information aboutpurity and quality of foodstuffs. This general information aboutproximate composition of *A. muricata* and *A. squamosa* may be crucial because it can find use in the manufacture offood supplements with good PE or PC contents that can used supportive therapy in leishmaniasis. The proximate and nutritional composition data signify the potential of *Annona muricata* and *A. squamosa* fruits in contributing to the nutrient needs of malnourished communities with leishmaniasis. The peels being an agrowaste are usually discarded by people can be explored as a viable source of natural antioxidants for the functional food and development of antileishmanial drugs as one of the pharmaceutical applications.

The results of this study showed that *L. major* infected BALB/c mice groups fed with formulated AFPP subjected to the same experimental condition as the RP fed groups (IT-AFPP vs IT-RP, NI-AFPP vs NI-RP and INT-AFPP vs INT-RP) exhibited almost the same growth performance and nutrients utilization rate. Therefore, the good performance and nutrients utilization rate of AFPP coupled with proximate and nutritional composition of *Annona muricata* and *A. squamosa* fruits highligth their potential for use in animal diets and manufacturing of nutritional supplement to be used for supportive management of leishmaniasis.Alkaloids, phenols, saponins and terpenoids were present in higher amounts in the seeds as compared to the pulp and peel. Flavonoids, tannins, glycosides and steroids were abundant in the pulp, peel and seed of *A. muricata* and *A. squamosa*. The *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts possess significant *in vitro* and *in vivo* antioxidant activities in *L. major* infected BALB/c mice. The conducted phytochemical screening coupled with the*in vitro* and *in vivo* analysis in this study support the majority of the traditional uses of A.muricata and A. squamosa in fighting various

diseases such as respiratory tract, heart and kidney related conditions, animal bites, stings and obesity besides antileishmanial activity as conducted in other countries.

It can also be concluded from the present data that oral administration of *A. muricata* and *A. squamosa* herbal extracts due to its pharmaceutical chemical entities had the most therapeutic effect on leision sizes and LHFD swelling due to the *L. major* amastigote parasites in susceptible BALB/c mice. The high LD_{50}values obtained in this study is an indication of safety of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds extractsfor internal and external use in the management of leishmaniasis. Further, the results on low cytotoxicty or non-cytotoxicity both *in vitro* and *in vivo* coupled with DNA binding and DNA Topo I inhibitory activities, have value in rational drug design of efficacious, safe and low cost antileishmanial agents. The mode of action of *A. muricata* and *A. squamosa* fruits pulp, peel and seeds extracts can be concluded from this study. Cytotoxicity implies disruption of mitochondrial membrane to arrest cells in G0/G1 phase, and the induction of apoptosis, the inhibition of multiple signaling pathways that regulate metabolism. Mechanism of action of antioxidant activity is by hydrogen donation, while antileishmamalial action may be due to some phytochemicals having the ability to bind with DNA and inhibiting RNA synthesis and by glycosidase inhibition lacking cytoplasmatic membrane function.

Overall, the results of this study contribute to the chemotaxonomic understanding of the family Annonaceae, especially the genusAnnona growing in Kenya. Besides, the results obtained confirms the importance of the selection of plant extracts used in folk medicine in screening programs in the search for new antileishmanial agents. The knowledge and information from this study highlight the potential usefulness of *A. muricata* and *A. squamosa* fruits, which is paramount as it adds urgency to the search for new leishmaniasis fighting strategies such as good PE or PC as supportive therapy for the disease.

6.2 Recomendations

The study was an attempt to determine the efficacy and safety, nutritional and antioxidant activity of Kenyan *Annona muricata* (L.) *and Annona squamosa* (L.) fruits extracts growing in the coast region of Kenya in BALB/c mice model of *L.*

*major*leishmaniasis. From the results of the study, the following may be recommended

- Although the AFPP showed good gwowth performance and nutrient utilization rate in *L. major* infected BALB/c mice, there is need to study these parameters in a higher animal model to validate the findings of this study. Feed utilization probably could have been adversely affected by the presence of antinutrients such as phytate, thus the need of more research to identify the possible anti-nutrient factor.
- Investigation on the crude extracts from *A. muricata* and *A. squamosa* fruits to focus on isolation and characterization of the constituents present with the aim of understanding mechanisms of action, and encourage continued *invitro* and *in vivo* investigations. Besides, metabolic studies are also necessary to determine whether digestive processes decrease or increase activity of the active compounds.
- Future studies on genotoxicity to establish mutagenic effcts of *A. muricata* and *A. squamosa* fruits extracts on organs such as liver, kidney, spleen, heart need to be investigated and results reported.
- Due to toxic nature of some of the *A. muricata* and *A. squamosa* fruits extracts on normal mammalian cell lines (Vero cells),studies may conducted for insecticidal and larvicidal properties forcusing on sandfly, the vector of leishmaniasis.
- Due to the slight variation in data in this study compared to others studies, future studies may be conducted in consideration of extraction process, temperature and different solvents used during analytical processes, storage, ecological and environmental conditions to establish their possibility in accounting for the variation in the results from this study and ealier studies.

REFERENCE

Abdel-Moneim, A.E. (2014a). *Azadirachta indica* attenuates cisplatin-induced neurotoxicity in rats. *Indian J. Pharmacol*, *46*: 316-321.

Abdel-Moneim, A.E. (2014b). Prevention of carbon tetrachloride (CCl_4)-induced toxicity in testes of rats treated with *Physalis peruviana* L. fruit. *Toxicol. Ind. Health*, *32*: 1064-1073.

Abdel-Moneim, A.E. & El-Khadragy, M.F. (2013). The potential effects of pomegranate (*Punica granatum*) juice on carbon tetrachloride-induced nephrotoxicity in rats. *J. Physiol. Biochem*, *69*: 359-370.

Abubakar, S.M. & Aburahman, E.M. (1998). "Useful plants in Control of insect pest." *Journal of Herbs, spices and medicinal plants*, *6*(2): 49-54.

Achlyia, G.S., Wadodkar, S.G. & Dorle, A.K. (2004). Evaluation of hepatoprotective effect of *Amalkadi ghrita* against carbon tetrachloride-induced hepatic damage in rats. *J Ethnopharmacol*, *90*(2-3): 229-232.

Adebayo, O.L., Suleman, D. & Samson, A.A (2013). Natural products in antilshmanial drug discovery: A review. *Journal of Asian Scientific Research*, *3*(2): 157-173.

Adewole S.O. & Ojewole J.A.O. (2009). Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. *Afr. J. Trad. CAM*, *6*(1): 30 - 41.

Aguiar, M.G., Pereira, A.M.M., Fernandes, A.P. & Ferreira, L.A.M. (2010). Reductions in skin and systemic parasite burdens as a combined effect of topical paromomycin and oral miltefosine treatment of mice experimentally infected with leishmania (*Leishmania*) amazonensis. *Antimicrob. Agents Chemother*, *54*: 4699-4704.

Ajaiyeoba, E., Mofolusho, F., Ogbole, O., Okpako, L. & Akinboye, D. (2006): *In vivo* antimalarial and cytotoxic properties of *Annona senegalensis* extract. *African Journal of Traditional, Complementary and Alternative Medicines*, *3*(1): 137-141.

Akiyama H., Fujii, K., Yamasaki, O., Oono, T. & Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *J. Antimirob Chemother*, *48*(4): 487- 491.

Al-Musayeib, N.M., Mothana, R.A., Al-Massarani, S., Matheeussen, A., Cos, P. & Maes, L. (2012). Study of the *in Vitro* Antiplasmodial, Antileishmanial and Antitrypanosomal Activities of Medicinal Plants from Saudi Arabia. *Molecules*. *17*: 11379-11390.

Alawa, J.N. (2008). Effects of three Nigerian plants on the histopathology of cutaneous leishmaniasis (*Leishmania major*) and immunotherapy of leishmaniasis using DNA/protein vaccine in BALB/c mice. Zaria, Nigeria, PhD Thesis, Department of Human Anatomy Faculty of Medicine, Ahmadu Bello University,

Alba, R., Payton, P., Fei, Z.J., McQuinn, R., Debbie, P., Martin, G.B, Tanksley SD. & Giovannoni, J.J. (2005). Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell*, *17*: 2954–2965.

Alkathiri, B., El-Khadragy, M.F., Metwally, D.M., Al-Olaya, E.M. Bakhrebah, MA. & Abdel-Moneim, A.E. (2017). Pomegranate (*Punica granatum*) Juice Shows Antioxidant Activity against Cutaneous Leishmaniasis-Induced Oxidative Stress in Female BALB/c Mice. *Int. J. Environ. Res. Public Health*, *14*, 1592.

Alvar, J., Cañavate, C., Gutiérrez-Solar, B., Jiménez, M., Laguna, F., López-Vélez, R., Molina, R. & Moreno, J**.** (1997). *Leishmania* and Human Immunodeficiency Virus co-infection: the first 10 years. *Clinical Microbiology Reviews*, *10*: 298-319.

Alvar, J., Velez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J. & WHO Leishmaniasis Control Team. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PloS one*, *7*(5): e35671.

Alvar, J., Yactayo, S. & Bern, C. (2006). Leishmaniasis and poverty. *Trends in Parasitology*, *22*: 552–557.

Ameen, M. (2007). Cutaneous leishmaniasis: therapeutic strategies and future directions. *Expert Opin Pharmacotherapy*, *8*: 2689-99.

Ameen, M. (2010). Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. *Clinical and Experimental Dermatology: Clinical dermatology*, *35*(7): 699-705.

Anderson, J.W., Baird, P., Davis, R.H., Jr Ferreri, S., Knudtson, M., Koraym, A., Waters, V. & Williams, C.L. (2010). Health benefits of dietary fiber. *Nutrition Reviews, 67*(4): 188 – 205.

Anstead, G.M., Chandrasekar, B., Zhao, W., Yang, J., Perez, L.E. & Melby, P.C. (2001). Malnutrition alters the innate immune response and increases early visceralization following *Leishmania donovani* infection. *Infect Immun, 69*: 4709-4718.

AOAC, (2000). *Official Methods of Analysis*. Association of Official Analytical Chemists, Washington DC.

Aouni, K., Amri, F., Chouihi, E., Haouas, N., Bedous, K., Benikhlef, R., Ghrab, J., Babba, H., Chabed, M.K., Harrat, Z. & Bouratbine, A. (2008). Epidemiology of *Leishmania (L) infantum, L. major* and *L. Killicki* in Tunisia: results and analysis of the identification of 226 human and canine isolates. *Bulletin de la societe de pathologieexotique*, *101*(4): 323-328.

Appiah, F., Oduro, I. & Ellis, W.O. (2011). Proximate and mineral composition of *Artocarpus altilis* pulp flour as affected by fermentation. *Pakistan Journal of Nutrition*, *10*(7): 653-657.

Arthur, F.K.N., Woode, E., Terlabi, E.O. & Larbie, C. (2011). Evaluation of acute and subchtonic toxicity of *Annona muricata* (Linn.) aqueous extract in animals. *Eur. J. Exp. Biol*, *1*(4): 115-124.

Arthur, F.K.N., Woode, E., Terlabi, EO. & Larbie, C. (2012b). Bilirubin lowering potential of *Annona muricata* (Linn.) in temporary jaundiced rats. *American journal of pharmacology and toxicology*, *7*(2): 33-40.

Arthur, F.K.N., Woode, E., Terlabi, O. & Larbie, C. (2012a). Evaluation of Hepatoprotective Effect of Aqueous Extract of *Annona muricata* (Linn.) Leaf Against Carbon Tetrachloride and Acetaminophen Induced Liver Damage. *Journal of Natural Pharmaceuticals*, *3*(1): 25 – 30.

Asford, R.W. (1997). The leishmaniases as model of zoonoses. *Ann Trop Med Parasitol*, *91*: 93-701.

Ashford, R.W. & Bates, P.A. (1998). Leishmaniasis in the world. *Parasitology, 5*: 215-242.

Atawodi, S.E., Bulus, T., Ibrahim, S., Ameh, D.A., Nok, A.J., Mamman, M. & Galadima, M. (2003): *In vitro* trypanocidal effect of methanolic extract of some Nigerian Savannah plants. *African Journal of Biotechnology, 2*(9): 317-321.

Attard, E. & Pacioni, P. (1996). The Phytochemical and *in Vitro* Pharmacological Testing of Maltese Medicinal Plants. *Bioactive Compounds in Phytomedicine*, *5*: 94-112.

Awasthi, A., Mathur, R.K. & Saha, B. (2004). Immune response to *Leishmania* infection. *Indian. J. Med. Res*, *119*, 238-258.

Aziz, K., Fakoorziba, MR., Jalali, M. & Moemenbellah-Fard, M.D. (2012a). First molecular detection of *Leishmania major* within naturally infected *Phlebotomus* salehi from a zoonotic cutaneous leishmaniasis focus in southern Iran. *Trop Biomed*, *29*(1):1–8.

Aziz, K., Moemenbellah-Fard, M.D, Kalantari, M. & Fakoorziba, M.R. (2012b). Molecular detection of *Leishmania major* kDNA from wild rodents in a new focus of zoonotic cutaneous leishmaniasis in an oriental region of Iran. *Vector-Borne Zoonotic Dis*, *12*(10):844–850.

Badaro, R., Jones, T.C., Lorenço, R., Cerf, B.J., Sampaio, D., Carvalho, E.M., Rocha, H., Teixeira, R. & Johnson, W.D. (1986). A prospective study of visceral leishmaniasis in an endemic area of Brazil. *J Infect Dis, 154*: 639-649.

Badrie, N. & Schauss, A.G. (2009). *Sopursop (Annona muricata, L) composition, nutritional value, medicinal use and toxicology*. In: Ronald Ross Watson and Victor R. Preedy (Eds). *Bioactive foods in promoting health*. UK, Oxiford academic press, pg 621-463.

Banerjee, M., Farahat, A.A., Kumar, A., Wenzler, T., Brun, R., Munde, MM., Wilson, W.D., Zhu, X., Werbovetz, K.A.&Boykin,D.W. (2012). Synthesis,

DNA binding and antileishmanial activity of low molecular weight bis-arylimidamides. *Eur J Med Chem*, 55: 449–454.

Barnett, H.A. & O'Gara, G. (2003). *Diabetes and the heart. Clinical Practice Series*. Edinburgh, UK, Churchill Livingstone, pg 11-32.

Basu M.K. & Lala S. (2004). Macrophage specific drug delivery in experimental leishmaniasis. *Curr Mol Med J*, *4*(6): 681–9.

Beattie, L. & Kaye, P. (2011). Leishmania-host interaction: what has imaging tought us? *Cell Microbiol*, *13*: 1659-1667.

Beauchamp, C. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem*, *44*, 276e7.

Beaver, P.C., June, R.C. & Cupp, E.W. (1984). *Clinical Parasitology*. 9th Edn. Lea and Febiger, Philadelphia, pg 70 - 77.

Berger, J.M. (2001). Isolation, Characterization, and Synthesis of Bioactive Natural Products from Rainforest Flora. PhD Thesis, Virginia Tech Department of Chemistry, Virginia Polytechnic Institute and State University.

Berman, J. & Dietze, R. (1999). Treatment of visceral leishmaniasis with Amphotericin- B colloidal dispersion. *Chemotherapy*, *45*: 54-66.

Bern, C., Maguire, J.H. & Alvar, J. (2008). Complexities of Assessing the Disease Burden Attributable to Leishmaniasis. *PLoS Neglected Tropical Diseases, 2*(10): 1-8.

Bhardwaj, A., Satpathy, G. & Gupta, R.K. (2014). Preliminary screening of nutraceutical potential of *Annona squamosa*, an underutilized exotic fruit of India and its use as a valuable source in functional foods. *Journal of Pharmacognosy and Phytochemistry*, *3*(2): 172-180.

Bhowmik, S., Majumdar, T., Snha, R. & Ali, N. (2010). Comparison of liposome based antigen delivery system for protection against *Leshmania denovani*. J. *Control. Release*, *141*: 199-207.

Biba, V.S., Lakshmi, S., Dhanya, G.S. & Remani, P. (2013). Phytochemical analysis of *Annona squamosa* seed extracts. *Int. Res J Pharm. App Sci,* 5: 29-31.

Binu, P. & Harikumaran, N.R. (2015). Screening of Eugenol - A Monoterpene, as an antioxidant. *International Journal of Advanced Research*, *3*(11): 1527-1533.

Blossner, M. & Onis, M. (2005). Malnutrition: Quantifying the health impact at national and local levels. *World Health Organization*, Geneva. pg 51.

Boake, A.A., Wireko-Manu, F.D., Agbenorhevi, J.K. & Oduro, I. (2014). Dietary fibre, ascorbic acid and proximate composition of tropical underutilized fruits. *Afr. J. Food Sci*, *8*(6): 305-310.

Boakye, D., Wilson, M. & Kweku, M. (2005). A review of leishmaniasis in West Africa. *Ghana Medical Journal,39*(3): 94-97.

Boelaert, M., Criel, B., Leeuwenburg, J., Damme van, W., Le Ray, D. & Van der Stuyft, P. (2000). Visceral leishmaniasis control: A public health perspective. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 94*: 465-471.

Bogdan, C. & Rollinghoff, M. (1999). How do Protozoan Parasites Survive inside Macrophages? *Parasitology Today*, *15*: 22-26.

Bolard, J., Legrand, P., Heitz, F. & Cybulska, B. (1991). One-sided action of amphotericin B on cholesterol-containing membranes is determined by its self-association in the medium. *Biochemistry*, *30*: 5707-5715.

Bozan, B. & Temelli, F. (2008). Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. *Bioresource Technology*, *99*(14): 6354-6359.

Bradley, D.J. & Kirkley, J. (1977). Regulations of Leishmania population within the host. The variable course of Leishmania donovani infection in mice. *Clinical Experimental Immunology*, *30*: 119-129.

Brajtburg, J. & Bolard, J. (1996). Carrier effects on biological activity of amphotericin B.*Clin. Microbiol. Rev, 9*(4:): 512–531.

Brajtburg, J., Elberg, S., Schwartz, D.A., Vertut-Croquin, A., Schlessinger, D., Kobayashi, G.S. & Medoff, G. (1985). Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. *Antimicrob. Agents. Chemother*, *27*: 172-176.

Burnett, M.S., Durrani, S., Stabile, E., Saji, M., Lee, CW., Kinnaird T.D., Hoffman, E.P. and Epstein, SE. (2004) Murine cytomegalovirus infection increases aortic expression of proatherosclerotic genes. *Circulation*, *109*(7): 893–897.

Burns, J., Fraser, P.D. & Bramley, P.M. (2003). Identification and quantification of carotenoids, tocopherol and chlorophylls in commonly consumed fruits and vegetables. *Phytochemistry, Plant Metabolomics*, *62*(6): 939–947.

Burres, N.S., Frigo, A., Rasmussen, R.R. & Mc Alpine, J.B. (1992). A colorimetric microassay or the detection of agents that interact with DNA. *Journal of Natural Products*, *55*(11): 1582–1587.

Camacho, M., Forero, M.E, Fajardo C., Niño, A., Morales, P. & Campos, H. (2008). *Leishmania amazonensis* infection may affect the ability of the host macrophage to be activated by altering their outward potassium currents. *Exp. Parasitol, 120*: 50-56.

Cao, R., Peng, W., Chen, H., Ma, Y., Lui, X., Hou, X., Guan, H. & Xu, A. (2005). DNA binding properties of 9-substituted harmine derivatives. *Biochem Biophys Res Commun*, *338*: 1557–1563.

Carrillo, E., Jimenez, M., Sanchez, C., Cunha, J., Martins, C.M., Seva, A.P. & Moreno, J. (2014). Protein Malnutrition Impairs the Immune Response and Influences the Severity of Infection in a Hamster Model of Chronic Visceral Leishmaniasis. *PLoS ONE*, *9*(2): e89412.

Cassady, J.M. (1990). Natural products as a source of potential cancer chemotherapeutic and chemopreventative agents. *Journal of natural products*, *53*(1): 23-41.

Castes, M., Agnelli, A. & Rondun, A.J. (1984). Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. *Clinical and Experimental Immunology, 37*: 295-309.

Cerf, B.J., Jones, T.C., Badaró, R., Sampaio, D., Teixeira, R & Johnson, W.D.J.R. (1987). Malnutrition as a risk factor for severe visceral leishmaniasis. *J Infect Dis, 156*: 1030-1033.

Chakraborty, A.K. & Majumder, H.K. (1988). Mode of action of pentavalent antimonials: specific inhibition of type I DNA topoisomerase of *Leishmania donovani*. *Biochem Biophys Res Commun*, *152*: 605-711.

Chakraborty, P. & Basu, M.K. (1997). Leishmania Phagolysosome: Drug Trafficking and Protein Sorting Across the Compartment. *Crit. Rev. Microbiol*, *23*: 253-268.

Champp, M., Langkilde, A., Brouns, F., Kettlitz, B. & Collet, Y.B. (2003). Advances in dietary fibre characterization. Definition of dietary fibre, physiological relevance, health benefits and analytical aspects. *Nutrition Research Reviews*, *16*: 7182.

Chang, F.R., Wu, Y.C. & Duh, C.Y. (1993). Studies of the acetogenins of Formosan Annonaceous plants. II. Cytotoxic Acetogenins from *Annona muricata*. *Journal of Natural products*, *56*(10): 1688-1694.

Chang, K.P., Chaudhuri, G. & Fong, D. (1990). Molecular determinants of Leishmania virulence. *Annu. Rev. Microbial*, *44*: 499-529.

Chang, K.P., Fong, D. & Bray, R.S. (1985). *Biology of leishmania and leishmaniasis*. In: Chang, K.P, Bray, R.S., Eds. *Leishmaniasis: Human Parasitic Diseases*. Philadelphia: Elsevier. 1: 1-30.

Choi, M., Scholl, U.I., Yue, P., Bjorklund, P., Zhao, B., Nelson-Williams, C., Ji, W., Cho, Y.; Pael, A., Men, C.J., Lolis, E., Wisgerhof, M.V., Geller D.S. Mane, S., Hellman, P., Westin, G., Åkerström, G., Wang W., Carling, T. &Lifton, R.P.(2011). K^+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*, *331*: 768–772.

Chokshi, D. (2007). Food and Chemical. *Toxicol*, *45*: 32 – 40.

Chukwuka, K.S, Ikheloa, J.O., Okonko, I.O., Moody, J.O. & Mankinde, T.A. (2011). The antimicrobial activities of some medicinal plants on *Escherichia coli* as an agent of diarrhea in livestock. *Advances in Applied Science Research*, *2*(4): 37–48, 2011.

Cohen, B.E. & Gamargo, M. (1987). Concentration and time dependence of amphotericin B-induced permeability changes across plasma membrane vesicles from Leishmania sp. *Drugs. Exp. Clin. Res*, *13*: 539-546.

Correa, Y.M., Nino, J. & Musauera, O.M. (2007). DNA interaction of plant extracts from colombian flora. *Pharmaceutical Biology*, *45*(2): 1111-1115.

Cortes, D., Myint, S.H., Dupont B. & Davaoust, D. (1993). Bioactive acetogenins from the seeds of *Annona cherimola*. *Phytochemistry*, *32*(6): 1475-1482.

Cragg, G. M &Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta*, *1830*(6): 3670–3695.

Croft, S.L. & Coombs, G.H. (2003). Leishmaniasis - current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol, 19*(11): 502 - 508.

Croft, S.L. & Olliaro, P. (2011). Leishmaniasis chemotherapy-challenges and opportunities. *Clin Microbiol Infect*, *17*(10): 1478-1483.

Croft, S.L. & Yardley, V. (2002). Chemotherapy of Leishmaniasis. *Curr Pharm Des*, *8*: 319-342.

Croft,S.L., Seifert, K. & Yardley, V. (2006b). Current scenario of drug development for leishmaniasis. *Indian journal of Medical Research*, *123*(3): 399 – 410.

Croft,S.L., Sundar, S. & Fairlamb, A.H. (2006a). Drug resistance in leishmaniasis. *Clinical Microbiology Reviews*, *19*(1): 111 – 126.

Crook, M.A. (2006). *Clinical Chemistry and Metabolic Medicine*. 7th Edn. Hodder Arnold, London, pg 426.

Cruz, I., Nieto, J., Moreno, J., Cañavate, C., Desjeux, P. & Alvar, J. (2006). *Leishmania*/HIV co-infections in the second decade. *Indian Journal of Medical Research*, *123*: 357-388.

Daramola, A.O., Oladimwji M.O., Oshodi A.A. & Popoola A.V. (2000). Investigation of nutrient composition of selected vegetables and banana samples. Federal University of Technology, Akure, Institutional repository. Retreaved from: http://hdl.handle.net/123456789/2433.

de Lima, J.P.S., Pinheiro, M.L.B., Santos, A.M.G., Pereira, J.L.S., Santos, D.M.F., Barison, A., Siva-Jardim, I. & Costa, E.V. (2012). *In vitro* antileshimanial and cytotoxic Activities of *Annona mucosa*. (Annonaceae). *Rev. Virtual. Quim*, *4*(6): 692-702.

Dembitsky, V.M., Poovarodom, S., Leontowicz, H., Leontowicz, M., Vearasilp, S., Trakhtenberg, S. and Gorinstein, S. (2011). The multiple nutrition properties of some exotic fruits: Biological activity and active metabolites. *Food Research International*, *44*: 1671-1701.

Desjardins, M. & Descoteaux, A. (1997). Inhibition of Phagolysosomal Biogenesis by the Leishmania Lipophosphoglycan. *J. Exp. Med*, *185*: 2061-2068.

Desjardins, M. & Descoteaux, A. (1998). Survival strategies of Leishmania donovani in mammalian, host macrophages. *Res. Immunol*, *149*: 689-692.

Desjeux P, Meert J, Piot B, Alvar J, Medrano F, Portus M, Munoz C, Laguna F, Velez RL, Salas A, Sirera G, Cisterna R, Montalban C, Quero H, Gradoni L, Gramiccia M, Russo R, Dedet J, Pratlong F, Dereure J, Deniau M, Izri A, Matheron S, Farault F, Marty P, Rosenthal E, Antunes F, Abranches P, Pradinaud R (2000). Leishmania/HIV co-infection in south-western Europe 1990–1998: Retrospective analysis of 965 cases. WHO/LEISH/2000.42

Desjeux, P. (1996). Leishmaniasis. Public health aspects and control. *Clinical Dermatology*, *14*: 417-423.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*: 239-243.

Desjeux, P. & Alvar, J. (2003). Leishmania⁄ HIV co-infections: epidemiology in Europe. *Ann Trop Med Parasitol*, *97*: 3–15.

Dkhil, M.A., Al-Quraishy, S., Abdel-Moneim, A.E. & Delic, D. (2013). Protective effect of *Azadirachta indica* extract against eimeria papillata-induced coccidiosis. *Parasitol. Res*, *112*: 101–106.

Dkhil, M.A., Moneim, A.E. and Al-Quraishy, S. (2016). *Indigofera oblongifolia* ameliorates lead acetate-induced testicular oxidative damage and apoptosis in a rat model. *Biol. Trace Elem. Res*, *173*: 354–361.

Doenhoff, M.J., Stanley, R.G., Griffiths, K. & Jackson, C.L. (2002). An anti-atherogenic effect of *Schistosoma mansoni* infections in mice associated with a parasite-induced lowering of blood total cholesterol. *Parasitology*, *125*(5): 415–421.

Drotman, R. & Lawhan, G. (1978). Serum enzymes are indications of chemical induced liver damage. *Drug Chem Toxicol*, *1*: 163–171.

Dumonteil, E., McMohan-Pratt, D. & Price, V.L. (2001). Report on the forth TRD/IDRI meeting on second generation vaccines against leishmaniasis. TRD, TDR/PRD/LEISH/VAC/01.1, Geneva, Swirtzerland.

During, A. & Harrison, E.H. (2004). Intestinal absorption and metabolism of carotenoids: insights from cell culture. *Arch Biochem Biophys*, *430*(1): 77-88.

Edeoga, H.O., Okwu, D.E. & Mbaebie, B.O. (2005). Phytochemical constituents of some Nigerian medicinal plant. *African Journal of Biotechnology*, *4*: 685–688

EFSA, (2009). Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern on request of European Food Safety Authority (EFSA). *EFSA Journal*, *7*(9): 100-123.

Eissa, M.M., Amer, E.I., Mossallam, S.F. & Gomaa, M.M. & Baddour, N.M. (2012) Miltefosine for old world cutaneous leishmaniasis: An experimental study on *Leishmania major* infected mice. *Alexandria J. Med, 48*: 261–271.

Eloff, J.N. (1998). *Conservation of Medicinal Plants: Selecting Medicinal Plants for research and gene banking*. In: Robert P. Adams and Janice E. Adams, Eds., *Conservation of plants Genes III: Conservation and utilisation of African plants*. Monographs in systematic Botany from the Missouri Garden. Missouri Botanical Garden Press, St. Louis, USA pg 209-222.

Enrique, M., Esperanza, G., José L.R. & Juan, M. (2005). Old World eyelid cutaneous leishmaniasis: A case report. *Dermatology Online Journal*, *11*: 31-43.

Et-Touys, A., Bouyahya, A., Fellah, H., Mniouil, M., El Boury, H., Dakka, N., Sadak, A. & Bakri,Y. (2017). Antileishmanial activity of medicinal plants from Africa: A review. *Asian Pac J Trop Dis, 7*(12): 826-840.

Etebu, E. (2012). Differences in Fruit Size, Postharvest Pathology and Phytochemicals between *Irvingia gabonensis* and *Irvingia wombolu*. *Sustainable Agriculture Research*, *2*(1): 52-61.

Evans, W.C. (2002). *Trease and Evans Pharmacognosy*. 15th Edn. London: W.R. Saunders, pg 137–140.

Ezeonu C.S. & Ejikeme. C. M. (2018). Qualitative and Quantitative Determination of Phytochemical Contents of Indigenous Nigerian Softwoods.*New Journal of Science*, *2016*: 5601327.

FAO, (2004). *Trade in medicinal plants. In: Economic and Social Department, Food and Agricultural Organization of the United Nation*, Rom. 2-3

FAO/WHO, (2004). Fruit and Vegetables for Health. *Report of a joint FAO/WHO workshop 1-3 Sept 2004*, Kobe, Japan.

Faria, A., Monteiro, R., Mateus, N., Azevedo, I. & Calhau, C. (2007). Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress. *Eur. J. Nutr*, *46*: 271–278.

Fendall, N.R. (1961). The spread of kala-azar in Kenya. *East Africa Medical Journal, 38*: 417-419.

Fernandes, L.R., Ribeir, A.C.C., Segatt, M., Santos, L.F.F.F., Amaral, J., Portugal, L.R. & Leite, J.I.A., (2013) Leishmania major Self-Limited Infection Increases Blood Cholesterol and Promotes Atherosclerosis Development. *Cholesterol*, *754580*.

Florence, A.R., Joselin, J., Shynin, P., Brintha, T.S., Sukumaran, S. & Jeeva, S. (2014). Preliminary phytochemical studies of select members of the family Annonaceae for bioactive constituents. *Bioscience Discovery*, *5*(1): 85-96.

Folorunso, A.E. & Modupe, O.V. (2007). Comparative study on the biochemical properties of the fruits of some Annona species and their leaf architectural study. *Not. Bot. Hort. Agrobot. Cluj, 35*(1): 15-19.

Francis, G., Kerem, Z., Makkar, H.P.S. & Becker, K. (2002). The biological action of saponins in animal systems: A review. *British Journal of Nutrition*, *88*: 587–605.

Frei, E., Bieler, CA., Arlt, V.M., Wiessler, M. & Stiborova, M. (2002). Covalent binding of the anticancer drug ellipticine to DNA in V79 cells with human cytochrome P450 enzymes. *Biochem Pharmacol*, *64*: 289–295.

Gamboa-Leon, M.R, Aranda-Gonzalez, I., Mut-Martin, M., Garcia-Miss, M.R. & Dumonteil, E. (2007). *In vivo* and *in vitro* control of Leishmania mexicana due to garlic-induced NO production. *Scandinavian J. Immunol*, *66*: 508–614.

Gatto, M., de Abreu, M.M., Tasca, K.I., Simão, J.C., Fortaleza, Carlos, M.C.B., Pereira P.C.M. & Calvi, S.A. (2013).Biochemical and nutritional evaluation of patients with visceral leishmaniasis before and after treatment with leishmanicidal drugs. *Evista da Sociedade Brasileira de Medicina Tropical*, *46*(6): 735-740.

Gazanion, E., Vergnes, B., Seveno, M., Garcia, D., Oury, B., Ait-Oudhia, K., Ouaissi, A. & Sereno, D. (2011). *In vitro* activity of nicotinamide/antileishmanial drug combinations. *Parasitol. Int,60*: 19-24.

George, A.P., Broadley, R. & Nissen, R. (2006). Preliminary review of the health and medicinal benefits of Annona spp. possible paths to commercialization. *A confidential report for the Australian Custard Apple Growers Association*. *Asian. Pac. J. Cancer. Prev. 2*: 99-124

George, V.C, Kumar, D.R., Rajkumar, V., Suresh, P.K. & Ashok, K., (2012). Quantitative Assessment of the relative antineoplastic potential of the n-butanolic leaf extract of *Annona muricata* Linn. In normal and immortalized human cell lines. *Asian. Pac. J. Cancer. Prev. 13*: 699-704.

Gershkovich, P., Wasan, E.K., Lin, M., Sivak, O., Leon, C.G., Clement, J.G. & Wasan, K.M. (2009). Pharmacokinetics and biodistribution of amphotericin B in rats following oral administration in a novel lipid-based formulation. *J. Antimicrob. Chemother*, *64*: 101-108.

Ghani A. (2003). *Medicinal plants of Bangladesh with chemical constituents and uses*. Asiatic society of Bangladesh, Dhaka, Bangladesh.

Ghate, N.B., Hazra, B., Sarkar, R. and Mandal, N. (2014b). Heartwood extract of *Acacia catechu* induces apoptosis in human breast carcinoma by altering Bax/Bcl-2 ratio. *Pharmacogn Mag*, *10*: 27-33.

Ghate, N.B., Hazra, B., Sarkar, R., Chaudhri, D & Mandal, N. (2014a). Alteration of Bax/Bcl-2 ratio contributes to Terminalia belerica induced apoptosis in human lung and breast carcinoma. *In Vitro Cell Dev Biol Anim*, *50*: 527-537.

Ghosh, J., Lal, C.S., Pandey, K., Das, V.N., Das, P., Roychoudhury, K. & Roy, S. (2011). Human visceral leishmaniasis: decrease in sérum cholesterol as a function of splenic parasite load. *Ann Trop Med Parasitol*, *105*: 267-271.

Ghosh, M.N. (1984). *Fundamentals of experimental pharmacology*. 2nd Edn. Scientific Book Agency, Calcutta.

Giovannucci, E. (2002a). Lycopene and prostate cancer risk. Methodological considerations in the epidemiologic literature. *Pure and Applied Chemistry*, *74*: 1427–1434.

Giovannucci, E. (2002b). A review of epidemiologic studies of tomatoes, lycopene, and prostate cancer. *Exp Biol Med (Maywood)*, *227*(10):852-859.

Giovannucci, E., Ascherio, A., Rimm, E.B., Stampfer, M.J., Colditz, G.A. & Willett, W.C. (1995). Intake of carotenoids and retinol in relation to risk of prostate cancer. *J Natl Cancer Inst*, *87*(23):1767-1776.

Giskes, K., Turrell, G., Patterson, C. & Newman, B. (2002). Socio-economic differences in fruit and vegetable consumption among Australian adolescents and adults. *Pub Health Nutr*, *5*(5): 663-669.

Goda, F.E. & Badria, F.A. (2005). Synthesis and Biological Evaluation of Certain New Substituted Pyrido-[2,3-D]-Pyrimidin-4(1h)-One and Pyrido [2,3-D]-Triazolo-[3,4- B]-Pyrimidine Analogs. *Saudi Pharmaceutical Journal*, *13*(2-3): 65-73.

Green, L.C., Wagner, D.A., Glogowaki, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Anal Biochem*, *126*: 131-143.

Griensven, J.V., Balasegaram, M., Meheus, F., Alvar, J., Lynen, L. & Boelaert, M. (2011). Combination therapy for visceral leishmaniasis. *Lancet. Infect. Dis*, *10*: 184-194.

Grimaldi, G., & Tesh, R. B. (2012). Leishmaniasis of the New World: Current concepts and implications for future research. *Clinical Microbiology Review*, 6: 230-250.

Grimm, F., Brun, R., & Jenni, L. (2011). Promastigotes infectivity in *Leishmania infantum*. *Parasitology Research*, *11*(1): 185–191.

Grogl, M., Thomason, T.N. & Franke, E.D. (1992). Drug resistance in leishmaniasis: Its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. *Am. J. Trop. Med. Hyg*, *47*: 117-126.

Gross, R.L. & Newberne, P.M. (1980). Role of nutrition in immunologic function. *Physiol Rev*, *60*: 188–302.

Guerin, P.J., Olliaro, P., Dar, S., Boelaert, M., Croft, S.L., Desjeux, P., Na, M.K. & Bryceson, A.D (2002). Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect. Dis*, *2*: 494-501.

Gulçin, I., Bursal, E., Şehitoglu, H.M., Bilsel, M. & Goren, A.C. (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum. *Turkey Food and Chemic. Toxicol*, *48*(8-9), 2227−2238.

Gupta, N., Goyal, N. & Rastogi, A.K. (2001): *In vitro* cultivation and characterization of axenic amastigotes of *Leishmania*. *Trends in Parasitology*, *17*: 150-153.

Gupta, S., Pal, A. & Vyas, S.P. (2010). Drug delivery strategies for therapy of visceral leishmaniasis. *Expert. Opin. Drug Deliv*, *7*: 371-402.

Habtemariam, S. (2003). *In vitro* antileishmanial effects of antibacterial diterpenes from two Ethiopian Premna species, *P. schimperi* and *P. oligotricha*. *BMC Pharmacology, 3*: 6-18.

Halliwell, B. & Gutteridge, J.M.C. (1981). Formation of thiobarbituric acid reactive substances from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBSLett*, *128*: 347e52.

Handman, E. (2001). Leishmaniasis. Current status of vaccine development. *Clin. Microbial. Rev, 14*(2): 229.

Harborne, J.B. (1973). *Photochemical methods: A guide to modern techniques of plant analysis*. London, Chapman A. and Hall, 279-297

Hartsel, S. & Bolard, J. (1996). Amphotericin B, new life for an old drug. *Trends. Pharmacol. Sci*, *17*: 446-449.

Heidarpour, M., Soltani, S., Mohri, M. & Khoshnegah, J. (2012). Canine visceral leishmaniasis: Relationships between oxidative stress, liver and kidney variables, trace elements, and clinical status. *Parasitol. Res*, *111*: 1491–1496.

Heisch, R.B., Wijers, D.J. & Minter, D.M. (1962). In pursuit of the vector of kala-azar in Kenya. *BMJ,5298*: 1456-1458.

Herwaldt, B.L. (1999). Leishmaniasis. *Lancet, 354*: 1191-1199.

Hide, G. and Tait, A. (1991). The molecular epidemiology of parasites. *Experientia*; **47**: 128-142.

Hocking, P.J. (1994). Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *Journal of Plant Nutrition*, *17*(8):1289-1308.

Hofman, P.J., Vuthapanich, S., Whiley, A.W., Klieber, A. & Simons, D.H. (2002). Tree yield and fruit minerals concentrations influence “Hass” avocado fruit quality. *Sci. Hort*, *92*:113–123.

Hotta, M., Ogata, K., Nitta, A., Hoshikawa, K., Yanagi, M. & Yamazaki, K. (1989). *Useful plants of the world*. Heibonsha LTD, Tokyo, Japan.

Hussain, K., Ismail, Z., Sadikun, A. & Ibrahim, P. (2009). Proximate and qualitative analysis of different parts of *Piper sarmentosum*, and quantification of total amides in various extracts. *Phcog. Res,1*: 113–119.

ICUC, (2002). *Fruits for future*. International Centre for underutilized Crops Southampton, UK. University of Southampton. March Institute of airrigation and Developmental studies.

Igwe, A.C. & Onabanjo, A.O. (1989). Chemotherapeutic effects of *Annona senegalensi* in *Trypanosoma brucei brucei*. *AnnTrop med parasitol,83*(5): 527-534.

Ilavenil, S. (2012). Hepatoprotective mechanism of Crinum asiaticum L. and lycorine in carbon tetrachloride induced oxidative stress in Swiss albino mice. India, Thanjavur, PhD thesis, Prist University.

Inacio, J.D., Gervazoni, L., Canto-Cavalheiro, M.M. & Almeida-Amaral, E.E. (2014). The effect of (+)-epigallocatechin 3-o-gallate *in vitro* and *in vivo* in leishmania braziliensis: Involvement of reactive oxygen species as a mechanism of action. *PLoS Negl. Trop. Dis. 8*: e3093.

Indumathi, C., Durgadevi, G., Nithyavani, S. & Gayathri, P.K. (2014). Estimation of terpenoid content and its antimicrobial property in *Enicostemma litorrale*. *Int. J. Chem.Tech. Res. 6* (9): 4264 – 4267.

Iqbal, K., Iqbal, J., Staerk, D. & Kongstad, K.T. (2017).Characterization of antileishmanial compounds from *Lawsonia inermis* L. Leaves using semi-high resolution antileishmanial profiling combined with HPLC-HRMS-SPE-NMR. *Front. Pharmacol*, *8*: 337.

Ishida, K. & Asao, T. (2002). Self-association and unique DNA binding propreties of the anti-cancer agents TAS-103, a dual inhibitor of topoisomerases I and II. *Biochem Biophys Acta*, *1587*:155–163.

Ito, M., Murakami, K. & Yoshino, M. (2005). Antioxidant action of eugenol compounds: role of metal ion in the inhibition of lipid peroxidation. *Food and chemical toxicology*, *43*(3): 461- 466.

Iwu, M.M., Jackson J.E. & Schuster, B.G. (1994). Medicinal plants in the fight against leishmaniasis. *Parasitol. Today, 10*: 65-68.

Iwu, M.M., Jackson, J.E., Tally, J.D. & Klayman, D.L. (1992). Evaluation of plant extracts for antileishmanial activity using a mechanism-based radiorespirometric microtechnique (RAM). *Planta Medica, 58*:436-441.

Jacob, R.A. (1995). The integrated antioxidant system. *Nutr. Res*, *15*: 755-766.

Jafari, M., Shirbazou, S. & Norozi, M. (2014). Induction of oxidative stress in skin and lung of infected BALB/c mice with iranian strain of *Leishmania major* (MRHO/IR/75/ER). *Iran. J. Parasitol*, *9*: 60–69.

Jafari, M., Shirbazou, S., Sadraie, S.H., Kaka, G. & Norozi, M. (2015). The role of apoptosis in the cellular response of liver and spleen of BALB/c mice in cutaneous leishmaniasis. *Iran. J. Med. Sci*, *40*: 133–142.

Jarallah, H.M. (2015). Dissemination of Canine Visceral Leishmaniasis to Different Organs of Jackals Experimentally Infected with *Leishmania donovani*. *Pak Vet J*, *35*(1): 98-100.

Jarallah, H.M. (2016) Pathological Effects of *Leishmania Donovani* Promastigotes on Liver and Spleen of Experimentally Infected BALB/c Mice *Medical Journal of Babylon*. *13*(1): 134 – 140.

Jaramillo, M., Arango, G., Gonzalez, M., Robledo, S. & Velez, I.D. (2000). Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp. *Fitoterapia*, *71*(2): 183-186.

Jha, T.K. (1983). Evaluation of diamidine compound (Pentamidine isethionate) in the treatment of resistant cases of kala-azar occurring in North Bihar, India. *Trans. R. Soc. Trop. Med. Hyg.77*: 167.

Johnson, E.J. (2002). The role of carotenoids in human health. *Nutr Clin Care*, *5*: 56–65.

Johnson, E.J., Chung, H.Y., Caldarella, S.M. & Snodderly, D.M. (2008). The influence of supplemental lutein and docosahexaenoic acid on serum, lipoproteins, and macular pigmentation. *Am. J. Clin. Nutr, 87*: 1521–1529.

Kachmar, J.F. & Grant, G.H. (1982). *Proteins and Amino Acids*. In: Tietz, N.W. (Ed.) *Fundamentals of Clinical Chemistry*. 2nd Edn. W.B. Saunders Company, Philadelphia, USA, pg 849-944.

Kar, I. & Chattopadhyaya, R. (2016). Effect of seven Indian plant extracts on Fenton reaction-mediated damage to DNA constituents. *J Biol Struct Dyn*, *10*: 1-15.

Kar, I. Majumder, H.K. & Chattopadhyaya, R. (2017). Extracts of Seven Indian Plants Inhibit Human Topoisomerase I and Partially Inhibit Human Topoisomerase II. *Mol Enz Drug Tar*, *2*: 5-11.

Karimi, A., Hanafi-Bojd, A.A., Yaghoobi-Ershadi, M.R., Akhavan, A.A. & Ghezelbash, Z. (2014). Spatial and temporal distributions of phlebotomine sand flies (Diptera: Psychodidae), vectors of leishmaniasis, in Iran. *Acta Trop*, 132: 131– 9.

Kaur, S., Kaur, G., Sachdeva, H. & Kaur, J.(2013). *In Vivo* Evaluation of the Antileishmanial Activity of Two Immunomodulatory Plants, *Emblica Officinalis* and *Azadirachta Indica* in BALB/c Mice. *International Journal of Ayurvedic and Herbal Medicine*, *3*(1): 1066-1079.

Kaye, P. & Scott, P. (2011). Leshmaniasis complexity at the host-pathogen interface. *Nat. Rev. Microbiol*, *9*: 604-615.

Kedzierski, L. (2010**)**. Leishmaniasis vaccine: Where are we today? *J. Glob. Infect. Dis*, *2*, 177–185.

Khademvatan, S., Gharavi, M.J., Rahim, F. & Saki, J. (2011).Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains. *Korean J. Parasitol*, *49*: 17–23.

Killick-Kendrick, R. (1999).The biology and control of *Phlebotomine* sandflies. *ClinicalDermatology, 17*: 279-289.

Kinuthia, G.K., Anili, C.O., Gikonyo, N.K., Kigondu, E.M., Ingonga, J.M. & Kabiru, E.W. (2013). *In vitro* and *in vivo* activities of blends of crude aqueous extracts from *Allium sativum* L, *Callistemon citrinus* (Curtis) Skells and *Moringa stenopetala* (Barker F) cufodontis against *Leishmania major*. *Int. J. Med. Arom*. Plants, *3*(2): 234-246.

Kirk, H. & Sawyer, R. (1998). *Frait Pearson Chemical Analysis of Food*. (8th Ed.) Edinburgh, Longman Scientific and Technical, pg 211-212.

Kluza, J., Baldeyrou, B., Colson, P., Rasoanaivo, P., Mambu, L., Frappier, F. & Bailly, C. (2003): Cytotoxicity and DNA binding properties of the plant alkaloid burasaine. *Eur J Pharm Sci*, *20*: 383–391.

Kokwaro, J.O. (1993). Medicinal plants of East Africa. 2nd Edn. East Africa Literature Bureau. Kampala, Nairobi and Dares Salam.

Krey, A.K. & Hahn, F.E. (1975). Studies on the methyl green-DNA complex and its dissociation by drugs. Biochemistry, *14*(23): 5061 – 5067.

Krinsky, N.I. & Johnson, E.J. (2005) Carotenoid actions and their relation to health and disease. *Mol Aspects Med*, *26*: 459–516.

Kumar, R., Roopan, S.M., Prabhakarn, A., Khanna, V.G. & Chakroborty, S. (2012a). Agricultural waste *Annona squamosa* peel extract: biosynthesis of silver nanoparticles. *Spectrochim Acta A Mol Biomol Spectrosc*, *90*: 173e6.

Kumar, V., Akinleye, A.O., Makkar H.P.S., Angulo-Escalante, M.A. & Becker, K. (2012b). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing Jatropha platyphylla kernel meal as a protein source. *J Anim Physiol Anim Nutr*, *96*(1): 37-46.

Kurnick, B. & Foster, M. (1950). Methyl green. III. Reaction with deoxyribonucleic acid: Stoichiometry and behavior of the reaction product. *Journal of General Physiology*, *34*: 147-159.

Kurnick, N. (1950). The determination of desoxyribonuclease activity by methyl green: Application to Serum. *Archives of Biochemistry*, *29*: 41-53.

Lal, C.S., Verma, N., Rabidas, V.N., Ranjan, A., Pandey, K., Verma, R.B., Singh, D., Kumar, S. & Das, P. (2010). Total serum cholesterol determination can

provide understanding of parasite burden in patients with visceral leishmaniasis infection. *Clin Chim Acta*, *411*: 2112-2113.

Lampronti, I., Khan, M.T.H., Borgatti, M., Bianch, N. & Gambari, R. (2008). Inhibitory Effects of Bangladeshi Medicinal Plant Extractson Interactions between Transcription Factors and Target DNA Sequences. *eCAM*. *5*(3): 303–312.

Lim, T.K. (2012). Fruits: *Cucurbita moschata. The encyclopedia of earth. Edible medicinal and non-medicinal plants*. 2nd Edn. Springer, Netherlands, pg 266-294.

Lorke, D.A. (1983). A new approach to practical acute toxicity testing. *Arch. Toxicol*, *54***:** 275-287.

Lozano, J. E. (2006). *Chemical composition of fruits and its technological importance: In Fruit Manufacturing*. 2nd Edn. Springer, NewYork, United States.

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., ... Memish, Z. A. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, *380*: 2095–2128.

Luciana, I., Felipe, M., Eliane, D.M., Teixeira, S., Bruna, D.S., Lima, R., Verônica, V. & Ana, R. (2012). Validation of quantitative real-time PCR for the in vitro assessment of antileishmanial drug activity. *Experimental Parasitology,131*: 175–179.

Lugwisha, E.H., Fabian, C. and Othman, O.C. (2016). Postharvest changes in physicochemical properties and level of some inorganic elements of sugar apple (*Annona squamosa* L.) of coast region Tanzania. *Journal of food and Nutrition Science*. *4*(3): 41- 48.

Maciel, B.L., Lacerda, H.G., Queiroz, J.W., Galvão, J., Pontes, N.N., Dimenstein, R., McGowan, S.E., Pedrosa, L.F. & Jerônimo, S.M. (2008). Association of nutritional status with the response to infection with *Leishmania chagasi*. *Am J Trop Med Hyg, 79*: 591-598.

Makwali, J.A., Wanjala, F.M.E., Kaburi, J.C., Ingonga, J., Byrum, W.W. & Anjili, C.O. (2012). Combination and monotherapy of *Leishmania major* infection in BALB/c mice using plant extracts and herbicides. *Vector Borne Dis, 49*: 123–130.

Malafaia, G. (2009). Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review. *Parasite Immunol, 31*: 587-596.

Malafaia, G., Serafim, T.D., Silva, ME., Pedrosa, M.L. & Rezende, S.A. (2009). Protein-energy malnutrition decreases immune response to *Leishmania chagasi* vaccine in BALB/c mice. *Parasite Immunol, 31*: 41-49.

Malik, S.K., Ahmad,M.& Khan, F. (2017). Qualtitative and quantitative estimation of terpenoid contents in some important plants of punjab, Pakistan.*Pakistan Journal of Science*, *69*(2): 127-139.

Mandle, V.S., Salunke, S.D., Gaikwad, S.M., Dande, K.G. & Patil, M.M. (2012). Studies on Nutritional Value of Fruits in Latur District of Maharashtra State (India) with Special Reference to Iron Mineral. *Journal of Animal Science Advances.2*(2): 344-346.

Marinova, D., Ribarova, F. & Atanassova, M. (2005). Total phenolic and total flavonoids in Bulgarian fruits and vegetables. *Journal of the University of Chemical Technology and Metallurgy*, *40*(3): 255-260.

Mariod, A.A., Abdelwahab, S.I., Elkheir, S., Ahmed Y. M., Fauzi, P.N.M. & Chuen, C.S. (2012). Antioxidant activity of different parts from *Annona squamosa*, and *Catunaregam nilotica* methanolic extract. *Acta Sci. Pol., Technol. Aliment*, *11*(3): 249-257.

Mariod, A.A., Elkheir, S., Ahmed, Y.M. & Matthaus, B. (2010). *Annona squamosa* and *Catunaregam nilotica* seeds, the effect of the extraction method on the oil composition. *J. Am. Oil Chem. Soc*, *87*: 763–769.

Marrapu, V.K., Mittal, M, Shivahare, R., Gupta, S. & Bhandari, K. (2011). Synthesis and evaluation of new furanyl and thiophenyl azoles as antileishmanial agents. *Eur. J. Med. Chem*, *46*: 1694-1700.

Marsden, P.D. (1986). Mucosal leishmaniasis (“espundia” Escomel,1911). *TransR Soc Trop Med Hyg*, *80*: 859-876.

Marsella, R. & de Gopegui, R.R. (1998). Leishmaniasis: a re-emerging zoonosis. *International Journal of Dermatology*, *37*: 801-814.

Mathur, P., Samantaray, J.C. & Samanta, P. (2008). High prevalence of functional liver derangement in visceral leishmaniasis at an Indian tertiary care centre. *Clin Gastroenterol Hepatol*, *6*(10): 70-1172.

Maundu, P.M., Ngugi, G.W. & Kabuye, C.H.S. (1999). *Traditional Food plants of Kenya.* National museums of Kenya, Nairobi, Kenya, KENRIK.

McConville, M.J., Souza, D., Saunders, E., Likic, V.A.& Naderer, T. (2007). Living in a phagolysosome; metabolism of Leishmania amastigotes. *Trends Parasitol*, *23*: 368.

Meghashri, S., Kumar, H.V. & Gopal, S. (2010). Antioxidant properties ofa novel flavonoid from leaves of Leucasaspera. *Food Chem*,*122*: 105e10.

Metwally, D.M., Al-Olayan, E.M., El-Khadragy, M.F. & Alkathiri, B. (2016).Anti-leishmanial activity (*in vitro* and *in vivo*) of allicin and allicin cream using *Leishmania major* (Sub-strain Zymowme LON4) and BALB/c mice. *PLoS ONE*, *11*: e0161296.

Meurman, H., Sanz, M. & Janket, S.J. (2004). Oral health, atherosclerosis, and cardiovascular disease. *Critical Reviews in Oral Biology and Medicine*, *15*: 403–413.

Miao, L., Stafford, A., Nir, S., Turco, S.J., Flanagan, T.D. & Epand, R.M. (1995). Potent Inhibition of Viral Fusion by the Lipophosphoglycan of *Leishmania donovani. Biochemistry*, *34*: 4676-4683.

Mishra, B.B., Kale, R.R., Prasad, V., Tiwari, V.K. & Singh, R.K. (2011a). Scope of natural products in fighting against leishmaniasis. *Oportunity, challenge and scope of natural products in medicinal chemistry*, *4*: 121-154.

Mishra, B.B., Saxena, A. & Singh, S. (2011b). Chemotherapy of leishmaniasis: Past, present and future. *Curr Med Chem J*, *14*:1153–1169.

Moghadamtousi, S.Z, Kamarudin, M.N.A., Cha, C.K., Goh, B.H. & Kadir, H.A. (2014) Phytochemistry and biology of *Loranthus parasiticus* Merr, a commonly used herbal medicine. *Am. J. Chin. Med, 42*: 23–35.

Moghadamtousi, S.Z., Goh, B.H., Chan, C.K., Shabab, T. & Kadir, H.A. (2013). Biological activities and phytochemicals of *Swietenia macrophylla* king. *Molecules, 18*: 10465–10483.

Moncada, A., Palmer, R.M. & Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev*, *43*: 109-142

Monzote, L. (2009). Current Treatment of Leishmaniasis: A Review. *The Open Antimicrob. Agents. J*, *1*: 9-19.

Morgan, J.B. & Connolly, E.L. (2013). Plant-Soil Interactions: Nutrient Uptake. *Nature Education Knowledge,* 4(8): 21-33.

Moskaug, J.O., Carlsen, H., Myhrstad, M.C. & Blomhoff, R. (2005).Polyphenols and glutathione synthesis regulation. *Am. J. Clin. Nutr*, *81*: 277S–283S.

MS, (2016a). Ministério da Saúde. Letalidade de Leishmaniose Visceral. Brasil, Grandes Regiões e Unidades Federadas. 2000 a 2016.

MS, (2016b). *Ministério da Saúde Casos de Leishmaniose Tegumentar*. Brasil: Grandes Regiões e Unidades Federadas. 1990 a 2016.

Mshana, N.R., Abbiw, D.K., Addae-Mensah, I., Adjanouhoun, E., Ahyi, M.R., Ekpere, J.A., Enow-Orock, E.G., Gbile, Z.O., Noamesi, G.K., Odei, M.A., Odunlami, H., Oteng-Yeboah, A.A., Sarpong, K., Sofowora, A. & Tackie, A.N. (2000). Traditional Medicine and Pharmacopoeia; Contributionto the Revision of Ethnobotanical and Floristic Studies in Ghana. *OAUSTRC*, 12: 57-58.

Muigai, R., Githure, J.I., Gachihi, G., Were, J.B.O., Leuweenburg, J. and Perkins, P.V. (1987). Cutaneous leishmaniasis caused by *Leishmania major* in Baringo District, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 81*: 600-602.

Murray, C. J. L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., Ezzati, M., Shibuya., … Memish, Z. A. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, *380*, 2197–2223.

Murray, H.W. (2001). Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Agents Chemother*, *45:* 2185–97.

Murray, H.W., Pepin, J., Nutman, T.B., Hoffman, S.L. & Mahmoud, A.A. (2000). Tropical medicine. *British Medical Journal*, *320*: 490-494.

Murray, L.J, Bamford, KB, Reilly, D.P.J.O., McCrum, E.E. & Evans, A.E. (1995). *Helicobacter pylori* infection: relation with cardio- vascular risk factors, ischaemic heart disease, and social class". *British Heart Journal*, *74*(5): 497–501.

Mussa, F.F., Chai, H., Wang, X., Yao, Q., Lumsden, A.B. & Chen, C. (2006). *Chlamydia pneumoniae* and vascular disease: ''an update". *Journal of Vascular Surgery*, *43*: 1301–1307.

Muthaura, C.N., Rukungaa G.M., Chhabra S.C., Mungai G.M. & Njagi E.N.M. (2007). Traditional antimalarial phytotherapy remedies used by the Kwale community of the Kenyan Coast. *Journal of Ethnopharmacology,114*: 377–386.

Mutinga, M.J. (1975a). *Phlebotomus* fauna in the cutaneous leishmaniasis focus of Mt. Elgon, Kenya. *East African Medical Journal*; *52*: 307-317.

Mutinga, M.J. (1975b). The animal reservoir of cutaneous leishmaniasis on Mt. Elgon, Kenya. *East African Medical Journal*, *52*: 142-151.

Mutiso, J.M., Macharia, J.C., Barasa, M., Taracha, E., Bourdichon, A.J. & Gicheru, M.M. (2011). *In vitro* and *in vivo* antileishmanial efficacy of a combination therapy of diminazene and artenuate against *Leishmania donovani* in BALB/c mice. *Rev. Inst. Med. Trop. Sao Paulo*, *53*(3): 129-32.

Naderer, T. & McConville, M.J. (2008). The *Leishmania*–macrophage interaction: a metabolic perspective. *Cell. Microbiol, 10*: 301-308.

Najmuddin, S.U.F.S., Romli, M.F., Hamid, M., Alitheen, N.B., Afizan, N.M. & Abdrahman, N. (2016). Anti-cancer effect of *Annona Muricata* Linn Leaves Crude Extract (AMCE) on breast cancer cell line. *BMC Complementary and Alternative Medicine*. *16*: 311-325.

Nandhakumar, E. & Indumathi, P. (2013). *In vitro* Antioxidant Activities of Methanol and Aqueous Extract of *Annona squamosa* (L.) Fruit Pulp. *Journal of Acupuncture and Meridian Studies*, *6*(3): 142 – 148.

NAS, (1995). *Nutritional requirements of laboratory animals*. 4th Edn. National academy press. Washington DC .

Nawwar, M., Ayoub, N., Hussein, S., Hashim, A., El-Sharawy, R., Wende, K., Harms, M. & Lindequist, U. (2012). Flavonol triglycoside and investigation of the antioxidant and cell stimulating activities of *Annona muricata* linn. *Arch. Pharm. Res*, *35*: 761–767.

Neal, R.A. & Croft, S.L. (1984). An *in vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. *Journal of Antimicrobial agents and Chemotherapy, 14*: 463-475.

Neupane, D.P., Majhi, S., Chandra, L., Rijal, S. & Baral, N. (2008). Erythrocyte glutathione status in human visceral leishmaniasis. *Indian J. Clin. Biochem*, *23*: 95–97.

Newman, D.J. & Craggy G.M. (2007). Natural products as sources of new drugs over the last 25 years. *Journal of natural products*, *70*: 461-477.

Ni, Y., Lin, D. & Kokot, S. (2006). Synchronous fluorescence, UV-visible spectophotometric, and voltametric studies of the competitive interaction of bis-(1,10-phenanthroline)-copper (II) complex and neutral red with DNA. *Anal Biochem*, *352*: 231–242.

Nolan, T.J. & Farrell, J.P. (1987). Experimental infections of the multimmate rat (Mastomys natalensis) with *L. donovani* and *L. major*. *American Journal of Tropical Medicine and Hygiene*, *36*(2): 264-269.

NRC, (1989). *Recommended Dietary Allowances*. National Research Council. National Academy Press, Washington D.C. USA.

Nwaka, S. & Hudson, A. (2006). Innovative lead discovery strategies for tropical diseases. *Nature review in drug discovery*, *5*: 941-955.

Obuntibejua, O.O., Truter, E.J. & Esterhuyse, A.J. (2013). The Role of Fruit and Vegetable Consumption in Human Health and Disease Prevention. *Diabetes Mellitus-Insights and Perspectives*, *3*(2): 172-180.

OECD, (2008). Guideline on acute oral toxicity (AOT). Environmental and safety monograph series on testing and adjustment. No. 425.

Ogbadoyi, E.O., Robinson, & Gull, K (2003). A high-order trans-membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. *Mol. Biol. Cell*, *14*: 1769–1779.

Ogbonnia, S.O., Nkemehule, F.E. & Anyika, E.N. (2009). Evaluation of acute and subchronic toxicity of *Stachytarpheta angustifolia* (Mill) Vahl (Fam. Verbanaceae) extract in animals. *African Journal of Biotechnology*, *8*(9): 1793 – 1799.

Ohkawa, H., Ohishi, N. & Yagi, K. (1978). Assay of lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem*, *95*: 351 - 365.

Oliveira LF, Schubacha, A.O., Martinsb, M.M., Passosa, S.L., Oliveiraa, R.V., Marzochia, M.C. & Andradea, C.A. (2011a). Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta. Tropica*, *118*: 87-96.

Oliveira, F.J. & Cecchini, R. (2000). Oxidative stress of liver in hamsters infected with *Leishmania* (L.) *chagasi*. *J. Parasitol*, *86*: 1067–1072.

Oliveira, L.R., Cezário, G.A., de Lima, C.R., Nicolete, V.C., Peresi, E., de Síbio, M.T., Picka, M.C. & Calvi, S.A. (2011b). DNA damage and nitric oxide production in mice following infection with *L. chagasi*. *Mutat. Res*, *723*: 177-181.

Olivier, M., Gregory, D.J. & Forget, G. (2005). Subversion Mechanisms by which *Leishmania* Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clin. Microbiol. Rev*, *18*: 293-305.

Onike, R. (2010). Phytochemical screening tests and medicinal values of plants active properties. Retreved from:http://wrytestuff.com/swa599816-Phytochemical-Screening-Tests And-Medicinal-Values-Of-Plants-Active-Properties.htm

Onimawo, I.A. (2002). Proximate composition and selected physicochemical properties of the seed, pulp and oil of soursop (*A. muricata*). *Plant Foods Human Nutr*, *57*: 165–171.

Onyechi, U., Ibeanu, U., Nkiruka, V., Eme, E.P. & Madubike, K. (2012). Nutrient Phytochemical Composition and Sensory Evaluation of Soursop (*Annona muricata*) Pulp and Drink in South Eastern Nigeria. *International Journal of Basic and Applied Sciences*, *12*(6): 53-57.

Osorio, E., Arango, G.J., Jimenez, N., Alzate, F., Ruiz, G., Gutierrez, D., Paco, M.A., Gimenez, A. & Robledo, S. (2007). Antiprotozoal and cytotoxic activities *in vitro* of Colombian Annonaceae. *Journal of Ethno-Pharmacology*, *111*: 630-635.

Othman, M.S., Nada, A., Zaki, H.S. & Abdel-Moneim, A.E. (2014b). Effect of *Physalis peruviana* L. on cadmium-induced testicular toxicity in rats. *Biol. Trace Elem. Res*, *159*: 278–287.

Othman, O.C., Fabian, C. & Lugwisha, E. (2014a). Postharvest physicochemical properties of soursop (*Annona muricata* L.) fruits of Coast region, Tanzania. *J. Food Nutri. Sci*, *2*(5): 220-226.

Ou, H.C., Chou, F.I. & Lin, T.M. (2006). Protective effects of eugenol against oxidized LDL induced cytotoxicity and adhesion molecule expression in endothelial cell. *Food Chem Toxicol*, *44*(9): 1485-1495.

Owuor, B.O. & Kisangau, D.P. (2006). Kenyan medicinal plants used as antivenin: a comparison of plant usage. *Journal of Ethnobiology and Ethnomedicine*, *2*: 7.

Ozbilge, H., Aksoy, N., Kilic, E., Saraymen, R., Yazar, S. & Vural, H. (2005). Evaluation of oxidative stress in cutaneous leishmaniasis. *J. Dermatol*, *32*: 7-11.

PAHO, (2017). Pan-American Health Organization. Informe Epidemiológico das Américas Informe de Leishmanioses N° 5 - Abril, 2017. Leishmanioses.

Paiva, C.N. & Bozza, M.T. (2014). Are reactive oxygen species always detrimental to pathogens? *Antioxid. Redox Signal*, *20*: 1000–1037.

Pareek, S., Yahia, E.M., Pareek, O.P. & Kaushik, R.A. (2011). Postharvest physiology and technology of Annona fruits. *Food Res. Int*, *44*: 1741–1751.

Parry, J., Hao, Z., Luther, M., Su, L., Zhou, K. and Yu, L. (2006), “Characterization of cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin and

milk thistle seed oils”. *Journal of the American Oil Chemists' Society*, *83*(10): 847-854.

Pastor, N. & Cortes, F. (2002). DNA topoisomerase activities in Chinese hamster radiosensitive mutants after x-ray treatment. *Cell Biology International*, *26*(6): 547–555.

Peebles, K.A., Baker, R.K., Kurz, E.U., Schneider, B.J. & Kroll, D.J. (2001). Catalytic inhibition of human DNA topoisomerase II by hypericin, a naphthodianthrone from St. John’s wort (*Hypericum perforatum*). *Biochemical Pharmacology*, *62*(8): 1059-1070.

Pereira, P.C.M. (2003). Interaction between infection, nutrition and immunity in Tropical Medicine. *J Venom Anim Toxins Incl Trop Dis*. *9*: 163-173.

Pinto, A.C. de Q., Cordeiro de Andrade S.R.M., Ferreira F.R., Filgueiras H.A. de C., Alves R.E. & Kinpara D.I. (2005). *Annona species*. International Centre for Underutilised Crops, University of Southampton, Southampton, UK.

Piombo, G., Barouh, N., Barea, B., Boulanger, R., Brat, P., Pina, M. & Villeneuve, P. (2006), Characterization of the seed oils from kiwi (*Actinidia chinensis*), passion fruit (*Passiflora edulis*) and guava (*Psidium guajava*). *Ole ́agineux*, *13*(2): 195-9.

Pirmez, C. (1992). Immunopathology of American cutaneous leishmaniasis. *Memorias doInstituto Oswaldo Cruz*, *5*: 105-109.

Piscopo, T.V. & Mallia, A.C. (2006). Leishmaniasis. *Postgraduate Medical Journal*, *82*: 649-657.

Pommier, Y., Leo, E., Zhang, H. & Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol*, *17*: 421-433.

Portugal, L.R., Fernandes, L.R. & Cesaretal, G.C. (2004). “Infectionwith *Toxoplasma gondii* increases atherosclerotic lesion in ApoE- deficient mice.” *Infection and Immunity*, *72*: 3571– 3576.

Prieto, P., Pineda, M. & Aguilar, M. (1999). Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum

complex: specific application to the determination of vitamin E. *Anal Biochem*, *69*: 337e41.

Pyne, R.W., Murray, D.A., Harding, S.A., Baird, D.B. & Soutar, D.M. (2011). *An introduction to GenStat for Windows*. 14th Edn. VSN International, Hemel Hempstead, UK.

Qin, Y., Pang, J.Y., Chen, W.H., Cai, Z. & Jiang, Z.H. (2006). Synthesis, DNA binding affinities, and binding mode of berberine dimmers. *Bioorg Med Chem,14*: 25–32.

Quintana, E., Torres, Y., Alvarez, C., Rojas, A., Forero, M.E. & Camacho, M. (2010). Changes in macrophage membrane properties during early *Leishmania amazonensis* infection differ from those observed during established infection and are partially explained by phagocytosis. *Exp. Parasitol*, *124*: 258-264.

Rana, V.S. (2015). Fatty Oil and Fatty Acid Composition of *Annona squamosa* Linn. Seed Kernels. *Biocontrol Science and Technology*, *15*(1): 34-48.

Rates, S.M.K. (2001). Plants as sources of drugs. *Toxicon*, *39*: 603-13.

Ravaomanarivo, L.H.R., Razafindraleva, H.A., Raharimalala, F.N., Rasaohantaveloniaina, B., Ravelonandro, P.H. & Mavingui, P. (2014). Efficacy of seeds extracts of *Annona squamosa* and *Annona muricata* (Annonaceae) for the control of *Aedes albopictus* and *Culex quinquefasciatus* (Culicidae). *Asian Pacific Journal of Tropical Biomedicine*, *4*(10): 787-795.

Ready, P. D. (2014). Epidemiology of visceral leishmaniasis. *Clin Epidemiol*, *6*:147–54.

Ready, P.D (2013) Biology of phlebotomine sand flies as vectors of disease agents. *Annu Rev Entomol, 58*: 227-250.

Reithinger, R., Brookerb, S. & Kolaczinski, J.H. (2007). Visceral leishmaniasis in eastern Africa: current status. *Trans. R. Soc. Trop. Med. Hyg*, *101:* 1169 –70

Roberts, M.T.M. (2006).Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *British Medical Bulleting, 75/76*:115-130.

Rodriguez-Amaya, D.B., Rodriguez, E.B. &Amaya-Farfan, J. (2006). Advances in food carotenoids research: Chemical and technological aspects, implication in human health. *Mal. J. Nutr*, *12*(1): 101-121.

Roopana, S.M., Bharathi, A., Kumar, R., Khanna, V.G. & Prabhakarn, A. (2012). Acaricidal, insecticidal, and larvicidal efficacy of aqueous extract of *Annona squamosa* L peel as biomaterial for the reduction of palladium salts into nanoparticles. *Colloids Surf B Biointerfaces*, *92*, 209e21.

Roy, N., Laskar, R.A., Sk, I., Kumari, D., Ghosh, T. & Begum, N.A. (2011). A detailed study on the antioxidant activity of the stem bark of *Dalbergia sissoo* Roxb, an Indian medicinal plant. *Food Chem. 126*: 1115e21.

Roy, P., Das, S., Auddy, R.G. & Mukherjee, A. (2012). Biological targeting and drug delivery in control of Leishmaniasis. *Journal of Cell and Animal Biology*, *6*(6): 73-87.

Roy, P., Das, S., Bera, T., Mondol, S. & Mukherjee, A. (2010). Andrographolide nanoparticles in leishmaniasis: characterization and *in vitro* evaluations. *Int. J. Nanomed*, *5*: 1113-1121.

Rupprecht, J.K., Hui, Y.H. & McLaughlin, J.L. (1990). Annonaceous acetogenins: a review. *J. Nat. Prod*, *53*: 237-278.

Sackow, M.A., Danneman, P. & Brayton, C. (2005). *The laboratory Mouse. A Volume in the Laboratory Animal Pocket Reference Series*. CRC Press, Boca Raton London New York, Washington, D.C.

Sakthianandeswaren, A., Elso, C.M., Simpson, K., Curtis, J.M., Kumar, B., Speed, T.P., Handman, E. & Foote, S.J. (2005). The wound repair response controls outcome to cutaneous leishmaniasis. *Proc. Natl. Acad. Sci. USA*, *102*: 15551-15556.

Salawu, O.A., Chindo, B.A., Tijani, A.Y., Obidike, I.C., Salawu, T.A. & Akingbasote, A.J. (2009). Acute and Sub-Acute Toxicological Evaluations of the Methanolic Stem Bark Extract of *Crossopteryx febrifuga* in Rats. *African Journal of Pharmacy and Pharmacology*, *3*: 621-626.

Salgado, F.N., Ferreira, T.M. & Costa, J.M. (2003). Involvement of the renal function in patients with visceral leishmaniasis (kala-azar). *Rev Soc Bras Med Trop*, *36*(2): 217-21.

Samuagam, L., Khoo, H.E., Akowuah, G.A., Okechukwu, P.N. & Yim, H.S. (2014). HPLC analysis of antioxidant compounds in some selected tropical fruits' peel. *Innovative Romanian Food Biotechnology*, *14*: 61-68

Sang, D.K. & Chance M.L. (1993). Cutaneous leishmaniasis due to *Leishmania aethiopica*, on Mount Elgon, Kenya. *Annals of Tropical Medicine andParasitology*, *87*(4): 349-357.

Santos, D.O., Coutinho, C.E.R., Bottino, C.G., Viera, R.T., Nascimento, S.B., Bourguignon, S.C., Castro, H.C., Madeira, M.F., Bernardino, A., Corte-Real, S., Pinho, R.T. & Rodrigues, C.R. (2008). Leishmaniasis treatment - a challenge that remains; a review. *Parasitology Research*, *103*: 1 – 10.

Santos, E.W., Oliveira, D.C., Hastreiter, A., Silva, G.B., Beltran, J.S.O., Tsujita, M., Crisma, A.R., Neves, S.M.P., Fock, R.A. & Borelli, P. (2016). Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. *Braz. J. Vet. Res. Anim. Sci*, *53*(2): 138-145.

Santos, H.R., Aguar, I.A., De Souza, E.P., De Lima, M.F. & Palatnik, M. (2003). Immunotherapy against murine experimental visceral leishmaniasis with the FML. *Vaccine*, *21*(32): 4668-4676**.**

Saraiva, E.M., Pinto-Da-Silva, L.H., Wanderley, J.L.M., Bonomo, A.C., Barcinski, M.A. & Moreira, M.E.C. (2005). Flow cytometric assessment of Leishmania spp. metacyclic differentiation: validation by morphological features und specific markers. *Exp. Parasitol*, *110*: 39.

Sarkar, R. & Mandal, N. (2011). *In vitro* cytotoxic effect of hydroalcoholic extracts of medicinal plants on Ehrlich's Ascites Carcinoma (EAC). *Int. J. Phytomed*, *3*: 370-380.

Savithramma, N., Linga Rao, M. & Suhrulatha, D. (2011). Screening of medicinal plants for secondary metabolites. *ME. J. Sci. Res*, *8*(3): 579- 584.

Schindler, J.J., Warren R.P., Allen, S.D. & Jackson, M.K. (1993). Immunological effects of amphotericin B and liposomal amphotericin B onsplenocytes from

immune-normal and compromised mice. *Antimicrob. Agents Chemother*, *37*: 2716-2721.

Schmid-Hempel, P. (2009). Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena' such as virulence. *Phil. Trans. R. Soc. B,364*: 85-98.

Schnur, L.E., Zuckerman A. & Montillio, B. (1973) Dissemination of leishmaniasis to the organs of Syrian hamsters following intrasplenic inoculation of promastigotes. *Exp. Parasitol*, *34*: 432-447.

Seddon, J.M.,Ajani, UA., Sperduto, R.D., Hiller, R., Blair, N., Burton, T.C., Farber, M.D., Gragoudas, E.S., Haller, J., Miller, D.T., Yannuzzi, L.A. & Willett, W.(1994). Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. *JAMA*, *272*(18): 1413-1420.

Sen, G., Mukhopadhyay, R., Ghosal, J. & Biswas, T. (2001). Oxidative damage of erythrocytes: A possible mechanism for premature hemolysis in experimental visceral leishmaniasis in hamsters. *Ann. Hematol*, *80*: 32–37.

Sen, N., Banerjee, B. & Majumder, H.K. (2008). *Molecular analysis of programmed cell death by DNA topoisomerase inhibitors in Kinatoplatid parasite Leishmania. In Martin, M.P. Eds: Programmed cell death in protozoa*. India, Landes Bioscience and Springer Science + Bussiness Media.

Sen, R., Ganguly, S., Saha, P. & Chatterjee, M. (2010). Efficacy of artemisinin in experimental visceral leishmaniasis. *Int. J. Antimicrob. Agents*, *36*: 43-49.

Serafim, T.D., Malafaia, G., Silva,M.E., Pedrosa, M.L. & Rezende, S.A. (2010). Immune response to *Leishmania* (*Leishmania*) *chagasi* infection is reduced in malnourished BALB/c mice.*Mem Inst Oswaldo Cruz*, *Rio de Janeiro*, *105*(6): 811-817.

Serarslan, G., Yilmaz, H.R. & Sogut, S. (2005). Serum antioxidant activities, malondialdehyde and nitric oxide levels in human cutaneous leishmaniasis. *Clin. Exp. Dermatol*, *30*: 267–271.

Shapaz, S., Bories, C., Loiseau, P.M., Cortes, D., Hocquemiller, R., Laurens, A. & Cave, A. (1994). Cytotoxic and antiparasitic activity from *Annona senegalensis* seeds. *Planta Medica, 60*(6): 538-540.

Shapaz, S., Gonzalez, M.C., Hocquemiller, R., Zafra-Polo, M.C. & Cortes, D. (1996). Annosenegalin and Annogalene: two cytotoxic monotetrahydrofuran acetogenins from *Annona senegalensis* and *Annona cherimolla*. *Phytochemistry, 42:* 106-107.

Sheikholeslami, M., Gorji-Bandpy, M., Ganji, D.D. & Soleimani, S. (2014). Heat flux boundary condition for nanofluid-filled enclosure in presence of magnetic field. *J. Mol. Liq*, *193*, 174–184.

Shiraz, J. K. & Syed, M. (2007). Cutaneous leishmaniasis in Pakistan. *DermatologyOnline Journal*, *11*: 4.

Shirbazou, S. & Jafari, M. (2012). The multiple forms of *Leishmania major* in BALB/c mice lung in Iran. *Iran. J. Parasitol*, *7*: 99–102.

Singodia, D., Verma, A., Verma, R.K. & Mishra, P.R. (2011). Macrophages using 4-sulfated N-acetyl galactosamine more efficiently in comparison mannose-decorated liposome. An application in drug delivery. *Nanomedicine.Epub*, *10*: 1061.

Soares, N.M., Leal, T.F., Fiúza, M.C., Reis, E.A.G., Souza, M.AL, Dos-Santos, W.L. &Pontes-de-Carvalho L. (2010). Plasma lipoproteins in visceral leishmaniasis and their effect on *Leishmania*-infected macrophages. *Paras Immunol*, *32*: 259-266.

Sofowora, E.A. (1993). *Phytochemical screening of medicinal plants and traditional medicine in Africa*. 2nd Edn. Nigeria, Spectrum Books Limited, pg 50-156.

Soni, H., Sharma, S., Patel, S.S., Mishra, K. & Singhai, A.K. (2011). Preliminary phytochemical screening and HPLC analysis of flavenoids from methenolic extract of leaves of *Annona squamosa*. *Int. Res. J. Pharma*, *2*(5): 242-246.

Souza, L.W., Souza, S.V. & Botelho, A.C. (2012). Comparative analysis of the geographic distribution of the histopathological spectrum and leishmania species of American cutaneous leishmaniasis in Brazil. *Anais Bras. Dermatol*, *87*: 369–374.

Spanos, G.A. & Wrolstad, R.E. (1990). Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. *Journal of Agricultural and Food Chemistry*, *38*: 1565–1571.

Straub, D.A. (2007). Calcium supplementation in clinical practice: A review of forms, Doses and indications. *Nutr. Clin. Pract*, *22*: 286–296.

Sun, T. & Zhang, Y. (2008). Pentamidine binds to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation. *Nucleic. Acids. Res*, *36*: 1654-1664.

Sundar, S. & Chakravarty, J. (2015).An update on pharmacotherapy for leishmaniasis. *Expert Opin. Pharmacother*, *16*: 237–252.

Sundar, S., Jha, T., Thakur, C., Sinha, P. & Bhattacharya, S.K. (2007). Injectable paromomycin for visceral leishmaniasis in India. *N Engl J Med*, *356*: 2571–2581.

Sundar, S., Jha, T.K., Sindermann, H., Junge, K., Bachmann, P. & Berman, J. (2003). Oral miltefosine treatment in children with mild to moderate Indian visceral leishmaniasis. *Pediatr J Infect Dis*, *22*: 434–448.

Tayeb, H., Salerno, M., Priebe, W., Kozlowski, H. & Garnier-Suil-lerot, A. (2003). New findings in the study of the intercalation of bisdaunorubicin and its monomeric analogues with naked and nucleus DNA. *Chem Biol Interact*, *145*: 349–358.

Taylor, L. (2002). *Technical data report for Graviola* (*Annona muricata*). *In Herbal Secretes of the Rainforest*. 2nd Edn. Sage Press, Austin.

Tempone, A. G., da Silva, A. C., & Brandt, C. A. (2005). Synthesis and antileishmanial activities of novel 3-substituted quinolines. *Antimicrobial Agents and Chemotherapy*, *49*: 1076-80.

Tempone, A.G., de Oliveira, C.M. & Berlinck R.G.S. (2011). Current approaches to discover marine antileishmanial natural products. *Planta Med*, *77*: 572–585.

Thakur, C.P. (1984). Epidemiological, clinical and therapeutic features of Bihar kala-azar (including post kala-azar dermal leishmaniasis). *Trans. R. Soc. Trop. Med. Hyg*, *78*: 391-398.

Thakur, C.P., Narayan, S. & Ranjan, A. (2004). Epidemiological, clinical and pharmacological study of antimony-resistant visceral leishmaniasis in Bihar India. *Indian J Med Res*, *120*: 166-172.

Thakur, C.P., Narayan, S. & Ranjan, A. (2004). Epidemiological, clinical and pharmacological study of antimony-resistant visceral leishmaniasis in Bihar India. *Indian J Med Res*, *120*: 166-72.

Thakur, C.P., Singh, R.K., Hassan, S.M., Kumar, R., Narain, R.K. & Kumar, S.A. (1999).Amphotericin B deoxycholate treatment of visceral leishmaniasis with newer modes of administration and precautions: a study of 938 cases. *Trans. R. Soc. Trop. Med. Hyg, 93*: 319.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Filho, B.P. & Nakamura, C.V. (2011). Recent advances in leishmaniasis treatment. *Int. J. Infect. Dis*. *15*: e525-e532.

Tonui, W.K. (2006).Situational Analysis of *Leishmania*ses Research in Kenya. *African Journal of Health Sciences, 13*: 7-21.

Tonui, W.K. & Titus, R.G. (2006). *Leishmania major* soluble exo-antigens (LmSEAgs) protect neonatal BALB/c mice from a subsequent challenge with L. major and stimulate Cytokine production by Leishmania naïve Human Peripheral blood mononuclear cells. *Journal of parasitology*, *92*: 971-976.

Trease, G.E. & Evans W.C., (2002). *A textbook of pharmacognosy*. (15th ed.) London Academic Press, pg 578-678.

Trease, G.E. & Evans, W.C. (1989*). Pharmacology*. 11th Edn. Bailliere Tindall Ltd, London, pg 60-75.

Turco, S.J. & Descoteaux, A. (1992). The lipophosphoglycan of *Leishmania* parasites. *Annu. Rev. Microbiol*, *46*: 65-92.

Ugwu, B.U., Okogun, J.I., Kabiru, A.Y. and Ogbadoyi, E.O. (2011). Evaluation of Therapeutic Potentials of Stem Bark Extracts of *Annona senegalensis*in Experimental *Trypanosoma bruceibrucei* Infection in Mice. *British Journal of Pharmacology and Toxicology,* 2(2): 63-70.

USDA, (2009). *National Nutrient Database for Standard Reference*. Agricultural Research Service, Release 22. United States Department of Agriculture (USDA), USA.

Usunomena, U. (2012). A Review of some African medicinal plants. *International Journal of Pharma and Bio Sciences*, *3*(4): 1-11.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1), 44-84.

Van de Van, H. Vermeersch, M., Matheeussen, A., Vandervoort, J., Weyenberg, W., Apers, S., Cos, P., Maes, L. & Ludwig, A. (2011). PLGA nanoparticles loaded with the antileishmanial saponins β-aescin factor influence study and in vitro efficacy evaluation. *Int. J. Pharm*, *420*: 122-132.

Van Duyn, M.A. & Pivonka, E. (2000). Overview of the health benefits of fruit and vegetable consumption for the dietetics professional. *J Am Diet Assoc*, *99*(10): 1241-1248.

Van Het-Hof, K.H., West, C.E., Weststrate, J.A. & Hautvast, J.G. (2000). Dietary factors that affect the bioavailability of carotenoids. *J Nutr*, *130*(3): 503-506.

Van Rooyen, J., Esterhuyse, A.J., Engelbrecht, A.M. & Du-Toit, E.F. (2008) Health benefits of natural carotenoid rich oil: a proposed mechanism of protection against ischaemia/reperfusion injury. *Asia Pacific Journal of Clinical Nutrition*, *17*(1): 316– 319.

Vannier-Santos, M.A., Martiny, A. & De Souza, W. (2002). Cell biology of *Leishmania* spp.: invading and evading. *Curr Pharm Des*, *8*: 297-318.

Varadharajan, V.,Janarthanan, U.K. & Krishnamurthy, V. (2012). Physicochemical, phytochemical screening and profiling of secondary metabolites of *Annona squamosa* leaf extract. *World Journal of Pharmaceutical research*, *1*(4): 1143-1164.

Vertut-Do, A., Hannaert, A. & Bolard, J. (1988). The polyene antibiotic amphotericin B inhibits the Na1/K1 pump of human erythrocytes. *Biochem. Biophys. Res. Commun*, *157*: 692-697.

Vila-Nova, N.S., de Morais, S.M., Falcão, M.J.C., Machado, L.K.A., Beviláqua, C.M.L., Costa, I.R.S., de Sousa-Brasil, N.V.G.P. & de Andrade-Júnior. H.F. (2011). Leishmanicidal activity and cytotoxicity of compounds from two

Annonacea species cultivated in Northeastern Brazil. *Revista da Sociedade Brasileira de Medicina Tropical*. *44*(5): 567-571.

Villa, S.Z., Borjas, D.R., Carrero, J.C. & Ortiz, L.O. (2002). How protozoan parasites evade the immune response. *Trends. Parasitol*, *18*: 272-278.

Vinet, A.F., Jananji, S., Turco, S.J., Fukuda, M. & descoteaux, AS. (2011). Exclussion of synaptotagmoin V at the phagocytotic cup by the *Leshmania denovani* lipophosphoglycan results in decreased promastigote internalization. *Microbiology*, *157*: 2619-2628.

Vural, H., Aksoy, N. & Ozbilge, H. (2004). Alterations of oxidative-antioxidative status in human cutaneous leishmaniasis. *Cell. Biochem. Funct*, *22*: 153–156.

Wabwoba, B.W., Anjili, C.O., Ngeiywa, M.M., Ngure, P.K., Kigondu, E.M., Ingonga J. & Makwali, J. (2010). Experimental chemotherapy with *Allium sativum* (Liliaceae) methanolic extract in rodents infected with *Leishmania major* and *Leishmania donovani*. *J Vector Borne Dis*, *47*: 160–167.

Walker, J., & Saravia, N.G. (2004). Inhibition of *Leishmania donovani* promastigote DNA topoisomerase I and human monocyte DNA topoisomerases I and II by antimonial drugs and classical antitopoisomerase agents. *J Parasitol*, *90*(5): 1155-62.

Walters, L.L. (1993).*Leishmania* differentiation in natural and unnatural sandfly hosts. *Journal of Eukaryotic Microbiology, 40*: 196-206.

Wang, Y., Bentley, M.E., Zhai, F. & Popkin, B.M. (2002). Tracking dietary intake patterns of Chinese from childhood to adolescence over a six-year follow-up period. *J Nutr*, *132*(93): 430-438.

Wardlaw, G.M. & Smith, A.M. (2009). *Contemporary Nutrition*. 7thEdn. McGraw Hill, New York. pg 163-199.

Wardlaw, J., Permenter, K. & Waller, J. (2000). Nutrition knowledge and food intake. *Appet, 34*(3): 269-275.

Wasan, K.M., Najafi, S., Wong, J. & Kwong, M. (2001).Assessing Plasma Lipid Levels, Body Weight, and Hepatic and Renal Toxicity Following Chronic

Oral Administration of a Water Soluble Phytostanol Compound, FM-VP4, to Gerbils. *J. Pharm. Sci*, *4*(3): 228 – 233.

Wasunna, M.K., Rashid, J.R., Mbui, J., Kirigi, G., Kinoti D., Ldenyo H., Felton, J.M., Sabin, A.J. & Horton J. (2005). A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya, *Am. J. Trop. Med. Hyg*. *73*: 871.

Watt, J.M. & Breyer-Brandwijk, G.M. (1962). *The medicinal and poisonous plants of southern and eastern Africa*. 2nd Ed. Livingstone, London, UK.

Wheeler, C.R., Salzman, J.A., Elsayed, N.M., Omaye, S.T., Korte, D.W. & Jr, R. (1990).Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. *Anal. Biochem*, *184*: 193-199.

Wheeler, R.J., Sunter J.D., & Gull, K (2016). Flagellar pocket restructuring through the *Leishmania* life cycle involves a discrete flagellum attachment zone. *J. Cell Sci*. *129*: 854–867.

WHO (2010). Control of the leishmaniasis. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis. World Health Organization technical report series, no. 949. World Health Organization, Geneva, Switzerland, Retreved from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf

WHO, (1990). Control of the Leishmaniasis. Technical Report Series 793. Geneva: *WHO*, pg 1-158.

WHO, (2001). Report on global surveillance of epidemic prone infectious diseases WHO/CDS/CSR/ISR/2000.

WHO, (2002). Reducing risks, promoting healthy life, The World Health Report, Chapter 4.

WHO, (2007). Leishmaniasis, background information. Retrieved from: http,//www.who.int/leishmaniasis/en/

WHO, (2014) *Leishmaniasis - A Brief History of the Disease*. Geneva, Switzerland World Health Organization. Retreved

from:http://apps.who.int/iris/bitstream/10665/111008/1/WHODCOWHD2014.1eng.pdf.

WHO, (2016). Fact sheet no. 375, update. Geneva, World Health Organization. Retreved from: http://www.who.int/mediacentre/factsheets/fs375/en/

WHO. (2008). Population nutrient intake goals for preventing diet-related chronic diseases. Retreved from:http://www.who.int/nutrition.

WHO. (2017). Leishmaniasis: the global trend. WHO; Geneva, Switzerland: Retrieved from:http://www.who.int/neglected_diseases/integrated_media_leishmaniasis/en/index.html.

Wijers, D.J. & Minter, D.M. (1966). Studies on the vector of kala-azar in Kenya. VL. The outbreak in Meru district. *Annals of Tropical Medicine and Parasitology*; *60*(1): 11-21.

Wink, M. (2007). Molecular modes of action of cytotoxic alkaloids from DNA intercalation, spindle poisoning, topoisomerase inhibition to apoptosis and multiple drug resistance. *Alkaloids*, *64*: 1- 48.

Wink, M. (2012). Medicinal plants: A source of anti-parasitic secondary metabolites. *Molecule*, *17*: 12771-12791.

Wink, M. & Schimmer, O. (2010). *Molecular modes of action of defensive secondary metabolites. In Function and Biotechnology of plant secondary metabolites*; Wink, M. Eds.Wiley-Blackwell, oxford, UK, pg 1621-1691.

Worrell, D.B.C., Seancarrington, C. & Hubber, D.J. (1994). Growth, maturation and ripening of soursop (*Annona muricata* L.) fruit. *Scientia Horticulturae*, *57*(1-2):7-15.

Wyllie, S., Cunningham, M.L. & Fairlamb, A.H. (2004). Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. *J Biol Chem*, *279*: 39925-39932.

Xin, L.T, Liu, L., Shao, C.L., Yu, R.L., Chen, F.L., Yue, F.J., Wang, U., Guo, Z.L., Fan Y.C., Guan, H.S. & Wang, C.Y. (2017). Discovery of DNA topoisomerase I inhibitors with low toxicity based on virtual screening from natural products. Marine drugs, *17*: 771-791.

Xu, X., Oliveira, F., Chang, B.W., Collin, N., Gomes, R., Teixeira, C., Raeynoso, D., Pharm, V., Elnaiem, D.F Kamhawi, S., Ribeiro, J.M., Valenzuela, J.G. & Andersen, J.F. (2011). Structure and function of a 'yellow' protein from saliva of the sandfly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. *J. Biol. Chem*, *286*: 32383-32393.

Yaghoobi-Ershadi, M.R. (2012) Phlebotomine sand flies in Iran and their role on leishmania transmission. *J Arthropod-Borne Dis*, *6*(1): 1–17.

Yamada, M., Hayashi, K., Shogo, I., Ken, T., Tsutsui, K., Ito, T., Iinuma, M. & Nozaki, H. (2006). Inhibitory Activity of Plant Stilbene Oligomers against DNA Topoisomerase II. *Biol. Pharm. Bull, 29*(7): 1504-1507.

Yen, G.C. & Chen, H.Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem*. *3*, 27e32.

Yoshida, T., Maulik, N., Engelman, R.M., Ho, Y.S., Magnenat, J.L., Rousou, J.A., Flack J.E., Deaton, D. & Das, D.K. (1997). Glutathione peroxidase knockout mice are susceptible to myocardial ischemia reperfusion injury. *Circulation*, *96*: 211-216.

YukIlhami, G. (2011). Antioxidant Activity of Eugenol: A Structure–Activity Relationship Study. *J Med Food, 14*(9): 975–985.

Zahraei-Ramazani, A.R., Kumar, D., Mirhendi, H., Sundar, S.H., Rajnikan, M., Moin- Vaziri, V., ... Yaghoobi-Ershadi, M.R. (2015). Morphological and genotypic variations among the species of the subgenus Adlerius (Diptera: Psychodidae, Phlebotomus) in Iran. *J Arthropod-Borne Dis*, *9*(1): 84–97.

Zahraei-Ramazani, A.R., Kumar, D., Yaghoobi-Ershadi, M.R., Naghian, A., Jafari, R., Shirzadi, M.R., ... Hanafi-Bojd, A.A. (2013). Sand flies of the subgenus Adlerius (Diptera: Psychodidae) in an endemic focus of visceral leishmaniasis and introduction of Phlebotomus (Adlerius) comatus as a new record for Iran. *J Arthropod-Borne Dis. 7*(1): 1–7.

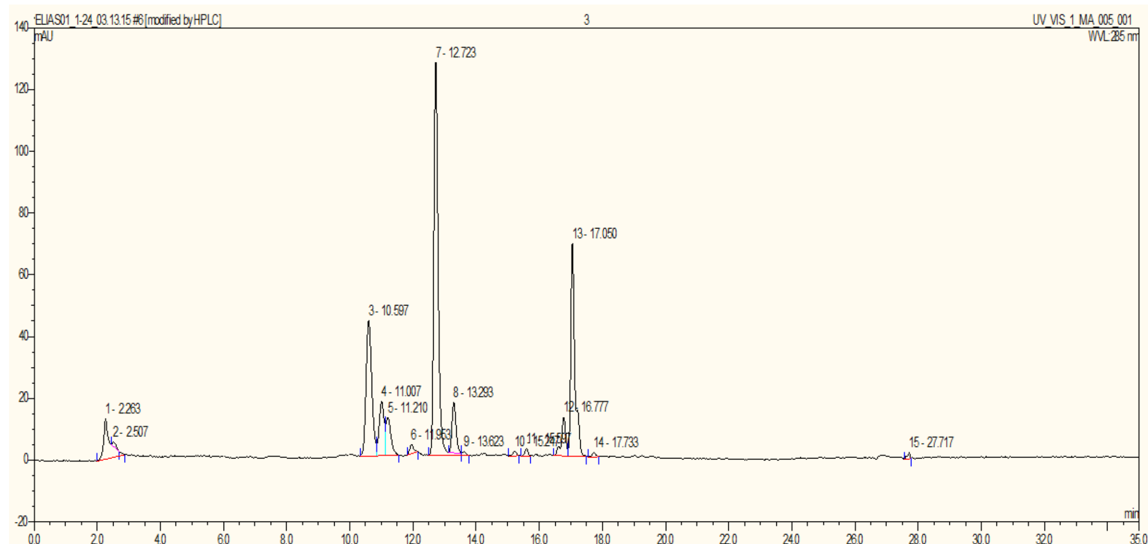
Zimmerman, M. & Snow, M.E. (2018). *Nutrition Basics: An Active Approach*. Flat World, Boston, MA.

APPENDICES

Appendix I: Properties of different solvents used in extraction of phytochemicals

Solvent	Formula	Boiling point ^oC	Melting point oC	Density (g/mL)	Solubility in H_2O (g/100g)	Relative polaity	Eluent strength	Threshold limit (ppm)	Vaporpressure at 20^oC (hPa)
Hexane	C_6H_{14}	69	-95	0.655	0.0014	0.009	0.01	50	160
Ethyl acetate	$C_4H_8O_2$	77	-83.6	0.894	8.7	0.228	0.58	400	97
Methanol	CH_4O	64.6	-98	0.791	M	0.762	0.95	200	128
Water	H_2O	100.00	0.00	0.998	M	1.000	>>1	N/A	N/A

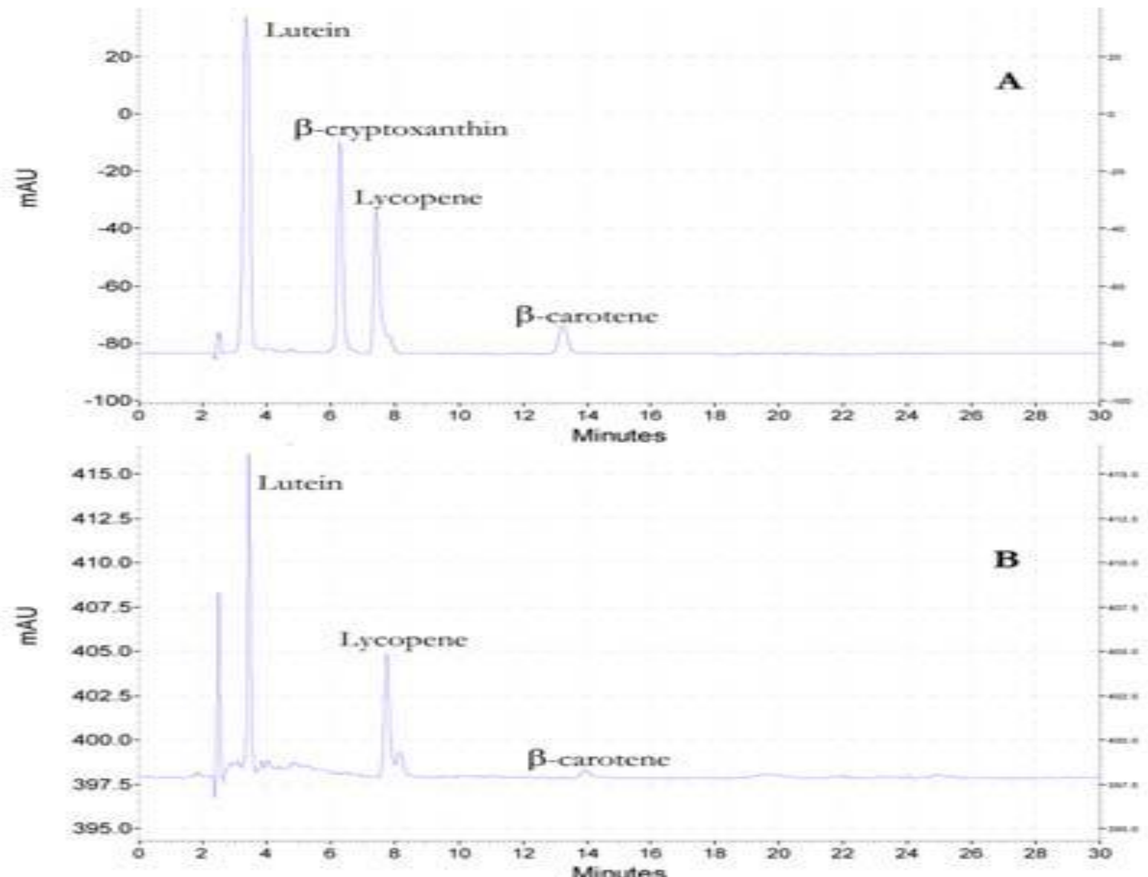
Appendix II: HPLC chromatogram of a sample showing different carotenoids eluted at different retention times



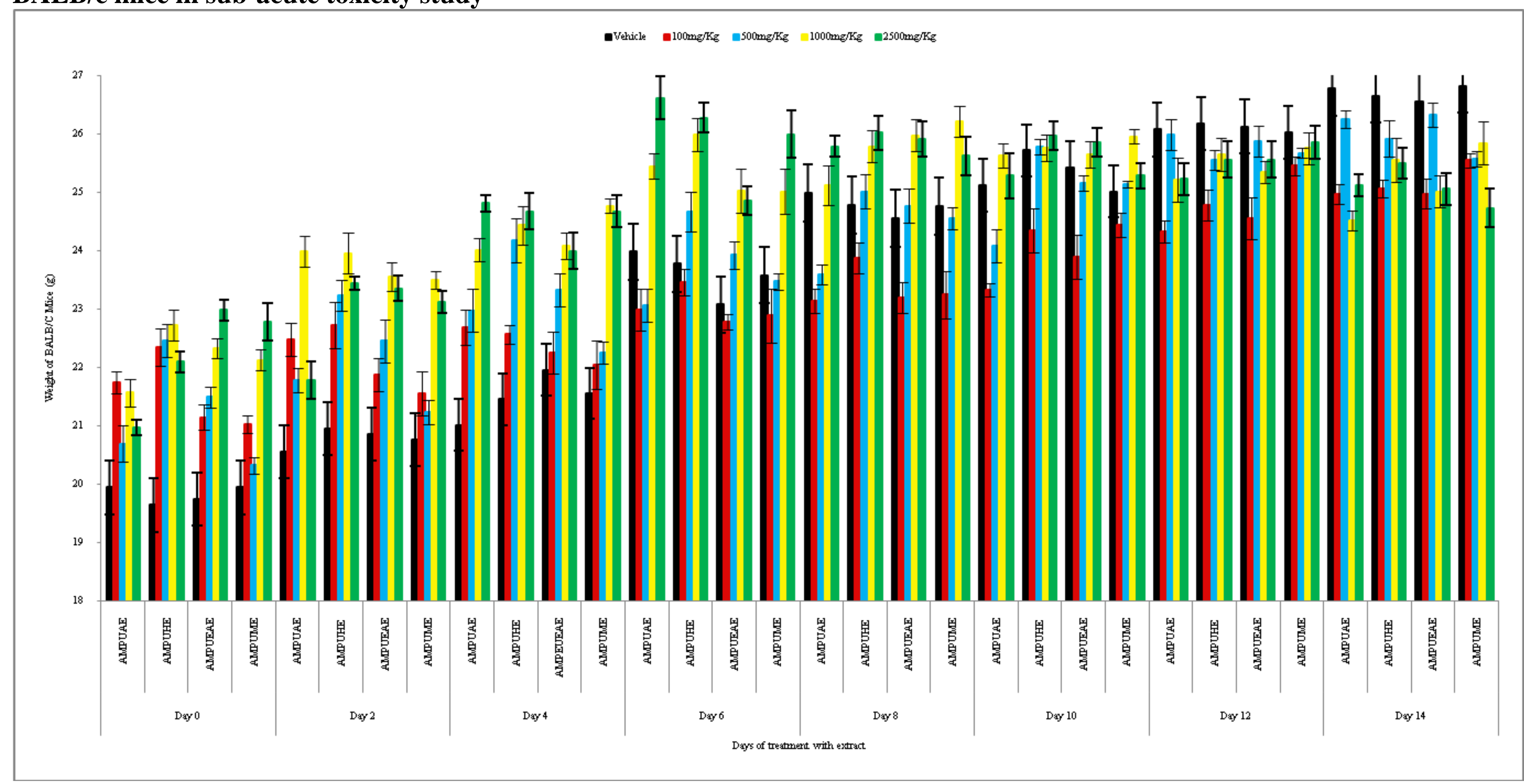
Appendix III: Different carotenoids from *A. muricata* and *A. squamosa* fruits peel, pulp and seeds separated on a reverse-phase C_{30} HPLC system and spectral characteristics used in identification from photodiode array detection

Carotenoid	Spectral characteristics (nm λ_{max})	Retention time (min)
Neoxanthin	466	6.99
Violaxanthin	433	7.6
Lycopene	440	8.67
Lutein	442	11
Antheraxanthin	442	14.93
Zeaxanthin	430	13.23
α-carotene	408	17.06
β-carotene	450	17.2
γ-carotene	444	17.6
Chlorophyll a	430	12.7
Chlorophyll b	465	10.6
Others	446, 441, 443	11.8, 15.9, 16.3

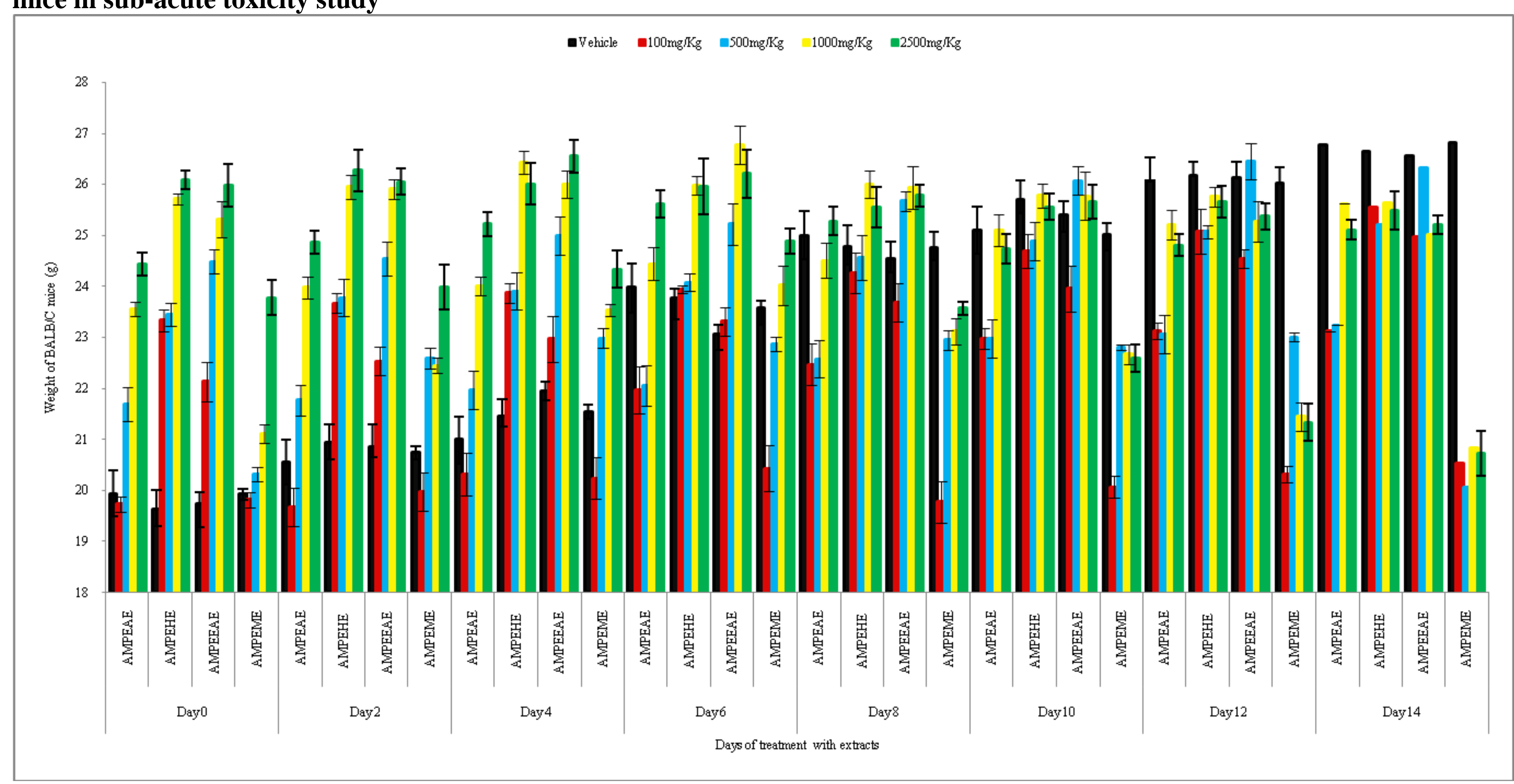
Appendix IV: (A) HPLC chromatogram for carotenoid standards; (B) HPLC chromatogram for carotenoids from *A. squamosa* (custard apple)



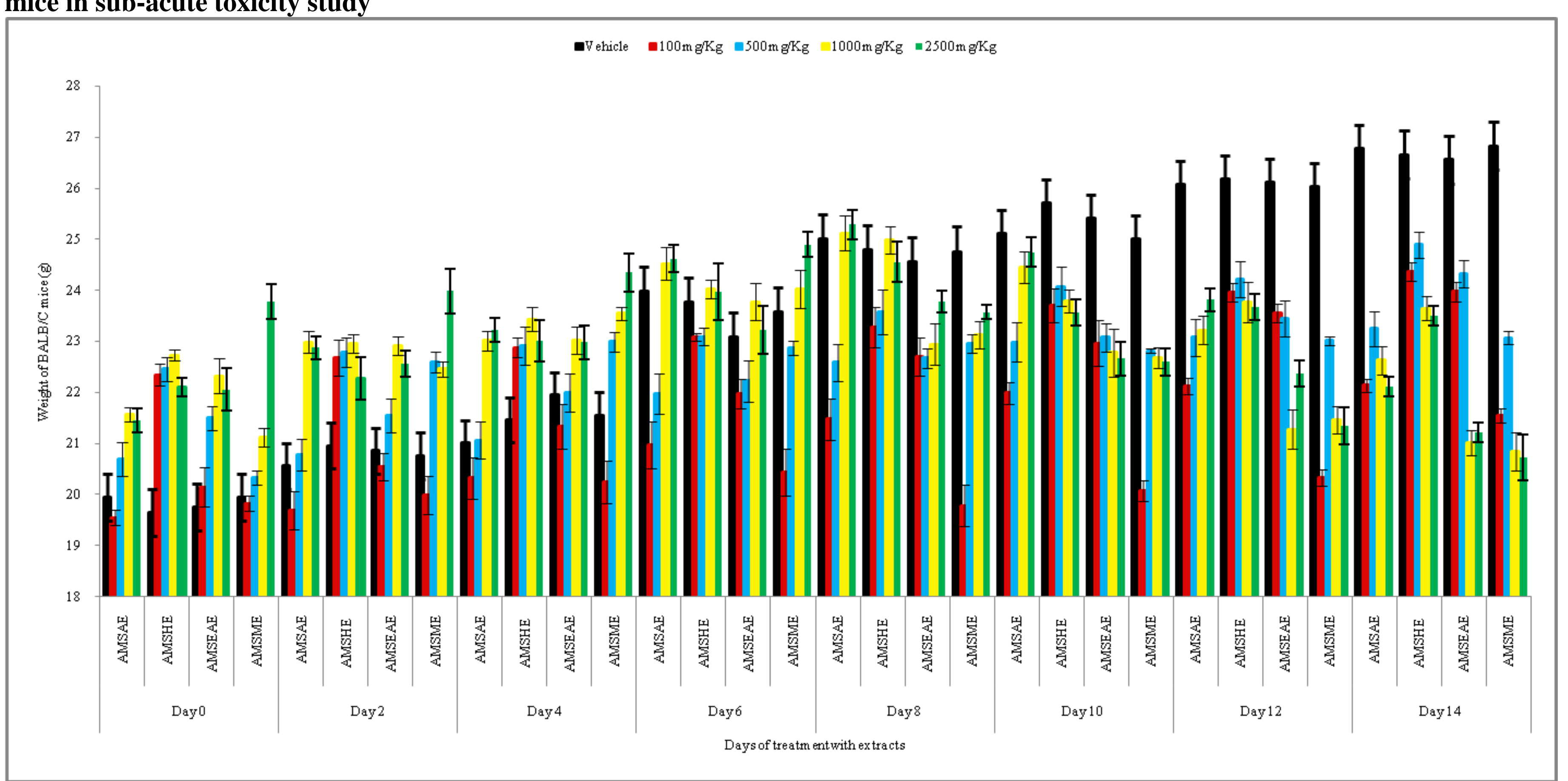
Appendix V: Effects of A. muricata pulp fruit aqueous, methanol, ethyl acetate and hexane extracts of on body weight of BALB/c mice in sub-acute toxicity study



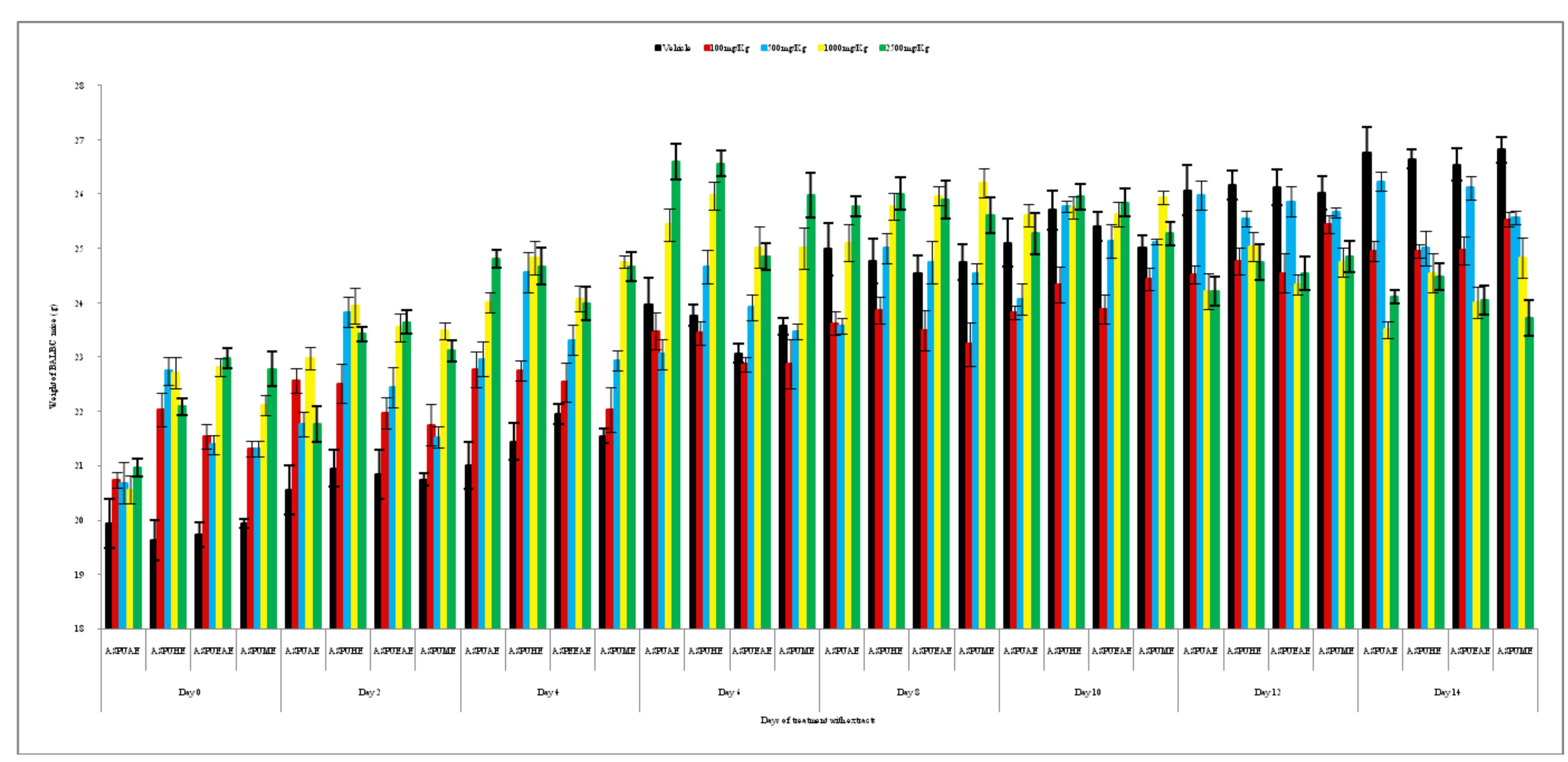
Appendix VI: Effects of A. muricata peel aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study



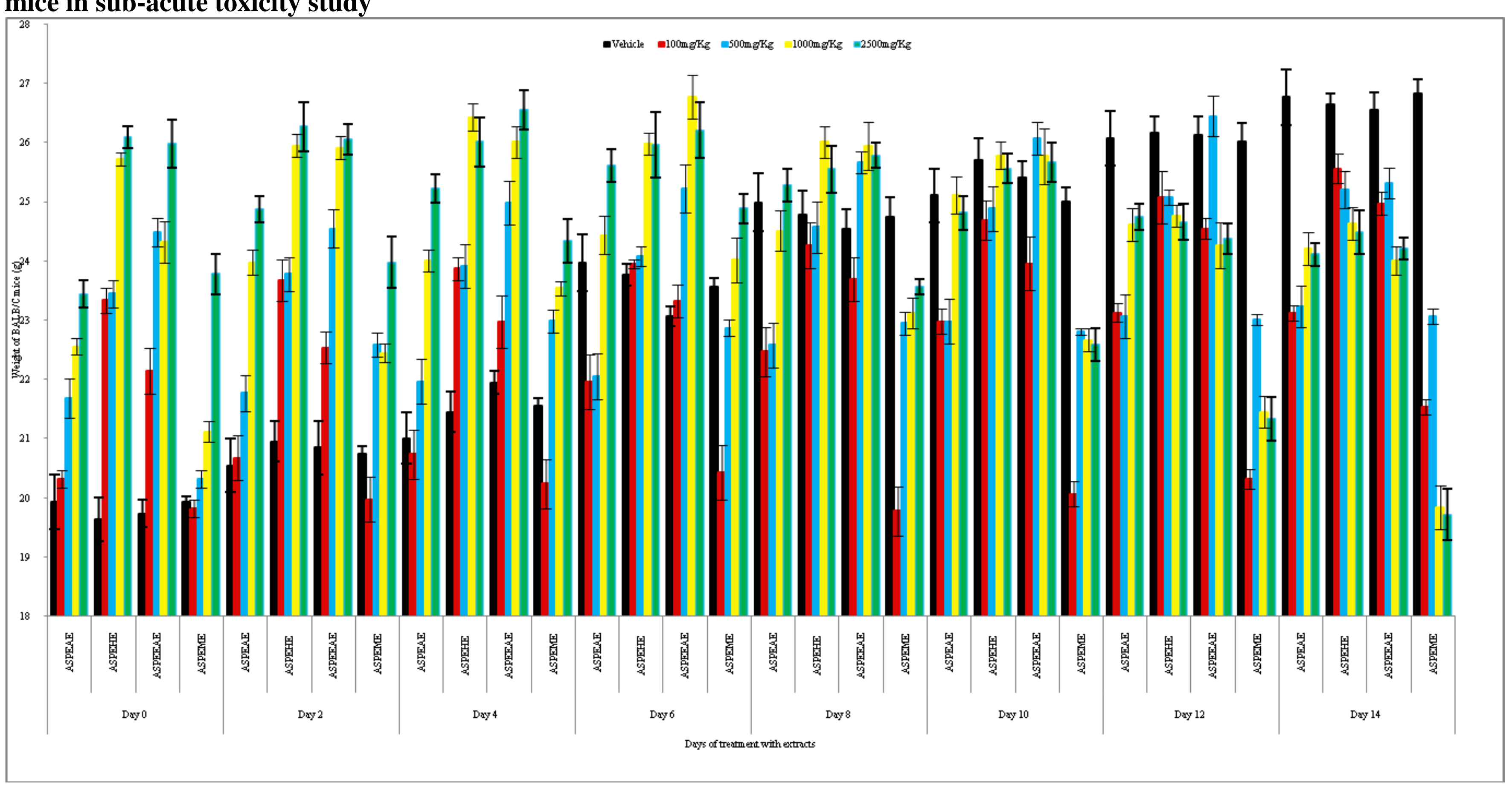
Appendix VII: Effects of A. muricata seeds aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study



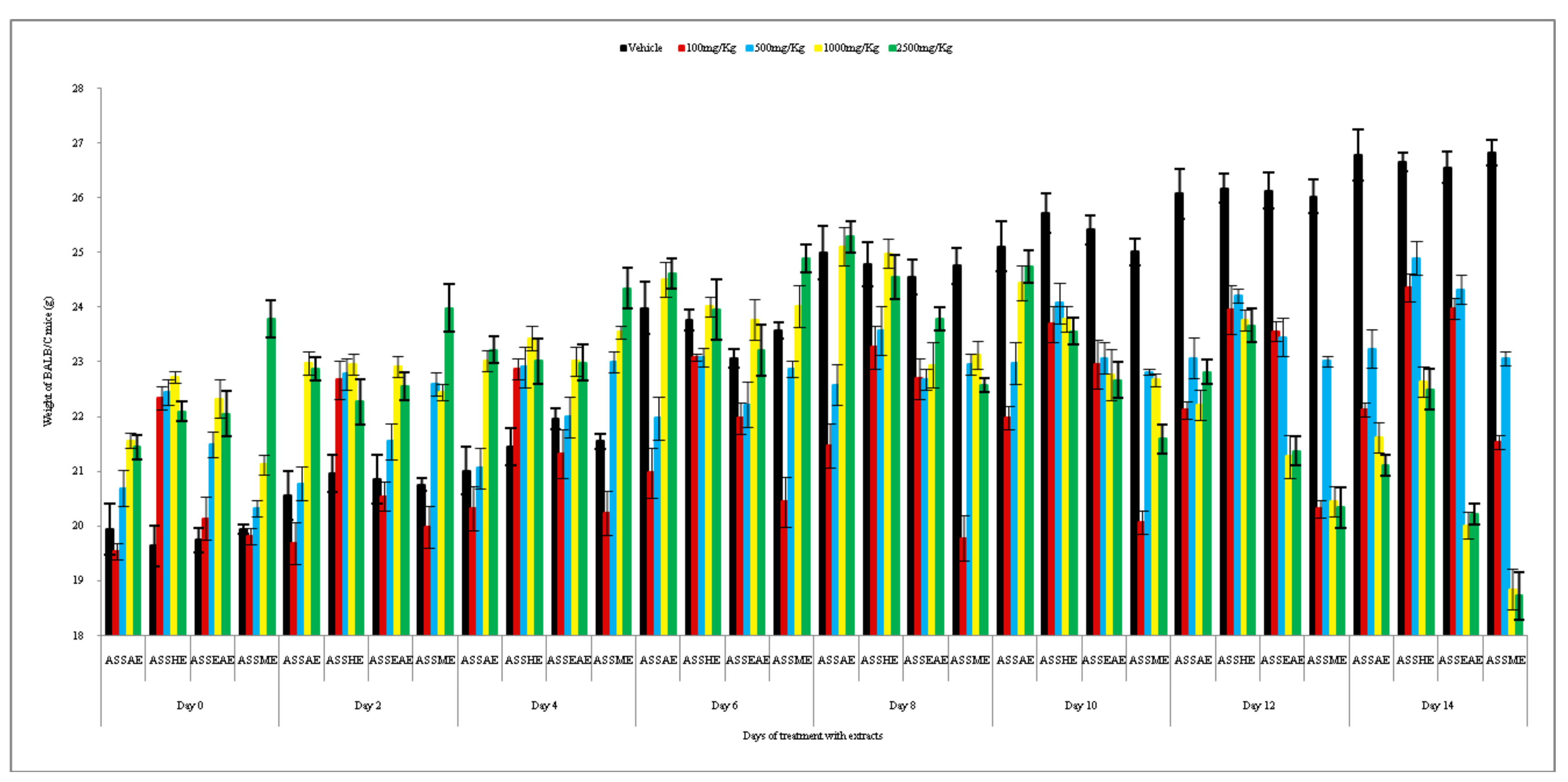
Appendix VIII: Effects of *A. squamosa* pulp aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study



Appendix IX: Effects of *A. squamosa* peel aqueous, methanolic, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study



Appendix X: Effects of A. squamosa seeds aqueous, methanol, ethyl acetate and hexane extracts on body weight of BALB/c mice in sub-acute toxicity study



Appendix XIa: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit pulp on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
AMPUAE	Liver	2.75±0.04	2.76±0.06	2.74±0.07	2.76±0.05
	Kidney	0.72±0.01	0.73±0.02	0.73±0.01	0.74±0.03
	Heart	0.38±0.02	0.39±0.01	0.40±0.01	0.41±0.02
	Spleen	0.24±0.03	0.25±0.03	0.25±0.04	0.24±0.01
	Stomach	0.80±0.05	0.85±0.04*	0.87±0.05*	0.86±0.03*
AMPUHE	Liver	2.70±0.05	2.75±0.04	2.76±0.06	2.74±0.07
	Kidney	0.73±0.03	0.75±0.04*	0.75±0.07*	0.74±0.05
	Heart	0.35±0.02	0.40±0.03	0.39±0.02	0.41±0.04
	Spleen	0.24±0.03	0.26±0.02	0.25±0.02	0.26±0.01
	Stomach	0.77±0.04	0.80±0.07	0.85±0.05*	0.87±0.06*
AMPUEAE	Liver	2.74±0.04	2.76±0.05	2.77±0.04	2.78±0.02
	Kidney	0.72±0.02	0.73±0.02	0.74±0.05	0.75±0.06*
	Heart	0.37±0.02	0.39±0.02	0.40±0.03	0.39±0.03
	Spleen	0.23±0.03	0.25±0.04	0.24±0.01	0.25±0.04
	Stomach	0.80±0.05	0.83±0.06	0.87±0.03*	0.86±0.04*
AMPUME	Liver	2.73±0.03	2.75±0.05	2.76±0.04	2.75±0.07
	Kidney	0.71±0.01	0.74±0.02	0.74±0.03	0.76±0.02*
	Heart	0.30±0.03	0.41±0.01	0.40±0.02	0.39±0.03
	Spleen	0.23±0.02	0.24±0.04	0.25±0.01	0.25±0.01
	Stomach	0.79±0.04	0.82±0.03	0.85±0.05*	0.89±0.05*
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs.Control (DMSO)					

Appendix XIb: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit peel on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
AMPEAE	Liver	2.69±0.08	2.79±0.09	2.74±0.09	2.76±0.07
	Kidney	0.68±0.02	0.69±0.02	0.67±0.01	0.64±0.03
	Heart	0.37±0.01	0.35±0.01	0.38±0.01	0.36±0.02
	Spleen	0.22±0.03	0.24±0.01	0.25±0.01	0.25±0.02
	Stomach	0.69±0.05	0.59±0.02**	0.74±0.03**	0.71±0.04**
AMPEHE	Liver	2.64±0.07	2.81±0.07	2.79±0.09	2.79±0.08*
	Kidney	0.63±0.05	0.73±0.03	0.75±0.01	0.74±0.03
	Heart	0.40±0.01	0.37±0.02	0.41±0.02	0.40±0.02
	Spleen	0.23±0.01	0.24±0.01	0.26±0.03	0.25±0.01
	Stomach	0.71±0.02*	0.79±0.02	0.82±0.02	0.81±0.03
AMPEEAE	Liver	2.71±0.09	2.76±0.07	2.75±0.08	2.77±0.09
	Kidney	0.75±0.01	0.74±0.03	0.73±0.02	0.74±0.03
	Heart	0.38±0.01	0.39±0.04	0.40±0.02	0.41±0.02
	Spleen	0.23±0.02	0.25±0.01	0.24±0.02	0.26±0.01
	Stomach	0.80±0.01	0.71±0.01	0.81±0.01	0.82±0.03
AMPEME	Liver	2.69±0.06	2.71±0.09	2.74±0.05	2.75±0.07
	Kidney	0.71±0.02	0.70±0.03	0.69±0.01	0.66±0.02
	Heart	0.37±0.02	0.39±0.01	0.38±0.01	0.39±0.03
	Spleen	0.22±0.01	0.25±0.02	0.26±0.02	0.25±0.01
	Stomach	0.69±0.03**	0.73±0.01**	0.74±0.02**	0.74±0.03**
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs.Control (DMSO)					

Appendix XIc: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. muricata* fruit seed on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
AMSAE	Liver	2.65±0.04	2.82±0.06	2.88±0.09	2.87±0.06
	Kidney	0.62±0.07	0.64±0.02	0.65±0.04	0.77±0.05
	Heart	0.37±0.01	0.40±0.03	0.42±0.02	0.41±0.01
	Spleen	0.30±0.02	0.35±0.02	0.31±0.01	0.31±0.01
	Stomach	0.70±0.01**	0.75±0.01**	0.72±0.02**	0.86±0.03*
AMSHE	Liver	2.7±0.08	2.76±0.05	2.75±0.07	2.77±0.04
	Kidney	0.69±0.04	0.73±0.01	0.74±0.03	0.76±0.02
	Heart	0.35±0.02	0.40±0.04	0.39±0.03	0.39±0.01
	Spleen	0.23±0.01	0.25±0.03	0.26±0.03	0.25±0.01
	Stomach	0.78±0.03*	0.82±0.01*	0.86±0.04**	0.88±0.02**
AMSEAE	Liver	2.70±0.03	2.75±0.05	2.77±0.05	2.75±0.07
	Kidney	0.70±0.01	0.72±0.03	0.75±0.01	0.74±0.02
	Heart	0.37±0.02	0.39±0.02	0.41±0.04	0.39±0.01
	Spleen	0.22±0.04	0.25±0.01	0.25±0.02	0.24±0.03
	Stomach	0.78±0.05*	0.78±0.03*	0.81±0.03	0.89±0.04**
AMSME	Liver	2.78±0.07	2.80±0.05	2.80±0.08	2.82±0.05
	Kidney	0.69±0.01	0.70±0.02	0.72±0.04	0.74±0.03
	Heart	0.36±0.02	0.39±0.02	0.40±0.03	0.40±0.01
	Spleen	0.24±0.01	0.25±0.03	0.24±0.02	0.26±0.03
	Stomach	0.79±0.02*	0.85±0.01*	0.86±0.02*	0.87±0.02**
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs.Control (DMSO)					

Appendix XIIa: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit pulp on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
ASPUAE	Liver	2.73±0.11	2.77±0.12	2.75±0.23	2.77±0.28
	Kidney	0.71±0.01	0.72±0.03	0.73±0.9	0.740±0.02
	Heart	0.37±0.03	0.39±0.04	0.39±0.01	0.40±0.03
	Spleen	0.25±0.02	0.24±0.02	0.25±0.02	0.25±0.01
	Stomach	0.82±0.08	0.86±0.05**	0.86±0.04**	0.86±0.04**
ASPUHE	Liver	2.71±0.17	2.76±0.08	2.76±0.29	2.75±0.19
	Kidney	0.70±0.01	0.74±0.03	0.75±0.03	0.74±0.02
	Heart	0.34±0.06	0.40±0.04	0.39±0.02	0.41±0.05
	Spleen	0.24±0.02	0.25±0.01	0.25±0.04	0.26±0.03
	Stomach	0.78±0.08*	0.79±0.04	0.84±0.06*	0.86±0.06**
ASPUEAE	Liver	2.75±0.14	2.75±0.16	2.77±0.19	2.78±0.11
	Kidney	0.72±0.02	0.74±0.02	0.74±0.05	0.75±0.03
	Heart	0.36±0.04	0.39±0.03	0.40±0.04	0.39±0.04
	Spleen	0.23±0.03	0.25±0.01	0.24±0.02	0.25±0.02
	Stomach	0.80±0.01	0.83±0.04*	0.87±0.03**	0.86±0.01*
ASPUME	Liver	2.74±0.12	2.76±±0.15*	2.76±0.18	2.75±0.17
	Kidney	0.71±0.04	0.73±0.05	0.74±0.02	0.76±0.04*
	Heart	0.35±0.05	0.40±0.02	0.40±0.01	0.39±0.02
	Spleen	0.24±0.03	0.24±0.03	0.25±0.04	0.25±0.01
	Stomach	0.79±0.03	0.83±0.05	0.86±0.05**	0.87±0.03**
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIIb: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit peel on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
ASPEAE	Liver	2.65±0.11	2.77±0.23	2.79±0.09	2.86±0.12
	Kidney	0.69±0.06	0.68±0.07	0.68±0.01	0.67±0.08
	Heart	0.36±0.01	0.34±0.07	0.39±0.03	0.37±0.04
	Spleen	0.23±0.01	0.24±0.02	0.250.01	0.25±0.03
	Stomach	0.67±0.05**	0.69±0.04**	0.79±0.02	0.83±0.05*
ASPEHE	Liver	2.68±0.12	2.82±0.29	2.80±0.23	2.79±0.09
	Kidney	0.67±0.04	0.74±0.05	0.76±0.04	0.75±0.03
	Heart	0.40±0.02	0.38±0.03	0.40±0.05	0.40±0.01
	Spleen	0.24±0.04	0.24±0.05	0.25±0.02	0.25±0.05
	Stomach	0.73±0.03**	0.80±0.06	0.84±0.03*	0.85±0.04**
ASPEEAE	Liver	2.72±0.39	2.74±0.17	2.76±0.15	2.78±0.12
	Kidney	0.75±0.04	0.76±0.03	0.75±0.04	0.75±0.05
	Heart	0.37±0.01	0.38±0.05	0.39±0.02	0.41±0.01
	Spleen	0.24±0.02	0.25±0.04	0.25±0.05	0.26±0.03
	Stomach	0.80±0.07	0.77±0.03*	0.82±0.04	0.83±0.04*
ASPEME	Liver	2.59±0.09	2.73±0.04	2.75±0.02	2.77±0.06*
	Kidney	0.72±0.02	0.72±0.03	0.69±0.03	0.74±0.04
	Heart	0.37±0.02	0.38±0.02	0.39±0.04	0.39±0.02
	Spleen	0.22±0.01	0.25±0.01	0.26±0.01	0.25±0.07
	Stomach	0.79±0.05*	0.83±0.13*	0.84±0.05*	0.84±0.06*
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIIc: Effects of aqueous, methanol, ethyl acetate and hexane extracts of *A. squamosa* fruit seeds on relative organ weights (organ [mg]/body weight [g])of BALB/c mice in sub-acute toxicity study

Extract	Organ	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	Liver	2.74±0.07	2.79±0.17	2.75±0.02	2.76±0.05
	Kidney	0.70±0.01	0.73±0.05	0.71±0.04	0.72±0.08
	Heart	0.39±0.01	0.37±0.02	0.35±0.03	0.38±0.01
	Spleen	0.24±0.11	0.22±0.12	0.24±0.01	0.23±0.14
	Stomach	0.81±0.04	0.80±0.02	0.79±0.01	0.82±0.04
ASSAE	Liver	2.88±0.21	2.71±0.28	2.77±0.18	2.88±0.01
	Kidney	0.45±0.08	0.65±0.07	0.65±0.05	0.64±0.06
	Heart	0.34±0.02	0.36±0.05	0.37±0.06	0.38±0.02
	Spleen	0.23±0.05	0.26±0.02	0.24±0.07	0.21±0.03
	Stomach	0.76±0.07*	0.79±0.04	0.83±0.03*	0.87±0.01**
ASSHE	Liver	2.71±0.08	2.84±0.09	2.96±0.02	2.94±0.30
	Kidney	0.70±0.07	0.69±0.04	0.74±0.03	0.75±0.05
	Heart	0.35±0.02	0.36±0.04	0.39±0.04	0.39±0.07
	Spleen	0.20±0.04	0.25±0.06	0.26±0.01	0.24±0.03
	Stomach	0.77±0.07*	0.79±0.06	0.83±0.02*	0.86±0.08**
ASSEAE	Liver	2.89±0.03	2.79±0.29	2.93±0.05	3.04±0.04*
	Kidney	0.71±0.01	0.74±0.05	0.75±0.05	0.74±0.04
	Heart	0.07±0.02	0.39±0.04	0.38±0.03	0.39±0o6
	Spleen	0.21±0.01	0.22±0.04	0.24±0.02	0.25±0.03
	Stomach	0.80±0.09	0.81±0.03	0.82±0.01	0.83±0.06*
ASSME	Liver	2.77±0.02	2.76±0.04	2.93±0.02	3.13±0.04*
	Kidney	0.72±0.01	0.75±0.04	0.76±0.02	0.77±0.06
	Heart	0.38±0.04	0.39±0.05	0.38±0.01	0.40±0.05
	Spleen	0.28±0.05	0.25±0.06	0.27±0.08	0.25±0.05
	Stomach	0.74±0.01**	0.79±0.08	0.84±0.03*	0.85±0.04*
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIIIa: Effects of of *A. muricata* fruit pulp aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
	Lymphocytes	74.62±0.35	73.82±0.25	74.92±0.67	75.02±0.15
AMPUAE	WBCs x 10^9L^{-1}	5.64±0.43*	6.84±0.47	6.16±1.33*	6.26±0.74*
	RBCs x $10^{12}L^{-1}$	7.45±0.14*	7.15±0.47*	7.44±0.77*	7.04±0.94*
	HGB (gdL^{-1})	11.46±0.17*	12.45±0.56*	11.38±0.54	12.74±0.72*
	HCT (PCV)	44.90±3.12*	46.02±2.27*	39.00±0.78*	41.04±1.16*
	MCV (FL)	55.28±2.84	53.16±1.14*	57.42±0.63*	54.10±0.99
	MCH (FL)	25.34±0.77*	28.67±0.28*	24.24±1.69**	23.34±0.70*
	MCHC (pg)	31.14±1.17	33.60±0.36	34.48±0.98*	34.44±1.52
	Lymphocytes	83.78±4.63*	87.30±3.27**	89.56±5.63**	87.64±3.59**
AMPUHE	WBCs x 10^9L^{-1}	7.75±0.14*	6.75±0.47	7.44±0.77*	8.04±0.74*
	RBCs x $10^{12}L^{-1}$	10.46±0.17*	11.45±0.56*	12.38±0.54*	11.74±0.72*
	HCT (PCV)	37.90±3.12*	41.02±2.27**	39.00±0.78**	40.04±1.17***
	MCV (FL)	55.08±2.84	54.16±1.14	57.42±0.63*	54.10±0.99
	MCH (FL)	28.34±0.97*	28.67±0.28*	26.24±1.69*	27.34±0.75*
	MCHC (pg)	30.14±1.17	32.60±0.36	34.48±0.98*	32.44±1.52
	Lymphocytes	83.78±4.63*	87.30±3.27**	89.56±5.63***	87.64±3.59**
AMPUEAE	WBCs x 10^9L^{-1}	6.76±0.89	5.92±0.91	5.80±0.76*	5.06±1.94**
	RBCs x $10^{12}L^{-1}$	5.96±0.27*	6.86±0.55	7.04±0.28*	7.66±1.20*
	HGB (gdL^{-1})	11.84±0.41*	12.20±1.74*	12.90±0.28*	11.08±1.36*
	HCT (PCV)	37.78±1.25*	35.64±2.53**	30.86±0.92**	31.34±0.89**
	MCV (FL)	49.68±1.66*	54.12±0.53	55.20±0.60	54.88±0.68
	MCH (FL)	27.40±0.15*	24.26±1.40*	25.36±0.19*	25.66±0.14*
	MCHC (pg)	30.20±0.73	32.68±2.48	35.36±0.23*	34.32±0.30
	Lymphocytes	79.38±3.22*	81.74±3.16*	87.46±2.11***	88.74±2.53***
AMPUME	WBCs x 10^9L^{-1}	6.06±0.49	5.62±0.51*	5.70±0.76*	5.26±0.94*
	RBCs x $10^{12}L^{-1}$	6.76±0.17*	7.76±0.45	7.54±0.28*	7.46±0.20*
	HGB (gdL^{-1})	12.74±0.31	12.10±1.34*	11.90±0.28	12.08±0.36*
	HCT (PCV)	33.78±1.25*	29.64±2.53**	30.86±0.92***	31.34±0.89***
	MCV (FL)	55.68±0.66*	53.12±0.53*	54.20±0.60	55.88±0.68
	MCH (FL)	27.10±0.15*	24.16±1.40*	25.06±0.19*	25.06±0.14*
	MCHC (pg)	32.20±0.43	33.68±2.48	36.36±0.23*	35.32±0.30
	Lymphocytes	81.38±2.22*	79.74±3.16*	88.46±2.11***	84.74±2.53**
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIIIb: Effects of of *A. muricata* fruit peel aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
	Lymphocytes	74.62±0.35	73.82±0.25	74.92±0.67	75.02±0.15
AMPEAE	WBCs x 10^9L^{-1}	7.58±0.18*	8.54±0.48**	8.71±0.16***	9.28±0.27***
	RBCs x $10^{12}L^{-1}$	6.94±0.27*	7.27±o.17*	7.11±0.53*	7.20±1.56*
	HGB (gdL^{-1})	14.45±1.54*	15.45±0.67*	14.27±1.47*	13.95±1.25*
	HCT (PCV)	41.45±1.22*	42.76±2.47*	39.23±3.45*	42.32±1.54**
	MCV (FL)	52.57±1.98*	54.64±1.47	53.55±1.67	52.97±1.94
	MCH (FL)	25.87±4.74*	20.77±0.94*	19.96±1.55**	20.15±3.87*
	MCHC (pg)	32.43±1.17	32.60±0.31	33.48±0.69	33.44±1.52
	Lymphocytes	79.89±1.77*	85.56±2.33**	87.77±2.47**	88.45±4.02**
AMPEHE	WBCs x 10^9L^{-1}	7.08±0.18**	9.54±0.48***	9.71±0.16***	9.18±0.27***
	RBCs x $10^{12}L^{-1}$	7.94±0.27*	7.97±0.17	8.11±0.53	8.20±1.56*
	HGB (gdL^{-1})	12.45±1.54	11.45±0.67*	12.27±1.47	11.95±1.25*
	HCT (PCV)	43.45±1.22*	42.76±2.47*	40.23±3.45*	41.32±1.54**
	MCV (FL)	50.57±1.98*	50.64±1.47*	51.55±1.67*	51.97±1.94*
	MCH (FL)	29.87±4.74*	27.77±0.94*	25.96±1.55*	23.15±3.87*
	MCHC (pg)	30.43±1.17	30.60±0.31*	31.48±0.69*	31.44±1.52*
	Lymphocytes	78.89±1.77*	86.56±2.33**	88.77±2.47**	89.45±4.02**
AMPEEAE	WBCs x 10^9L^{-1}	6.98±0.18*	7.94±0.48**	8.71±0.26***	9.28±0.47***
	RBCs x $10^{12}L^{-1}$	7.94±0.27*	6.27±1.17*	7.11±0.53*	7.20±1.16*
	HGB (gdL^{-1})	12.15±1.54	12.05±0.67*	12.07±1.47	12.15±1.25*
	HCT (PCV)	44.45±2.22*	46.76±2.47*	44.23±3.45*	46.32±2.54*
	MCV (FL)	52.57±3.98*	50.64±1.27*	50.55±1.77*	53.97±1.84*
	MCH (FL)	25.87±4.74*	27.77±0.94*	24.96±1.55**	25.15±3.87*
	MCHC (pg)	29.43±1.17*	30.60±0.31*	30.48±0.69*	31.44±1.52*
	Lymphocytes	77.89±1.77*	80.56±2.33*	89.77±2.47**	90.45±4.02**
AMPEME	WBCs x 10^9L^{-1}	6.58±0.18*	7.54±0.48*	9.71±0.26**	8.28±0.47**
	RBCs x $10^{12}L^{-1}$	6.94±0.27*	8.27±1.17*	8.11±0.53	8.20±1.16*
	HGB (gdL^{-1})	11.45±1.54*	12.45±0.67*	12.27±1.47*	12.95±1.25*
	HCT (PCV)	40.45±1.22*	43.76±2.47**	38.23±3.45**	41.32±2.54**
	MCV (FL)	50.57±3.98*	53.64±1.27*	52.55±1.77*	51.97±1.84*
	MCH (FL)	27.87±4.74*	22.77±0.94*	20.96±1.55**	21.15±3.87*
	MCHC (pg)	30.43±1.17	33.60±0.31	32.48±0.69	32.44±1.52
	Lymphocytes	79.78±4.63*	83.30±3.27**	79.56±5.63*	87.64±3.59***
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIIIc: Effects of of *A. muricata* fruit seeds aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
AMSAE	WBCs x 10^9L^{-1}	4.64±0.43**	5.84±0.47*	6.66±1.33**	7.26±0.94**
	RBCs x $10^{12}L^{-1}$	6.45±0.14*	6.15±0.47*	6.44±0.77*	6.04±0.948
	HGB (gdL^{-1})	12.46±0.17	11.45±0.56*	12.38±0.54*	11.74±0.72*
	HCT (PCV)	38.90±3.12*	35.02±2.27*	37.00±0.78*	35.04±1.16*
	MCV (FL)	60.28±2.84*	57.16±1.14*	57.42±0.63*	58.10±0.99*
	MCH (FL)	19.34±0.77*	18.67±0.28*	19.24±1.69*	19.34±0.70*
	MCHC (pg)	32.14±1.17*	32.60±0.36	33.48±0.98*	33.44±1.52
	%Lymphocytes	79.78±4.63*	83.30±3.27*	79.56±5.63*	87.64±3.59***
AMSHE	WBCs x 10^9L^{-1}	8.64±0.43**	7.94±0.47*	8.06±1.33**	9.26±0.14***
	RBCs x $10^{12}L^{-1}$	6.75±0.14*	7.15±0.47*	7.44±0.77*	7.04±0.94*
	HGB (gdL^{-1})	12.06±0.27*	12.45±0.56*	12.08±0.54*	12.74±0.72*
	HCT (PCV)	39.99±3.12*	37.02±4.27*	36.00±0.78*	36.04±1.16*
	MCV (FL)	55.28±2.84*	56.16±1.14*	56.42±0.63*	57.10±0.99*
	MCH (FL)	25.34±0.77*	26.67±0.28*	21.24±1.69*	24.34±0.70*
	MCHC (pg)	29.14±1.17*	30.60±0.36*	31.48±0.98*	32.44±1.52*
	%Lymphocytes	82.78±4.63*	85.30±3.27*	84.56±5.63**	89.64±3.59***
AMSEAE	WBCs x 10^9L^{-1}	8.86±0.49*	9.32±0.51**	9.70±0.76**	10.26±0.94**
	RBCs x $10^{12}L^{-1}$	4.76±0.17**	6.76±0.45*	7.54±0.28*	7.46±0.20*
	HGB (gdL^{-1})	10.74±0.31*	10.90±1.34*	11.97±0.28	12.08±0.36*
	HCT (PCV)	46.78±1.25*	41.64±2.53*	39.86±0.92*	41.34±0.89*
	MCV (FL)	55.68±0.66*	56.12±0.53*	55.20±0.60*	54.88±0.68*
	MCH (FL)	25.40±0.15*	26.26±1.40*	27.36±0.19*	26.66±0.14*
	MCHC (pg)	33.20±0.43*	34.68±2.48*	36.36±0.23*	35.32±0.30*
	Lymphocytes	83.38±2.22*	80.74±3.16*	85.46±2.11**	88.74±2.53***
AMSME	WBCs x 10^9L^{-1}	5.86±0.49*	5.32±0.51*	5.70±0.76*	6.26±0.94*
	RBCs x $10^{12}L^{-1}$	5.76±0.17**	5.76±0.45**	5.54±0.28**	5.46±0.20**
	HGB (gdL^{-1})	11.74±0.31*	10.10±1.34*	10.90±0.28*	11.08±0.36*
	HCT (PCV)	33.78±1.25*	29.64±2.53*	30.86±0.92*	31.34±0.89*
	MCV (FL)	59.68±0.66*	57.12±0.53*	57.20±0.60*	57.88±0.68*
	MCH (FL)	20.40±0.15*	19.26±1.40*	20.36±0.19*	20.66±0.14*
	MCHC (pg)	34.20±0.43*	33.68±2.48*	35.36±0.23*	35.32±0.30*
	%Lymphocytes	80.38±2.22*	78.74±3.16*	87.46±2.11**	83.74±2.53*
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIVa: Effects of *A. squamosa* fruit pulp aqueous, methanol, ethyl acetate and hexane extracts on haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
	%Lymphocytes	74.62±0.35	73.82±0.25	74.92±0.67	75.02±0.15
ASPUAE	WBCs x 10^9L^{-1}	6.97±0.45*	7.76±0.44*	7.89±0.91*	8.12±1.12**
	RBCs x $10^{12}L^{-1}$	6.56±0.05*	6.6.±0.06*	6.74±0.06*	6.21±0.04*
	HGB (gdL^{-1})	12.30±0.07	12.57±0.04*	11.44±0.17	12.30±0.33*
	HCT (PCV)	41.38±0.06*	46.97±0.04*	47.01±0.19*	45.90±0.28*
	MCV (FL)	53.23±0.44*	51.98±0.61*	53.06±0.82*	54.34±0.46*
	MCH (FL)	19.09±0.20*	19.03±0.21*	15.73±0.45*	14.00±0.55*
	MCHC (pg)	30.21±0.17*	30.70±0.08*	31.54±0.58*	32.63±1.10*
	%Lymphocytes	76.89±2.34*	80.05±0.12**	82.33±0.34**	85.44±0.77***
ASPUHE	WBCs x 10^9L^{-1}	7.77±0.77*	7.97±0.32*	8.09±1.23*	83.76±0.56**
	RBCs x $10^{12}L^{-1}$	6.11±±0.08*	6.09±0.03*	8.28±0.05*	8.67±0.03*
	HGB (gdL^{-1})	12.74±0.21	12.53±0.08*	7.23±0.07**	7.28±0.15**
	HCT (PCV)	44.38±0.10*	45.03±0.19*	45.15±0.20*	47.22±0.23*
	MCV (FL)	51.51±0.67*	54.10±0.49*	53.72±0.93*	53.37±0.91*
	MCH (FL)	30.87±0.23	30.58±0.13*	34.86±0.27*	33.62±0.36*
	MCHC (pg)	30.35±0.29*	30.10±0.28*	28.76±0.37*	29.75±1.19*
	%Lymphocytes	78.45±0.12**	79.57±0.47*	83.44±0.45**	85.78±0.34**
ASPUEAE	WBCs x 10^9L^{-1}	8.07±0.78**	7.97±0.44*	8.55±0.33**	8.92±1.03**
	RBCs x $10^{12}L^{-1}$	6.23±0.12*	6.18±0.02*	7.18±0.10*	7.76±0.08*
	HGB (gdL^{-1})	12.91±0.10*	12.70±0.18*	12.84±0.36*	11.91±0.85*
	HCT (PCV)	40.95±1.75*	49.85±1.03*	44.58±0.62*	47.64±2.89*
	MCV (FL)	54.16±0.74	54.47±0.58*	55.98±1.40*	55.08±0.62*
	MCH (FL)	27.75±0.13*	28.54±0.13*	29.43±0.61*	29.63±0.39*
	MCHC (pg)	32.34±0.49*	31.88±0.43*	27.84±0.72*	28.66±1.12*
	%Lymphocytes	75.25±0.25	79.67±0.77*	83.87±0.89**	85.46±0.67**
ASPUME	WBCs x 10^9L^{-1}	7.77±0.12*	8.34±0.47**	9.01±1.67**	10.23±1.78**
	RBCs x $10^{12}L^{-1}$	7.23±0.12*	8.18±0.02*	8.18±0.10	8.76±0.08*
	HGB (gdL^{-1})	11.91±0.10*	12.70±0.18*	12.84±0.36*	12.91±0.85*
	HCT (PCV)	45.95±1.75*	46.85±1.03*	45.58±0.62*	46.64±2.89*
	MCV (FL)	54.16±0.74	54.47±0.58*	55.98±1.40*	55.08±0.62
	MCH (FL)	29.75±0.13*	28.54±0.13*	29.43±0.61*	30.63±0.39*
	MCHC (pg)	30.34±0.49*	31.88±0.43*	29.84±0.72*	30.66±1.12*
	%Lymphocytes	77.45±1.78*	80.09±0.98**	81.17±1.12**	86.23±2.27***
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XIVb: Effects of *A. squamosa* fruit peel aqueous, methanol, ethyl acetate and hexane extracts on Haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
	%Lymphocytes	74.62±0.35	73.82±0.25	74.92±0.67	75.02±0.15
ASPEAE	WBCs x 10^9L^{-1}	6.65±0.48*	6.99±1.02*	7.09±0.12*	7.67±0.77*
	RBCs x $10^{12}L^{-1}$	6.99±0.67*	6.88±0.44*	7.09±0.88	7.77±0.44
	HGB (gdL^{-1})	10.87±1.47*	11.77±1.07*	12.34±0.68*	12.87±0.58*
	PCV (%)	45.67±2.34*	46.78±1.47*	47.67±1.08*	46.98±1.12*
	MCV (FL)	52.87±2.33*	53.98±1.11*	52.87±1.09*	53.94±1.05*
	MCH (FL)	28.78±0.67*	30.67±0.12*	29.85±0.23*	30.77±0.28*
	MCHC (pg)	30.98±0.95	31.33±0.33	30.09±0.12	32.05±0.47
	%Lymphocytes	76.66±1.07*	79.33±0.97**	80.44±1.45**	82.36±0.28**
ASPEHE	WBCs x 10^9L^{-1}	7.08±0.67*	7.65±0.47*	7.89±0.38*	7.95±0.38*
	RBCs x $10^{12}L^{-1}$	7.72±0.12*	6.98±0.77*	7.87±0.55*	8.07±0.37*
	HGB (gdL^{-1})	11.12±1.12*	10.98±0.97*	12.01±0.37*	12.64±0.33*
	HCT (PCV)	44.56±2.45*	46.78±1.12*	47.00±1.47*	47.98±1.11*
	MCV (FL)	50.12±1.11*	53.78±0.47*	52.67±1.67*	53.34±0.44*
	MCH (FL)	29.09±0.39*	28.78±0.28*	30.76±0.12*	29.44±0.47*
	MCHC (pg)	30.55±0.68	31.44±1.11	30.33±0.33	31.37±0.37
	%Lymphocytes	76.98±0.11*	78.76±1.11*	79.84±0.49**	80.91±0.29**
ASPEEAE	WBCs x 10^9L^{-1}	6.55±0.12	6.97±0.39*	7.45±0.28**	7.90±0.48**
	RBCs x $10^{12}L^{-1}$	7.11±0.11*	7.34±0.38*	7.78±0.28*	7.98±0.87*
	HGB (gdL^{-1})	10.67±0.67*	11.77±0.37*	12.43±1.12*	12.65±0.89*
	HCT (PCV)	46.77±1.11*	47.67±1.12*	46.98±0.58*	47.98±1.77*
	MCV (FL)	51.23±1.28*	52.55±1.57*	53.69±0.67*	54.00±1.44*
	MCH (FL)	30.03±0.12	31.27±0.27*	30.56±1.37*	29.56±0.39*
	MCHC (pg)	28.97±1.59*	29.87±1.33*	30.55±0.33	31.87±1.12*
	%Lymphocytes	75.87±1.39*	78.64±0.47**	80.44±0.39**	81.55±0.93**
ASPEME	WBCs x 10^9L^{-1}	6.89±0.12*	7.07±0.39*	7.34±0.34*	7.77±0.67*
	RBCs x $10^{12}L^{-1}$	7.09±0.17*	6.94±0.33*	7.67±0.39*	8.07±0.45*
	HGB (gdL^{-1})	12.09±1.23*	11.98±1.11*	12.77±0.12*	12.43±0.33*
	HCT (PCV)	46.67±1.12*	45.67±1.17*	47.34±0.87*	48.83±1.28*
	MCV (FL)	50.23±1.78*	51.44±1.47*	52.77±2.28*	53.76±1.19*
	MCH (FL)	29.67±1.13*	30.24±0.39*	30.68±0.47*	31.05±1.12*
	MCHC (pg)	30.22±0.39*	29.95±0.47*	30.77±0.29	31.00±0.29*
	%Lymphocytes	78.98±1.23*	79.43±0.45*	80.11±1.11**	82.23±0.44**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)

Appendix XIVc: Effects of *A. squamosa* fruit seeds aqueous, methanol, ethyl acetate and hexane extracts on Haematological profile of BALB/c mice in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	WBCs x 10^9L^{-1}	6.11±0.12	6.94±0.47	5.16±1.33	6.96±0.74
	RBCs x $10^{12}L^{-1}$	8.39±0.06	7.94±0.43	8.14±0.33	7.94±0.89
	HGB (gdL^{-1})	12.76±0.67	13.75±0.87	11.77±0.45	13.06±0.97
	HCT (PCV)	48.30±0.88	49.32±0.78	49.31±0.77	50.30±0.42
	MCV (FL)	54.28±0.97	55.28±1.17	54.89±0.44	55.28±0.77
	MCH (FL)	30.98±0.44	32.98±0.41	31.89±0.28	33.48±0.32
	MCHC (pg)	31.04±0.58	32.04±0.18	30.04±0.85	33.04±0.64
	%Lymphocytes	74.62±0.35	73.82±0.25	74.92±0.67	75.02±0.15
ASSAE	WBCs x 10^9L^{-1}	6.98±0.67*	7.09±0.75*	7.83±0.57*	8.67±0.67**
	RBCs x $10^{12}L^{-1}$	7.56±0.05*	7.66±0.06	7.74±0.06*	7.21±0.4*
	HGB (gdL^{-1})	12.30±0.07	12.57±0.04*	12.44±0.17	12.30±0.33*
	PCV (%)	46.38±0.06*	48.97±0.04	47.01±0.19*	48.90±0.28*
	MCV (FL)	53.23±0.44	51.98±0.61*	53.06±0.82*	54.34±0.46*
	MCH (FL)	30.09±0.20*	30.03±0.21*	31.73±0.45	31.00±0.55*
	MCHC (pg)	30.21±0.17*	30.70±0.08*	31.54±0.58	32.63±1.10*
	%Lymphocytes	75.78±0.12*	81.98±1.44**	83.55±0.55**	84.57±0.23***
ASSHE	WBCs x 10^9L^{-1}	6.99±0.33*	7.23±0.14*	7.87±0.68*	8.01±0.56**
	RBCs x $10^{12}L^{-1}$	7.11±±0.08*	7.09±0.03*	8.28±0.05	8.67±0.03*
	HGB (gdL^{-1})	12.74±0.21	12.53±0.08*	7.23±0.07*	7.28±0.15*
	PCV (%)	48.38±0.10	46.03±0.19*	47.15±0.20*	47.22±0.23*
	MCV (FL)	54.51±0.67	53.10±0.49*	53.72±0.93*	54.37±0.91*
	MCH (FL)	30.87±0.23	30.58±0.13*	31.86±0.27*	31.62±0.36
	MCHC (pg)	32.35±0.29*	32.10±0.28	30.76±0.37	33.75±1.19
	%Lymphocytes	78.67±0.13*	79.87±0.23*	81.44±0.55**	83.45±0.47***
ASSEAE	WBCs x 10^9L^{-1}	6.90±0.53*	7.23±0.44*	7.89±0.13**	7.94±0.17**
	RBCs x $10^{12}L^{-1}$	7.13±0.12*	6.98±0.02*	8.18±0.10	8.76±0.18*
	HGB (gdL^{-1})	10.91±0.10*	12.70±0.18*	11.84±0.36	11.91±0.45*
	PCV (%)	49.95±0.75	49.85±1.03	47.58±0.62*	47.64±0.89*
	MCV (FL)	51.16±0.74*	54.47±0.58*	55.98±1.40*	55.08±0.62
	MCH (FL)	28.75±0.13*	29.54±0.13*	31.43±0.61	31.63±0.39*
	MCHC (pg)	30.34±0.49*	31.88±0.43*	22.84±0.72**	31.66±1.12
	%Lymphocytes	79.32±1.12*	80.94±0.57**	84.14±0.28**	84.85±0.77***
ASSME	WBCs x 10^9L^{-1}	6.89±0.12*	7.04±0.35*	7.89±0.44**	7.94±0.13**
	RBCs x $10^{12}L^{-1}$	6.98±0.11*	7.45±0.55*	7.64±0.39*	8.01±0.37*
	HGB (gdL^{-1})	10.89±1.11*	11.67±0.17*	12.22±0.12*	12.33±0.39*
	HCT (PCV)	46.77±1.27*	47.56±1.47*	46.55±0.97*	48.07±0.88*
	MCV (FL)	51.23±1.33*	53.10±0.49*	52.45±0.99*	52.33±0.77*
	MCH (FL)	30.09±0.20	29.54±0.13*	30.76±0.37*	31.00±0.55*
	MCHC (pg)	30.34±0.49	32.10±0.28	31.54±0.58*	33.75±1.19
	%Lymphocytes	79.32±1.12*	81.98±1.44**	8.18±0.10**	84.57±0.23***
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XVa: Biochemical profile of BALB/c mice treated with *A. muricata*fruit pulp aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{-1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{-1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{-1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{-1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{-1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{-1}	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{-1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{-1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{-1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{-1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{-1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
AMPUAE	ALT (IUL^{-1})	75.45±10.64*	74.14±25.64*	77.77±16.77*	78.32±17.98*
	AST (IUL^{-1})	117.45±35.95*	124.33±37.07*	129.67±32.67*	137.22±21.78*
	ALkP (IUL^{-1})	127.47±5.06*	134.33±4.77	133.78±6.45	134.22±6.07
	Albumin (mgL-1)	26.45±0.78	26.01±0.37	26.85±1.93	26.87±1.02
	Urea (mgL^{-1})	7.55±1.17*	7.01±0.42*	7.77±0.47*	7.94±0.67
	Creatinine (mgL^{-1})	2.73±1.47*	3.20±0.23*	3.12±2.77*	3.88±0.80*
	Cholesterol (gdL^{-1}	3.08±0.47*	3.45±0.23*	3.74±0.77*	4.91±1.05*
	HDL (gdL^{-1})	0.84±0.04*	0.85±0.03*	0.84±0.02*	0.86±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.57±0.15*	1.57±0.47*	1.76±0.12*
	TAGs (gdL^{-1})	1.01±0.07*	0.91±0.09*	0.87±0.17*	0.67±0.12*
	Glucose (mgL^{-1})	164.52±19.77**	120.20±20.02	58.97±14.67***	60.57±31.77***
	Amylase (IUL^{-1})	1097.55±123.45**	1332.12±131.97***	1333.23±114.22***	1143±130.33***
AMPUME	ALT (IUL^{-1})	78.45±5.64*	78.14±3.64*	77.77±7.77*	75.32±8.98*
	AST (IUL^{-1})	113.45±15.95*	127.33±9.07*	129.67±4.67*	137.22±2.78**
	ALkP (IUL^{-1})	137.47±5.06*	136.33±4.77*	139.78±6.45*	143.22±6.07*
	Albumin (mgL-1)	26.45±0.78	26.01±0.37	26.85±1.93	26.87±1.02
	Urea (mgL^{-1})	7.55±1.17*	7.01±0.42*	7.77±0.47*	7.94±0.67
	Creatinine (mgL^{-1})	7.73±1.47***	9.20±0.23***	9.12±2.77***	10.88±0.80***
	Cholesterol (gdL^{-1}	2.08±0.47*	3.85±0.23*	3.74±0.77*	3.91±1.05*
	HDL (gdL^{-1})	0.79±0.04*	0.80±0.03*	0.84±0.02*	0.83±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.47±0.15*	1.47±0.47*	1.46±0.12*
	TAGs (gdL^{-1})	1.06±0.07*	0.91±0.09*	097±0.17*	097±0.12*
	Glucose (mgL^{-1})	164.52±19.77**	127.20±29.02**	148.97±44.67**	160.57±3.77*
	Amylase (IUL^{-1})	807.55±23.45*	1002.12±31.97**	1113.23±14.22**	1233±30.33**
AMPUEAE	ALT (IUL-1)	77.87±2.05*	78.54±21.64*	78.32±18.77*	86.76±3.59*
	AST (IUL-1)	111.12±3.47	125.15±27.09*	128.27±12.77*	127.97±31.64*
	ALkP (IUL-1)	137.98±41.88*	136.77±51.05*	141.43±32.76**	140.19±22.33**
	Albumin (mgL-1)	26.56±0.34	26.97±0.77	26.99±0.67	26.06±0.17
	Urea (mgL-1)	7.99±0.45	8.01±0.37*	7.99±0.33*	7.79±0.17
	Creatinine (mgL-1)	1.73±1.47	2.20±0.23	3.12±2.77*	4.88±0.80**
	Cholesterol (gdL-1)	2.09±1.09*	2.88±0.08*	3.23±0.05*	3.10±0.07*
	HDL (gdL-1)	0.88±0.04	0.79±0.07	0.81±0.07	0.90±0.02
	LDL (gdL-1)	1.26±0.19	1.28±0.14	1.34±0.12*	1.46±0.13*
	TAGs (gdL-1)	1.10±0.05	1.04±0.07	1.10±0.06*	1.02±0.02
	Glucose (mgL-1)	124.67±17.89*	126.33±3.44*	129±2.27*	132.09±4.77**
	Amylase (IUL-1)	897.55±23.45*	1232.12±31.97**	1123.23±14.22*	1433±30.33***
AMPUHE	ALT (IUL-1)	75.47±13.61*	74.87±5.77*	751.47±17.64*	76.00±25.50*
	AST (IUL-1)	108.05±11.23*	121.13±4.07	131.12±32.77**	138.11±21.54**
	ALkP (IUL-1)	133.77±16.05	135.89±77.78*	147.44±42.56**	142.09±33.86*
	Albumin (mgL-1)	26.97±1.12	26.45±1.78	26.66±1.47*	26.07±1.15*
	Urea (mgL-1)	7.83±0.87	8.00±0.23*	7.92±0.85*	7.97±1.17
	Creatinine (mgL-1)	2.73±1.47*	3.20±0.23*	4.12±2.77**	5.88±0.80**
	Cholesterol (gdL-1)	2.09±1.09*	3.88±0.08*	4.23±0.05**	5.10±0.07**
	HDL (gdL-1)	0.79±0.04	0.80±0.07*	0.81±0.07*	0.85±0.02*
	LDL (gdL-1)	1.26±0.19*	138±0.14*	1.44±0.12*	1.56±0.13*
	TAGs (gdL-1)	1.10±0.05*	1.04±0.07	1.11±0.06	1.12±0.02*
	Glucose (mgL-1)	127.67±17.89*	133.33±31.44**	136±12.27**	138.09±41.77**
	Amylase (IUL-1)	1001.55±123.45**	1032.101±31.97**	1023.23±104.22**	1033±130.33**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)

Appendix XVb: Biochemical profile of BALB/c mice treated with *A. muricata*peel aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{-1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{-1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{-1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{-1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{-1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{-1})	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{-1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{-1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{-1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{-1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{-1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
AMPEA	ALT (IUL^{-1})	71.45±5.64*	75.14±3.64*	78.77±7.77*	79.32±8.98*
	AST (IUL^{-1})	113.45±15.95*	121.33±9.07	123.67±4.67	127.22±2.78
	ALkP (IUL^{-1})	127.47±5.06*	133.33±4.77	135.78±6.45*	137.22±6.07*
	Albumin (mgL-1)	36.45±0.78	36.01±0.37	25.85±1.93	24.87±1.02
	Urea (mgL^{-1})	11.55±1.17*	8.01±0.42*	7.77±0.47*	7.94±0.67*
	Creatinine (mgL^{-1})	7.73±1.47**	9.20±0.23**	9.12±2.77**	10.88±0.80***
	Cholesterol (gdL^{-1})	2.08±0.47*	2.85±0.23*	3.74±0.77*	3.91±1.05
	HDL (gdL^{-1})	0.80±0.04*	0.82±0.03*	0.84±0.02*	0.83±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.57±0.15*	1.47±0.47*	1.66±0.12*
	TAGs (gdL^{-1})	1.06±0.07*	0.91±0.09*	0.87±0.17*	0.67±0.12*
	Glucose (mgL^{-1})	164.52±19.77*	120.20±2.02	58.97±4.67*	60.57±3.77*
	Amylase (IUL^{-1})	987.55±24.45*	1342.12±41.97**	1153.23±32.22**	1443±25.33***
AMPEM	ALT (IUL^{-1})	76.77±3.25	75.12±2.77	78.55±1.47*	79.03±2.87
	AST (IUL^{-1})	113.05±1.95*	119.93±2.25*	111.14±5.07*	116.75±3.24*
	ALkP (IUL^{-1})	130.13±0.93	129.34±3.21	131.21±4.74	134.75±1.47
	Albumin (mgL-1)	25.56±0.34	26.97±0.77	25.99±0.67	25.06±0.17
	Urea (mgL^{-1})	7.49±0.45*	7.67±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{-1})	3.76±0.09*	3.94±0.23*	4.00±0.39**	4.01±0.47**
	Cholesterol (gdL^{-1})	3.67±0.14	4.01±0.02*	3.94±0.23*	4.13±0.28*
	HDL (gdL^{-1})	0.75±0.06	0.79±0.07	0.78±0.08	0.81±0.08*
	LDL (gdL^{-1})	1.20±0.02*	1.67±0.05*	1.18±0.08*	2.05±0.06*
	TAGs (gdL^{-1})	1.13±0.01*	1.14±0.05*	1.13±0.03	1.01±0.01
	Glucose (mgL^{-1})	115.34±4.67*	97.29±2.77*	103.33±6.09*	109.27±5.67*
	Amylase (IUL^{-1})	897.55±23.45*	1232.12±31.97**	1123.23±14.22**	1433±30.33***
AMPEEA	ALT (IUL^{-1})	75.87±2.05*	79.54±2.64*	79.32±187*	86.76±3.59*
	AST (IUL^{-1})	111.12±3.47*	115.15±7.09*	108.27±2.77*	117.97±3.64*
	ALkP (IUL^{-1})	127.98±4.88*	126.77±5.05	131.43±2.76*	130.19±2.33*
	Albumin (mgL-1)	27.56±0.34*	26.97±0.77	25.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.09±0.45*	8.07±0.37*	7.94±0.33	7.55±0.17*
	Creatinine (mgL^{-1})	2.95±0.12*	2.88±0.05*	2.77±0.08*	3.01±0.06*
	Cholesterol (gdL^{-1})	3.05±0.12*	3.45±1.12*	3.88±0.14*	3.94±0.09*
	HDL (gdL^{-1})	0.81±0.07*	0.83±0.05*	0.85±0.04*	0.84±0.08*
	LDL (gdL^{-1})	1.33±0.06*	1.37±0.06*	1.19±0.04*	1.16±0.07*
	TAGs (gdL^{-1})	1.14±0.01*	1.12±0.05	1.13±0.03	1.09±0.01
	Glucose (mgL^{-1})	109.87±8.09*	112.21±3.45*	116.34±4.47*	111.13±5.07*
	Amylase (IUL^{-1})	887.55±13.45*	1532.12±51.97**	1133.23±22.22**	1333±35.33**
AMPEH	ALT (IUL^{-1})	55.47±3.61*	53.87±5.77*	51.47±3.64*	62.00±5.50*
	AST (IUL^{-1})	98.05±12.23*	111.13±4.07*	121.12±3.77*	108.11±2.54*
	ALkP (IUL^{-1})	121.77±6.05*	125.89±77.78*	127.44±4.56	120.09±3.86*
	Albumin (mgL-1)	24.97±1.12	22.45±1.78	24.66±1.47	25.07±1.15
	Urea (mgL^{-1})	7.73±0.87*	9.20±0.23*	9.12±0.85*	9.97±1.17*
	Creatinine (mgL^{-1})	5.73±1.47*	7.20±0.23*	9.12±2.77*	9.88±0.80*
	Cholesterol (gdL^{-1})	3.09±1.09*	2.88±0.08*	2.23±0.05*	2.10±0.07*
	HDL (gdL^{-1})	0.80±0.04*	0.79±0.07	0.75±0.07*	0.70±0.02*
	LDL (gdL^{-1})	1.16±0.19*	1.18±0.14*	1.24±0.12*	1.26±0.13*
	TAGs (gdL^{-1})	1.16±0.05	1.14±0.07*	1.10±0.06	1.12±0.02
	Glucose (mgL^{-1})	121.67±7.89	120.33±3.44	116±2.27*	108.09±4.77**
	Amylase (IUL^{-1})	997.55±17.45*	1132.12±19.97**	1122.23±26.22**	1444±45.33***

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)

Appendix XVc: Biochemical profile of BALB/c mice treated with *A. muricata*seeds aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{-1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{-1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{-1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{-1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{-1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{-1})	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{-1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{-1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{-1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{-1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{-1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
AMSA	ALT (IUL^{-1})	71.45±5.64*	75.14±3.64*	78.77±7.77*	79.32±8.98*
	AST (IUL^{-1})	148.45±15.95*	141.33±22.07*	114.67±24.67*	118.22±26.78*
	ALkP (IUL^{-1})	137.47±5.06*	143.33±4.77*	145.78±6.45*	147.22±6.07*
	Albumin (mgL-1)	27.45±0.78*	26.01±0.37	25.85±1.93	26.87±1.02
	Urea (mgL^{-1})	7.55±1.17*	8.01±0.42*	7.77±0.47*	7.94±0.67*
	Creatinine (mgL^{-1})	2.73±1.47*	3.20±0.23*	3.12±2.77*	3.88±0.80*
	Cholesterol (gdL^{-1})	1.08±0.47*	2.85±0.23*	3.74±0.77*	4.91±1.05*
	HDL (gdL^{-1})	0.82±0.04*	0.83±0.03*	0.85±0.02*	0.87±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.37±0.15*	1.47±0.47*	1.56±0.12*
	TAGs (gdL^{-1})	1.06±0.07*	0.91±0.09*	0.87±0.17*	0.67±0.12*
	Glucose (mgL^{-1})	114.52±19.77*	120.20±2.02	58.97±4.67**	60.57±3.77**
	Amylase (IUL^{-1})	1009.55±23.45**	1432.12±31.97**	1523.23±14.22***	1633±30.33***
AMSM	ALT (IUL^{-1})	75.87±2.05*	80.54±2.64	81.32±187	86.76±3.59*
	AST (IUL^{-1})	123.12±3.47*	120.15±7.09	128.27±2.77*	127.97±3.64
	ALkP (IUL^{-1})	137.98±4.88*	146.77±5.05*	151.43±2.76**	150.19±2.33**
	Albumin (mgL-1)	26.56±0.34	26.97±0.77	26.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.09±0.45*	8.07±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{-1})	2.63±0.19	3.04±013	2.99±0.28	2.87±0.13
	Cholesterol (gdL^{-1})	3.09±1.09*	3.88±0.08*	4.23±0.05*	4.10±0.07*
	HDL (gdL^{-1})	0.84±0.04*	0.89±0.07*	0.85±0.07*	0.87±0.02*
	LDL (gdL^{-1})	1.36±0.19	1.38±0.14	1.39±0.12*	1.39±0.13*
	TAGs (gdL^{-1})	1.01±0.05*	1.11±0.07*	1.11±0.06	1.01±0.02
	Glucose (mgL^{-1})	114.67±7.89*	101.33±3.44**	118±2.27*	102.09±4.77**
	Amylase (IUL^{-1})	897.55±23.45*	1232.12±31.97**	1333.23±14.22**	1544±30.33***
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XVIa: Biochemical profile of BALB/c mice treated with *A. squamosa*pulp aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{1})	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
ASPUA	ALT (IUL^{1})	70.45±3.14*	79.84±1.64	83.77±2.77*	85.12±4.98*
	AST (IUL^{1})	111.45±7.95*	101.33±1.07*	108.67±4.67*	112.22±2.78
	ALkP (IUL^{1})	137.47±2.06	144.33±5.77	141.78±2.45	147.22±1.07*
	Albumin (mgL-1)	26.45±0.78	26.01±0.37	26.85±1.93	26.87±1.02
	Urea (mgL^{1})	7.55±0.17*	7.01±0.42*	7.77±0.47*	7.94±0.67
	Creatinine (mgL^{1})	2.73±1.47*	3.20±0.23*	3.12±2.77*	2.88±0.80*
	Cholesterol (gdL^{1})	3.08±0.47*	3.85±0.23*	4.74±0.77*	5.91±1.05*
	HDL (gdL^{1})	0.90±0.04*	0.92±0.03*	1.04±0.02*	1.03±0.01*
	LDL (gdL^{1})	1.36±0.11*	1.57±0.15*	1.97±0.47*	1.86±0.12*
	TAGs (gdL^{1})	1.00±0.03*	0.91±0.09*	0.97±0.17*	1.07±0.12
	Glucose (mgL^{1})	155.52±39.77***	133.16±42.02**	114.97±24.67*	120.57±31.77*
	Amylase (IUL^{1})	987.55±63.45*	1252.12±31.97**	1323.23±143.22***	1473±130.33***
ASPUM	ALT (IUL^{1})	70.87±7.05*	74.54±4.64*	79.32±18.07*	80.76±3.59*
	AST (IUL^{1})	110.12±13.47*	119.15±17.09*	128.27±12.77*	131.97±31.64*
	ALkP (IUL^{1})	137.98±14.88*	146.77±15.05**	141.43±12.76**	1157.19±21.33***
	Albumin (mgL-1)	26.56±0.34	25.97±0.77	26.99±0.67	27.06±0.17
	Urea (mgL^{1})	7.59±0.45*	8.00±0.37*	7.74±0.33*	7.75±0.17
	Creatinine (mgL^{1})	3.73±1.47*	3.20±0.23*	5.12±2.77*	5.88±0.80*
	Cholesterol (gdL^{1})	3.09±1.09*	3.48±0.08*	5.23±0.05*	5.10±0.07*
	HDL (gdL^{1})	1.02±0.04*	1.09±0.07*	1.05±0.07*	1.07±0.02*
	LDL (gdL^{1})	1.16±0.19	1.18±0.14*	2.14±0.12*	2.06±0.13*
	TAGs (gdL^{1})	1.11±0.05*	1.13±0.07*	1.13±0.06*	1.01±0.02
	Glucose (mgL^{1})	129.59±31.15*	128.44±32.87*	136.45±45.17*	120.45±17.88*
	Amylase (IUL^{1})	977.55±23.45*	1332.12±231.97**	1423.23±140.22***	1553±180.33***
ASPUEA	ALT (IUL^{1})	75.87±2.05*	79.54±2.64*	79.32±187*	86.76±3.59*
	AST (IUL^{1})	111.12±3.47*	115.15±7.09*	108.27±2.77*	117.97±3.64*
	ALkP (IUL^{1})	127.98±4.88*	126.77±5.05*	131.43±2.76*	130.19±2.33*
	Albumin (mgL-1)	27.56±0.34*	26.97±0.77	25.99±0.67	26.06±0.17
	Urea (mgL^{1})	7.09±0.45*	8.07±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{1})	3.73±1.47*	3.20±0.23*	4.12±2.77*	4.88±0.80*
	Cholesterol (gdL^{1})	3.09±1.09*	3.48±0.08*	5.23±0.05*	5.10±0.07*
	HDL (gdL^{1})	1.00±0.04*	1.09±0.07*	1.25±0.07*	1.27±0.02*
	LDL (gdL^{1})	1.18±0.19*	1.19±0.14*	2.04±0.12*	2.26±0.13*
	TAGs (gdL^{1})	1.10±0.05*	1.15±0.07*	1.13±0.06	1.01±0.02
	Glucose (mgL^{1})	124.54±4482*	115.79±56.97*	117.64±37.49*	121.33±23.11*
	Amylase (IUL^{1})	1009.55±23.45*	1242.12±31.97**	1323.23±14.22**	1467±30.33***
ASPUH	ALT (IUL^{1})	65.47±11.61**	73.87±10.77*	69.47±7.64**	72.00±4.50**
	AST (IUL^{1})	108.05±32.23*	121.13±24.07	131.12±33.77*	148.11±42.54**
	ALkP (IUL^{1})	139.77±26.05*	145.89±47.78**	147.44±24.56**	157.09±43.86***
	Albumin (mgL-1)	27.97±1.12*	26.45±1.78	26.66±1.47	65.07±1.15*
	Urea (mgL^{1})	7.73±0.87	9.20±0.23*	9.12±0.85*	9.97±1.17*
	Creatinine (mgL^{1})	4.73±1.47	3.20±0.23	5.12±2.77	5.88±0.80
	Cholesterol (gdL^{1})	2.09±1.09*	2.88±0.08*	4.23±0.05*	5.10±0.07*
	HDL (gdL^{1})	0.90±0.04*	1.09±0.07*	1.15±0.07*	1.17±0.02*
	LDL (gdL^{1})	1.16±0.19	1.18±0.14*	1.24±0.12*	1.26±0.13*
	TAGs (gdL^{1})	1.13±0.05*	1.14±0.07*	1.10±0.06	1.02±0.02
	Glucose (mgL^{1})	148.67±45.89**	120.33±3.44	117.55±22.27*	118.12±26.77*
	Amylase (IUL^{1})	997.55±23.45*	1332.12±31.97**	1223.23±14.22**	1533±30.33***
Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)					

Appendix XVIb: Biochemical profile of BALB/c mice treated with *A. squamosa*fruit peel aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{-1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{-1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{-1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{-1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{-1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{-1})	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{-1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{-1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{-1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{-1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{-1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
ASPEA	ALT (IUL^{-1})	71.45±5.64*	75.14±3.64*	78.77±7.77*	79.32±8.98*
	AST (IUL^{-1})	113.45±15.95*	121.33±9.07	123.67±4.67	127.22±2.78*
	ALkP (IUL^{-1})	127.47±5.06*	133.33±4.77	135.78±6.45*	137.22±6.07*
	Albumin (mgL-1)	36.45±0.78*	36.01±0.37*	25.85±1.93	24.87±1.02
	Urea (mgL^{-1})	11.55±1.17*	8.01±0.42*	7.77±0.47*	7.94±0.67*
	Creatinine (mgL^{-1})	7.73±1.47*	9.20±0.23*	9.12±2.77*	10.88±0.80*
	Cholesterol (gdL^{-1})	2.08±0.47*	2.85±0.23*	3.74±0.77*	3.91±1.05*
	HDL (gdL^{-1})	0.80±0.04*	0.82±0.03*	0.84±0.02*	0.83±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.57±0.15*	1.47±0.47*	1.66±0.12*
	TAGs (gdL^{-1})	1.06±0.07*	0.91±0.09*	0.87±0.17*	0.67±0.12*
	Glucose (mgL^{-1})	164.52±19.77**	120.20±2.02	58.97±4.67***	60.57±3.77***
	Amylase (IUL^{-1})	797.55±23.45	1132.12±31.97*	1023.23±14.22**	1233±30.33**
ASPEM	ALT (IUL^{-1})	76.77±21.21*	74.23±12.37*	78.01±15.98*	77.95±32.76*
	AST (IUL^{-1})	109.87±45.47*	113.67±34.43*	116.99±27.09*	98.07±21.89**
	ALkP (IUL^{-1})	133.34±34.4*	139.44±40,97*	144.44±44.09*	141.24±34.43*
	Albumin (mgL-1)	25.56±0.34	25.97±0.77	25.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.19±0.45*	7.07±0.37*	7.94±0.33*	8.55±0.17*
	Creatinine (mgL^{-1})	2.47±0.25*	3.52±0.17*	3.51±0.14*	3.52±0.17*
	Cholesterol (gdL^{-1})	3.58±0.19*	3.60±0.12*	3.59±0.13*	3.33±0.23*
	HDL (gdL^{-1})	0.76±0.04*	0.79±0.07*	0.85±0.07*	0.80±0.02*
	LDL (gdL^{-1})	1.35±0.19*	1.37±0.14*	1.36±0.12	1.41±0.13*
	TAGs (gdL^{-1})	1.16±0.05	1.14±0.07*	1.10±0.06	1.12±0.02*
	Glucose (mgL^{-1})	109.67±7.89	101.33±3.44	99±2.27	110.09±4.77
	Amylase (IUL^{-1})	989.55±23.45	1202.12±31.97	1103.23±14.22	1033±30.33
ASPEEA	ALT (IUL^{-1})	50.87±12.05**	40.54±12.64**	47.32±14.87*	86.76±3.59*
	AST (IUL^{-1})	111.12±3.47*	115.15±7.09*	108.27±2.77*	117.97±3.64*
	ALkP (IUL^{-1})	127.98±4.88*	126.77±5.05*	131.43±2.76*	130.19±2.33*
	Albumin (mgL-1)	27.56±0.34*	26.97±0.77	25.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.09±0.45*	8.07±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{-1})	2.58±0.19*	2.60±0.12*	2.59±0.13*	3.33±0.23*
	Cholesterol (gdL^{-1})	2.58±0.19*	2.60±0.12*	2.59±0.13*	3.33±0.23*
	HDL (gdL^{-1})	0.81±0.04*	0.83±0.07*	0.85±0.07*	0.90±0.02*
	LDL (gdL^{-1})	1.26±0.19*	1.39±0.14*	1.46±0.12*	1.39±0.13*
	TAGs (gdL^{-1})	1.10±0.05	1.13±0.07*	1.12±0.06	1.12±0.02*
	Glucose (mgL^{-1})	120.67±7.89*	111.33±3.44**	109±2.27**	108.09±4.77**
	Amylase (IUL^{-1})	898.55±23.45*	1332.12±31.97**	1423.23±14.22***	1443±30.33***
ASPEH	ALT (IUL^{-1})	78.47±3.61*	77.87±5.77*	71.47±3.64*	72.00±5.50*
	AST (IUL^{-1})	108.05±12.23*	121.13±4.07	127.12±3.77*	128.11±21.54
	ALkP (IUL^{-1})	361.77±16.05***	145.89±27.78**	147.44±4.56**	140.09±3.86**
	Albumin (mgL-1)	26.07±1.12	27.45±1.78	26.66±1.47	26.07±1.15
	Urea (mgL^{-1})	7.33±0.87*	7.20±0.23*	8.12±0.85*	7.97±1.17
	Creatinine (mgL^{-1})	3.73±1.47*	3.20±0.23*	4.12±2.77*	3.88±0.80*
	Cholesterol (gdL^{-1})	2.09±1.09*	3.88±0.08*	4.23±0.05*	4.10±0.07*
	HDL (gdL^{-1})	0.86±0.04*	0.89±0.07*	0.95±0.07*	0.90±0.02*
	LDL (gdL^{-1})	1.37±0.19*	1.37±0.14*	1.36±0.12	141±0.13*
	TAGs (gdL^{-1})	1.16±0.05	1.14±0.07*	1.10±0.06	1.12±0.02*
	Glucose (mgL^{-1})	122.67±7.89	121.33±3.44	119±2.27	108.09±4.77**
	Amylase (IUL^{-1})	887.55±23.45*	1032.12±31.97**	1023.23±14.22**	1333±30.33***

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)

Appendix XVIc: Biochemical profile of BALB/c mice treated *with A. squamosa* seeds aqueous, methanol, ethyl acetate and hexane extracts in sub-acute toxicity study

Extract	Parameter	100mg/Kg	500mg/Kg	1000mg/Kg	2500mg/Kg
DMSO	ALT (IUL^{-1})	77.49±21.40	79.97±11.67	80.97±3.34	83.56±8.27
	AST (IUL^{-1})	119.87±32.16	121.87±12.17	123.87±20.56	128.87±9.147
	ALkP (IUL^{-1})	132.36±1.53	134.36±11.27	137.36±15.67	139.36±21.13
	Albumin (mgL-1)	26.46±0.49	25.46±0.37	24.97±0.77	24.06±0.11
	Urea (mgL^{-1})	7.88±0.11	6.08±0.12	7.08±0.19	7.98±0.16
	Creatinine (mgL^{-1})	2.47±0.16	2.57±0.12	2.77±0.17	2.67±0.19
	Cholesterol (gdL^{-1})	3.75±0.18	3.05±0.28	3.35±0.17	3.85±0.13
	HDL (gdL^{-1})	0.77±0.02	0.78±0.01	0.77±0.03	0.79±0.01
	LDL (gdL^{-1})	1.34±0.11	1.30±0.10	1.37±0.12	1.39±0.13
	TAGs (gdL^{-1})	1.16±0.04	1.06±0.14	1.11±0.01	1.10±0.06
	Glucose (mgL^{-1})	122.71±12.12	121.61±9.10	119.79±10.13	118.91±15.13
	Amylase (IUL^{-1})	796.97±17.47	894.97±14.47	967.97±27.17	969.93±10.47
ASSA	ALT (IUL^{-1})	71.45±5.64 *	75.14±3.64*	78.77±7.77*	79.32±8.98*
	AST (IUL^{-1})	113.45±15.95**	121.33±9.07	123.67±4.67	127.22±2.78
	ALkP (IUL^{-1})	127.47±5.06**	133.33±4.77	135.78±6.45**	137.22±6.07**
	Albumin (mgL-1)	36.45±0.78**	36.01±0.37**	25.85±1.93	24.87±1.02
	Urea (mgL^{-1})	11.55±1.17**	8.01±0.42*	7.77±0.47*	7.94±0.67
	Creatinine (mgL^{-1})	7.73±1.47*	9.20±0.23*	9.12±2.77*	10.88±0.80*
	Cholesterol (gdL^{-1})	2.08±0.47*	2.85±0.23*	3.74±0.77*	3.91±1.05
	HDL (gdL^{-1})	0.80±0.04*	0.82±0.03*	0.84±0.02*	0.83±0.01*
	LDL (gdL^{-1})	1.36±0.14*	1.57±0.15*	1.47±0.47*	1.66±0.12*
	TAGs (gdL^{-1})	1.06±0.07*	0.91±0.09*	0.87±0.17*	0.67±0.12*
	Glucose (mgL^{-1})	164.52±19.77***	120.20±2.02	58.97±4.67**	60.57±3.77***
	Amylase (IUL^{-1})	1121.55±23.45**	1532.12±31.97***	1523.23±14.22***	1633±30.33***
ASSM	ALT (IUL^{-1})	75.87±2.05*	80.54±2.64*	81.32±187*	86.76±3.59*
	AST (IUL^{-1})	123.12±3.47*	120.15±7.09	128.27±2.77*	127.97±3.64
	ALkP (IUL^{-1})	137.98±4.88*	146.77±5.05**	151.43±2.76**	150.19±2.33**
	Albumin (mgL-1)	26.56±0.34	26.97±0.77	26.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.09±0.45*	8.07±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{-1})	2.63±0.19	3.04±013*	2.99±0.28*	2.87±0.13*
	Cholesterol (gdL^{-1})	3.09±1.09*	3.88±0.08*	4.23±0.05*	4.10±0.07*
	HDL (gdL^{-1})	0.84±0.04*	0.89±0.07*	0.85±0.07*	0.87±0.02*
	LDL (gdL^{-1})	1.36±0.19*	1.38±0.14*	1.39±0.12*	1.39±0.13*
	TAGs (gdL^{-1})	1.01±0.05*	1.11±0.07	1.11±0.06	1.01±0.02*
	Glucose (mgL^{-1})	114.67±7.89**	101.33±3.44**	118±2.27**	102.09±4.77**
	Amylase (IUL^{-1})	1109.55±23.45**	1472.12±31.97***	1523.23±14.22***	1533±30.33***
ASSEA	ALT (IUL^{-1})	75.87±2.05*	79.54±2.64	79.32±187*	86.76±3.59*
	AST (IUL^{-1})	121.12±3.47**	111.15±7.09**	108.27±2.77**	127.97±3.64
	ALkP (IUL^{-1})	127.98±4.88*	126.77±5.05*	131.43±2.76*	130.19±2.33*
	Albumin (mgL^{-1})	26.56±0.34	26.97±0.77	26.99±0.67	26.06±0.17
	Urea (mgL^{-1})	7.09±0.45*	8.07±0.37*	7.94±0.33*	7.55±0.17*
	Creatinine (mgL^{-1})	2.52±0.17*	2.89±0.27*	3.47±0.12*	2.60±0.32*
	Cholesterol (gdL^{-1})	3.09±1.09*	3.88±0.08*	4.23±0.05*	4.10±0.07*
	HDL (gdL^{-1})	0.84±0.04*	0.89±0.07*	0.85±0.07*	0.87±0.02*
	LDL (gdL^{-1})	1.36±0.19*	1.38±0.14*	1.39±0.12*	1.39±0.13*
	TAGs (gdL^{-1})	1.00±0.05*	1.10±0.07	1.10±0.06	1.02±0.02*
	Glucose (mgL^{-1})	101.67±7.89 **	99.33±3.44**	108±2.27**	102.09±4.77**
	Amylase (IUL^{-1})	11201.55±23.45***	1532.12±31.97***	1423.23±14.22***	1533±30.33***
ASSH	ALT (IUL^{-1})	85.47±3.61*	78.87±5.77*	81.47±3.64*	82.00±5.50*
	AST (IUL^{-1})	118.05±12.23	171.13±4.07***	151.12±3.77***	178.11±2.54***
	ALkP (IUL^{-1})	141.77±6.05*	135.89±77.78*	147.44±4.56*	140.09±3.86*
	Albumin (mgL^{-1})	26.97±1.12	26.45±1.78	26.66±1.47	26.07±1.15
	Urea (mgL^{-1})	7.73±0.87	9.20±0.23*	9.12±0.85*	9.97±1.17*
	Creatinine (mgL^{-1})	1.73±1.47*	3.20±0.23*	4.12±2.77*	4.88±0.80*
	Cholesterol (gdL^{-1})	3.09±1.09*	3.88±0.08*	4.23±0.05*	4.10±0.07*
	HDL (gdL^{-1})	0.84±0.04*	0.89±0.07*	0.85±0.07*	0.87±0.02*
	LDL (gdL^{-1})	1.36±0.19*	1.38±0.14*	1.39±0.12*	1.39±0.13*
	TAGs (gdL^{-1})	1.00±0.05*	1.10±0.07	1.10±0.06	1.02±0.02*
	Glucose (mgL^{-1})	111.67±7.89*	100.33±3.44*	98±2.27*	108.09±4.77*
	Amylase (IUL^{-1})	1197.55±23.45**	1632.12±31.97***	1523.23±14.22***	1633±30.33***

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs. Control (DMSO)

Appendix XVII: A grant ward letter from National Commission for Science Technology and Innovation (NACOSTI) for funding the study

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471, 2241349, 310571, 2219420
Fax: +254-20-318245, 318249
Email: secretary@nacosti.go.ke
Website: www.nacosti.go.ke
When replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/RCD/ST&I 5th CALL PhD/066** Date: **5th June, 2014**

Lenny Mwagandi Chibevo
Jomo Kenyatta University of Agriculture & Technology
P.O. Box 62000 - 00200
NAIROBI

RE: SCIENCE, TECHNOLOGY AND INNOVATION RESEARCH GRANT (PhD)

I'm pleased to inform you that, you have been awarded the Science, Technology and Innovation (ST&I) grant for your **PhD research proposal**.

National Commission for Science, Technology and Innovation (NACOSTI) has approved an amount of Kenya shillings **One million, one hundred and ninety one thousand only (Kshs 1, 191, 000)** towards your PhD research proposal titled " **Antileishmanial, leishmania enzymes and genes inhibitory activities of Annonaceae fruits extracts".** Your grant will be disbursed in yearly instalments each not exceeding four hundred thousand shillings (Ksh 400,000).

Find the enclosed ***Research Grant Contract Form (NCST/ST&I/CONTRACT/FORM 1C)*** that should be duly completed. You should attach a certified copy of your ***national identity card, detailed work plan, breakdown of the yearly budget and a letter accepting the grant offered. Your recent passport size photograph and an abstract of your proposal, not exceeding 500 words should be submitted in soft copy (Ms Word format) to the email:- postgraduates@nacosti.go.ke***

Your duly signed contract form, acceptance letter and the abstract should be sent back to reach us not later than **18th June, 2014** for our further actions.

DR. MOSES K. RUGUTT, PhD, HSC.
Ag.SECRETARY/CEO

cc: Vice Chancellor,
Jomo Kenyatta University of Agriculture & Technology

National Commission for Science, Technology and Innovation is ISO 9001: 2008 Certified

Appendix XVIII: A letter of approval from Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (SSC) (KEMRI-SCC)

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/SSC/102854 **11th June, 2014**

Lenny Chimbevo

Thro'
Director, CTMDR
NAIROBI

Forwarded
13/6/2014

REF: SSC No. 2806 (Revised) – Determination of Safety, Efficacy, Nutritional activity and Protein/DNA Binding Interaction of Kenyan *Annona Muricata and Annona Squamosa* (Annonaceae) fruits Extracts in Balb/c Mice Model of Leishmaniasis

Thank you for your letter dated 23rd May, 2014 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix XIX: A letter of approval from Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) (KEMRI-ERC)

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/RES/7/3/1 **December 9, 2014**

TO: **LENNY MWAGANDI CHIMBEVO,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. PETER MWITARI,**
THE DIRECTOR, CTMDR,
NAIROBI

Forwarded 15/12/2014

Dear Sir,

RE: SSC PROTOCOL NO. 2806 (*RESUBMISSION 2*): DETERMINATION OF SAFETY, EFFICACY, NUTRITIONAL ACTIVITY AND PROTEIN/DNA BINDING INTERACTION OF KENYAN *ANNONA MURICATA* AND *ANNONA SQUAMOSA* (ANNONACEAE) FRUITS EXTRACT IN BALB/C MICE MODEL OF LEISHMANIASIS (*VERSION 1.5 DATED 3RD DECEMBER 2014*)

Reference is made to your letter dated 3rd December 2014 and received at the KEMRI Scientific and Ethics Review Unit on 8th December 2014.

This is to inform you that the Committee notes that the issues raised at the 229th meeting of the KEMRI ERC held on 22nd July, 2014 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this **9th December 2014** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 8, 2015.**

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **October 27, 2015.**

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix XX: A letter of approval from Kenya Medical Research Institute (KEMRI) - Animal Care and Use Committee (ACUC)(KEMRI-ACUC)

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 01.11.14 **27th November, 2014**

Lenny Mwagandi Chimbevo
P. O. Box 939 - 80108.
Kilifi, Kenya.

Chimbevo,

RE: Animal use approval for SSC 2806 - "Safety, Efficacy, Nutritional Activity and Protein/DNA Binding Interaction of Kenyan *Annona muricata* and *Annona squamosa* (Annonaceae) Fruits Extracts in Balb/c Mice Model of Leishmaniasis" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of two years starting from when the final ethical approval will be obtained. The committee expects you to adhere to all the 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
★ 27 NOV 2014 ★
ANIMAL CARE AND USE COMMITTEE
Signature: ....

Appendix XXI: A letter seeking for approval to carry out research in the Food Biochemistry Laboratory in the Department of Food Science and Technology at Jomo Kenyatta University of Agriculture and Technology (JKUAT)

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
CLINICAL SCIENCES DEPARTMENT

TEL: 067- 52181-4 Extn. 2226 FAX: 067-52030 Email: director@itromid.jkuat.ac.ke

14th July, 2014

To: CoD, Food Science and Technology Department

Dear Sir,

REF: LENNY MWAGANDI CHIMBEVO REG. NO TM405-2091-2012

The above named is a PhD student in the College of Health Sciences (COHES), Institute of Tropical Medicine and Infectious Diseases (ITROMID). He is undertaking a project titled "***Safety, efficacy, nutritional activity and protein/DNA binding interaction of Kenyan Annonaceae fruits in Balb/c mice model of leishmaniasis***" I am one of his University Supervisors.

As part of his work, Lenny needs to carry out proximate composition and nutritional analysis and formulate mice feeds in form of pellets from the fruits. This letter is a request to your department to allow him use the relevant equipment from you laboratories for the above analysis/work.

You favorable consideration of this request is highly appreciated.

Yours sincerely,

Dr. Simon M Karanja

Ag. CoD, Department of Public Health
College of Health Sciences (COHES)

Appendix XXII: A letter of approval to carry out research in the Food Biochemistry Laboratory in the Department of Food Science and Technology at Jomo Kenyatta University of Agriculture and Technology (JKUAT)

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY

INTERNAL MEMO

FROM: Chairman, FST **DATE:** 24TH July, 2014

TO: Chairman, Public Health **REF:** JKU/2/13/071/051

SUBJECT: USE OF LABORATORY

Please refer to your letter dated 14th July, 2014 on the above subject.

Permission to use our labs has been granted subject to the following conditions:

3. Provide all the consumables for required.
4. Adhere to all operating rules and regulations for our laboratory.

DR. ARNOLD N. ONYANGO
COD: FOOD SCIENCE AND TECHNOLOGY

ANO/mm

JKUAT is ISO 9001:2008 certified
Motto: Setting Trends in Training, Research and Innovation

Appendix XXIII:A letter of approval to carry out research in the Directorate of Research and Innovation laboratories (Mount Kenya University)

OFFICE OF THE DIRECTOR, MOUNT KENYA UNIVERSITY

TO WHOM IT MAY CONCERN 23RD MARCH 2018

RE: MR LENNY MWAGANDI CHIMBEVO

This is to confirm that Mr Lenny Mwagandi Chimbevo a postgraduate student at Jomo Kenyatta University of Agriculture and Technology (JKUAT) has been allowed to carry out his Laboratory experiments at our Research Centre for his PhD work. While in the Laboratory, the student will utilize the HPLC for phytochemical analysis.

Kindly accord him the necessary assistance.

Prof Francis Muregi, PhD
Director, Research and Development

Main Campus, General Kago Road, P.O. Box 342-01000 Thika. Tel: +254 67 2820 000,
Cell: +254 720 790 796, 0709 153 000
Email:info@mku.ac.ke, Web: www.mku.ac.ke
Chartered and ISO 9001 : 2008 Certified Institution.
Unlocking Infinite Possibilities

Appendix XXIV: List of publications arising from the study

1. ***Chimbevo, M.L.*** and Essuman, S. (2019). Preliminary Screening of Nutraceutical Potential of Fruit Pulp, Peel and Seeds from *Annona Squamosa* (L.) and *Annona Muricata* (L.) Growing in Coast Region of Kenya. ***American Journal of BioScience; 7(3): 58-70.***

2. ***Chimbevo, M.L.,*** Karanja, S.M., Orwa, J.A., and Anjili, C.O. (2018). An *in vitro* and *in vivo*model of free radical scavenging activity of methanolic and aqueous extract of fruit pulp from *Annona squamosa* (L.) and *Annona muricata* (L.) growing in Coast region of Kenya. ***Journal of Medical and Biological Research; 18(2): 1-14***

3. ***Chimbevo, M.L***., Karanja, S.M. Malala, J.B., Orwa, J.A., Anjili, C.O. and Essuman, S. (2018).Growth performance, metabolic efficiency and nutrient utilization of BALB/C mice infected with *L. major* fed with Annonaceae fruit pulp. ***International Journal of Tropical diseases and Health; 30(2): 1-14***

4. ***Chimbevo, M.L.,*** Malala J.B., Anjili, C.O, Orwa J.A., Mibei, E.K., Ndeti, C.M., Muchiri, F.W., Oshule P.S., Oginga F.O., Otundo, D.O. and Karanja, S.M. (2017). Annonaceae Fruits Growing in Coast Region of Kenya as an Alternative Source of Dietary Carotenoids. ***International Journal of Food Science and Biotechnology; 2(5): 114-120***