BIOASSAY- GUIDED ISOLATION AND CHARACTERIZATION OF A TRYPANOCIDEFROMMISTLETOE (*VISCUM ALBUM)* EXTRACTS

BY

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ZARIA

JULY, 2023

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BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,
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ZARIA, NIGERIA

JULY, 2023

DECLARATION

I declare that the work in this thesis entitled "**Bioassay-Guided Isolation and Characterization of a Trypanocide fromMistletoe (*Viscum album*)Extracts**" has been performed by me in the Department of Biochemistry,Ahmadu Bello University, Zaria. The information derived from the literature has been dulyacknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any otherInstitution.

John WASSAGWA _____ _____
(P17LSBC9008) Signature Date

CERTIFICATION

This thesis entitled "BIOASSAY- GUIDED ISOLATION AND CHARACTERIZATION OF A TRYPANOCIDEFROMMISTLETOE (*VISCUM ALBUM)* EXTRACTS" by John WASSAGWA meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D.) in Biochemistry of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and Literary presentation.

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DEDICATION

This work is dedicated to my wife Ruth Bitrus Ashifa and my children Samaila John, Tadaga John, Adam John and Joy John.

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ABSTRACT

Viscum album is a parasitic plant of immense medicinal significance and was evaluated for antitypanosomal potential. Fresh leaves and stemof *V. album*was obtainedfrom the following host plants;*Azadirachta indica*, *Psidium guajava*, *Acacia albida*, *Khaya senegalensis* and *Moringa oleifera*. The extracts were prepared by cold maceration using methanol. *In vitro* anti-trypanosomal screening of the extracts against *T. b. brucei* was carried out in 96 well microtiter plates at final concentrations of 0.8, 0.4, 0.2, 0.1 and 0.05 mg/mL. A bio-assay guided isolation of the active compound was carried out by repeated silica gel column chromatography, monitored by TLC. Structuralelucidation wasperformed by 1D and 2D NMR. The LD_{50} of HDN-one was evaluated in mice using OECD method. Body weights of mice were taken daily for two weeks and clinical signs of toxicity were observed. *In vivo* effect of the compound isolated was evaluated on *Trypanosoma bruceibrucei* infected mice. The results showed that leaves and stem of *V. album* harvested from *A. albida* and *A. indica* had*in vitro* activity against *Trypanosoma brucei brucei*. Leaves and stem of *V. album* from *A. albida* had 75% and 57.50% inhibition respectively while leaves and stem of *V. album* from *A. indica* had 61.21% and 59% respectively. *Viscum album* leaves from *A. albida* had the highest anti-trypanosomal activity (IC_{50} = 0.30 mg/mL). Qualitative phytochemicalscreening of the methanol extract of*V. album* leaves from *A. albida* showed the presence of alkaloids, tannins, saponins, anthraquinone, phenols, steroids, terpenes, courmarins, flavonoids, and glycosides. Quantitative phytochemistry of the phenolics, flavonoids, alkaloids, and saponins revealed 0.82, 0.04, 1.91, and 2.02% composition respectively.Sequenial extraction of the methanol extract of *V. album* leaves from *A. albida*with n-hexane, ethyl acetate, and n-butanol showed thatn-butanolfraction had

the highest yield of 45.26% and was themost active fraction against *Trypanosoma brucei brucei* (IC_{50} = 0.84 mg/mL). Bioassay- guided fractionation of n–butanol fraction and subsequentpurification of the resulting bioactive compound led to the isolation of [2-(4-hydroxyphenyl) ethyl]-2,6-dioxabicyclo [3.3.1] nonan-3-one (HDN-one)with *in vitro* anti-trypanosomal activity against *T. b. brucei* (IC_{50} = 0.26 mg/ mL). The compound did not show toxicity in mice at a dose level of 2000 mg/kg bw. There was a significant decrease in parasitemia of the group treated with the compound compared to infected untreated group. The compound had no inhibitory effect on the activity of trypanosome alternative oxidase (TAO) and trypanosome glycerol kinase (TGK). In conclusion, the results of this study revealed that HDN-one isolated from *V. album* leavesdid not show signs of toxicity in mice at a dose level of 2000 mg/kg body weight and had *in vivo* anti-trypanosomal activity against *T. b. brucei.*

TABLE OF CONTENTS

TITLE PAGE iii

DECLARATION iv

CERTIFICATION v

DEDICATION vi

ACKNOWLEDGEMENTS vii

ABSTRACT viii

TABLE OF CONTENTS x

LIST OF FIGURES xv

LIST OF TABLES xvii

LIST OF PLATES xviii

LIST OF APPENDICES xix

LIST OF ABBREVIATIONS xx

1.0 INTRODUCTION 1

1.1Background **1**

1.2Statement of the Problem **3**

1.3Justification of the Study **4**

1.4Aim and Objectives of the Study **4**

2.0 LITERATURE REVIEW **6**

2.1 Trypanosomiasis **6**

2.1.1 The life Cycle of Trypanosome 6

2.1.2 The Parasite 8

2.1.3 The Vector 9

2.1.4 Antigenic Variation in African Trypanosomes 10

2.1.5 Economic Significance of African Trypanosomiasis 11

2.1.6Transmission of African Trypanosomiasis 12

2.1.7Wild life as Reservoir Host 13

2.1.8 Trypanosome Infection in Laboratory Animals 14

2.1.9 Clinical Signs of Human African Trypanosomiasis 15

2.1.10 The Clinical Signs of African Animal Trypanosomiasis 16

2.1.11 Pathogenesis of *Trypanosoma brucei* Infections 17

2.1.12 Pathology of *Trypanosoma brucei* Infections 19

2.1.13 Biochemical Changes 20

2.1.14 Treatment for Animal Trypanosomiasis 23

2.1.15 Drug Resistance 25

2.1.16Trypanotolerance 26

2.1.17 Treatment of Human African Trypanosomiasis 27

2.1.18 Energy Metabolism of Trypanosome 30

2.1.19Inhibitors of Trypanasome Alternative Oxidase 31

2.1.20 Plants Potential for Anti-trypanosomal Activities 31

2.2 Mistletoe **34**

2.2.1 Biology of Mistletoe 35

2.2.2 Pollination 35

2.2.3 Toxicological Studies on Mistletoe Leaf Extracts 36

2.2.4 Ethnopharmacology of Mistletoe 38

3.0 MATERIALS AND METHODS **42**

3.1 Materials **42**

3.1.1 Chemicals and Reagents 42

3.1.2 Equipment 42

3.2 Methods 43

3.2.1 Plants Collection, Identification and Processing 44

3.2.2 Extraction of Plants Materials 48

3.2.3 Parasite Collection and Determination of Parasitaemia Load 50

3.2.4 *In vitro* Screening for Anti-Trypanosomal Activity 50

3.2.5 Qualitative Phytochemical Evaluation of the Methanol Extract of *Viscum album* Leaves of *A. albida* 51

3.2.6 Quantitative Phytochemical Analysis 51

3.2.7 Thin Layer Chromatography of the Methanol Extract of *Viscum album*Leaves of *Acacia albida* 52

3.2.8 Partitioning of Extracts into Fractions 52

3.2.9 Column Chromatography 53

3.2.10 Bio-GuidedAssay 54

3.2.11 Structural Determination of the Bioactive Compound 55

3.2.12 Acute Toxicity Testing 55

3.2.13 *In vivo* Assay 58

3.2.14 Haematological Studies 58

3.2.15 Biochemical Studies 58

3.2.16Enzyme Inhibition Assay 63

3.2.17 Statistical Analysis 65

4.0 RESULTS **66**

4.1Yield of the MethanolExtracts of *Viscum album* 66

4.2*In vitro* Anti-trypanosomal Activity of the Methanol Extract of *Viscum album* Leaves and Stems 68

4.3Qualitative Phytochemical Profile of the Methanol Extract of *Viscum album*Leaves of *A. albida* 70

4.4Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album*Leaves of *A. albida* 72

4.5 Thin Layer Chromatogram of the Methanol Extract of *Viscum album* Leavesof *A. albida* 74

4.6 Solvent Partition Yield of the Methanol Extract of *V. album* Leaves of *A. albida* 76

4.7 *In vitro* Antitrypanosomal Activity of the Partitioned Fractions of the Extract of *Viscum album* Leaves of *A. albida*Methanol 78

4.8 Thin Layer Chromatogram of n–Butanol Fraction of the Methanol Extractof *Viscum album* Leaves of *A. albida* 80

4.9 *In vitro* Anti-trypanosomal Activity of the n-Butanol Fractionof the Methanol Extractof *Viscum album* Leaves of *A. albida* 82

4.10 *In vitro* Anti-trypanosomal Activity of the Sub-Fractions (47-54) 84

4.11 Thin Layer Chromatogram of the sub-Fraction (24 – 35) 86

4.12 1D-Proton NMR (^{1}H-NMR) Spectra 88

4.13 1D-Carbon NMR (^{13}C-NMR) Spectra 88

4.14 2D ^{1}H, ^{1}H-COSY Correlation Spectroscopy 91

4.15 ^{1}H-^{13}C Heteronuclear Single Quantum Coherence Spectroscopy (HSQC)91

4.16 Heteronuclear Multiple Bond Correlation (HMBC) spectra 91

4.17 Chemical Shift Values of ^{1}H and ^{13}C NMR 95

4.18 The structure of HDN-one 97

4.19 Acute Toxicity Testing for HDN-one 99

4.20 Clinical Signs of Acute Toxicity Test in Mice 101

4.21 Mean Body Weight of Mice in Acute Toxicity Test of HDN-one 103

4.22 Relative Organ Weight of Mice in Acute Toxicity Test of HDN-one 105

4.23Haematological Parameters of Mice in Acute Toxicity Test of HDN-one 107

4.24Biochemical Parameters of Mice in Acute Toxicity Test of HDN-one 109

4.25Mean Parasite Count following Treatment with HDN-one and DiminazeneAceturate 111

4.26 Kaplan Meier Plot of Percentage Survival Days Post Infection of *T. b. brucei* Infected Mice 113

4.27Mean Body Weight of *T. b. brucei* Infected Mice treated with HDN-one and Diminazene Aceturate115

4.28Inhibitory effect of 50 µM of HND-one on Trypanosome Alternative Oxidase 117

4.29 Inhibitory effect of 50 µM of HND-one on *T. b. gambiense* glycerol kinase 117

5.0 DISCUSSION 120

6.0 SUMMARY, CONCLUSION AND RECOMMENATIONS 126

6.1 Summary 126

6.2 Conclusion 126

6.3 Recommendations 127

REFERENCES 128

APPENDICES 152

LIST OF FIGURES

Figure 2.1: Life Cycle of *Trypanosoma brucei* 7

Figure 3.1: Research Design 43

Figure 3.2: Procedure for Acute Toxicity Test using Starting Dose of 2000 mg/kg/bw 57

Figure 4.1: Yield of the Methanol Leaves and Stems Extract of *V. album*of*A. albida, A. indica, M. oleifera, P. guajava* and *K. senegelensis*67

Figure 4.2: Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leaf of *A. albida* 73

Figure 4.3: Solvent Partition Yield of the Methanol Extract of *V. album* Leaf of *A. albida* 77

Figure 4.4: 1D-Proton NMR (^{1}H-NMR) Spectra of HDN-one Isolated from the Methanol Extract of *Vsscum album* Leaves of *A. albida* 89

Figure 4.5: 1D-Carbon NMR (^{13}C-NMR) Spectra of HDN-one Isolated from theMethanol Extract of *Vsscum album* Leaves of *A. albida* 90

Figure 4.6: 2D ^{1}H,^{1}H-COSY (Correlated Spectroscopy) of HDN-one Isolated fromthe Methanol Extract of *Viscum album* Leaves of *A. abida*92

Figure 4.7: HSQC (Heteronuclear Single Quantum Correlation) Spectra of HDN-one Isolated from the Methanol Extract of *Viscum album* Leaves of *A. abida*93

Figure 4.8: HMBC (Heteronuclear Multiple Quantum Correlation) Spectra of HDN-oneIsolated from the Methanol Extract of *Viscum album*Leaves of *A. abida* 94

Figure 4.9: Chemical Structure of [2-(4-hydroxyphenyl) ethyl]-2,6-dioxabicyclo[3.3.1] nonan-3-one (HDN-one) 98

Figure 4.10: Mean Body Weight of Mice following the Intraperitoneal Administration of HDN-one 104

Figure 4.11: Mean Relative Organ Weight of Mice following the Administration ofHDN-one106

Figure 4.12: Mean Values of Haematological Parameters of Mice followingIntraperitoneal Administration of HDN-one108

Figure 4.13: Mean Values of Biochemical Parameter of Mice following Intraperitoneal Administration of HDN-one 110

Figure 4.14: Mean Parasite Count of *Trypanosoma bruce brucei* Infected Mice Treatedwith HDN-one and Diminazene Aceturate 112

Figure 4.15: Kaplan Meier Plot of Survival Time of *T. b. brucei* Infected Mice Treatedwith HDN-one and Diminazene Aceturate 114

Figure 4.16: Mean body Weight of *T. b. brucei* Infected Mice Treated with HDN-one and Diminazene Aceturate 116

Figure 4.17: Inhibitory Effect of 50 µM of HDN-one on Trypanosome Alternative Oxidase 118

Figure 4.18: Inhibitory Effect of 50 µM of HDN-one on *T. b. gambiense* Glycerol Kinase 119

LIST OF TABLES

Table 3.1: Identification of *Viscum album* and its Hosts 45

Table 4.1:*In vitro* Anti-trypanosomal Activity of *V. album*Leaves and Stems Extracts 69

Table 4.2: Qualitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leaves of *A. albida* 71

Table 4.3: *In vitro* Anti-trypanosomal Activity of the Partitioned Fractions of the Methanol Extract of *Viscum album* Leaves of *A. albida* 79

Table 4.4: *In vitro* Anti-trypanosomal Activity of n-Butanol Fractions of the Methanol Extract of *Viscum album* Leaves of *A. albida* 83

Table 4.5:*In vitro*Antitrypanosomal Activity of sub fractions of Fraction (47 – 54) 85

Table4.6: ^{1}H And ^{13}C NMR Chemical Shift of HDN-one Isolated from the Methanol Extract of *Viscum album* Leaves of *A. abida* 96

Table 4.7: Acute Toxicity Test of HDN-one on Mice 100

Table 4.8: The Clinical Signs of Acute Toxicity Test of HDN-one on Mice 102

LIST OF PLATES

Plate I:*Viscum album* on Different Host46

Plate II:Pulverized Samples of *V. album* Leaves and Stems from *Azadirachta indica*, *Psidium guajava*, *Acacia albida*, *Khaya senegalensis* and *Moringa oleifera*47

Plate III: Samples during Extraction49

Figure IV: Thin Layer Chromatogram of the Methanol Extract of *Viscum album*Leaves from *A. albida*75

Plate V: Thin Layer Chromatogram of n-Butanol Fraction of the Methanol Extract of *Viscum album* Leaves of *A. albida*81

Plate VI:TLC of the Purified Compound Developed in n-Hexane: Ethyl Acetate in the ratio 2:8 87

LIST OF APPENDICES

APPENNDIX I: Yield of the methanol Leaves and Stems Extract of *V. album*from Five Host Trees 152

APPENDIX II: IC_{50} Graphs of the Active Methanol Extracts of *Viscum album*Leaves and Stems of *A. albida* and *A. indica*153

APPENDIX III: Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leaves of *A. albida*154

APPENDIX IV:Solvent Partition Yield of the Methanol Extract of *V. album* Leaves of *A. albida*155

APPENDIX V: The IC_{50} Graph of the Active Fractions156

APPENDIX VI: Mean Body Weight of Mice in Acute Toxicity Test of HDN-one157

APPENDIX VII: Mean Relative Organ Weight of Mice in Acute Toxicity Test of HDN-one158

APPENDIX VIII: Mean Values of Haematological Parameters of Mice in Acute Toxicity Test of HDN-one159

APPENDIX IX: Mean Values of Biochemical Parameter of Mice in Acute Toxicity Test of HDN-one160

APPENDIX X: The Mean Parasite Count of *Trypanosoma brucei brucei*Infected Mice Treated with HDN-oneand Diminazene Aceturate161

APPENDIX XI: Mean Body Weight of *T. b. brucei* Infected Mice Treated with HDN-one and Diminazene Aceturate162

APPENDIX XII: Inhibitory Effect of 50 µM HDN-one on Trypanosome Alternative Oxidase163

APPENDIX XIII: Inhibitory Effect of 50 µM of HDN-one on *T. b. gambiense*glycerol kinase164

APPENDIX XIV: Certificate of Paper Presentation at a Conference 1165

APPENDIX XV: Certificate of Paper Presentation at a Conference 2166

APPENDIX XVI: Certificate of Conference Participation167

LIST OF ABBREVIATIONS

A	Albumin
AAT	African Animal Trypanosomiasis
AIDS	Acquired Immune Deficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	Analysis of Variances
AOX	Alternative Oxidase
ApoL1	Apolipoprotein 1
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
BBIQ	BisbenzylIsoquinoline
BESs	Bloodstream Expression Sites
BUN	Blood Urea Nitrogen
CATT	Card Agglutination Test for Trypanosomiasis
CC	Column Chromatography
CNS	Central Nervous System
COSY	Correlated Spectroscopy
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebrospinal Fluid
DBS	Dried Blood Spots
DE	Diosgenin Equivalents
DFMO	α-difluoro-methyl-ornithine

DHAP Dihydroxyacetone Phosphate

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic Acid

DW Dry Weight

EDTA Ethylene Diamine Tetra Acetic Acid

ELISA Enzyme Linked Immunosorbent Assay

EMR Electromagnetic Radiation

ESAGs Expression Sites Associated Genes

FAD Flavin Adenine Dinucleotide

FAO Food and Agriculture Organization

FC Flash Chromatography

G Globulin

GAE Gallic Acid Equivalent

GGT Gamma Glutamine Transaminase

GHS Globally Harmonized System

GPO Glycerol-3-Phosphate Oxidase

HAT Human African Trypanosomiasis

HCT Haematocrit Centrifuge Technique

HbHaemoglobin

HMBC Heteronuclear Multiple Quantum Correlation

HPLC High Performance Liquid Chromatography

HPTLC High-Performance Thin-Layer Chromatography

HSQC Heteronuclear Single Quantum Correlation

IC_{50}	Inhibitory Concentration at 50%
IgM	Immunoglobulin M
IL-6	Interleukines 6
ILCA	International Livestock Centre for Africa
ITS-1	Internal Transcribed Spacer
LAMP	Loop-mediated Isothermal Amplification
LC_{50}	Lethal Concentration at 50%
LD_{50}	Lethal Dose at 50%
LILIT	Low Inoculation Long Incubation Test
LtVA	Long-Term Viability Assay
mAECT	mini Anion Exchange Centrifugation Technique
MDR	Multiple-Drug Resistant
mG3PDH	mitochondrial FAD+-Dependent Glycerol-3-Phosphate
mHCT	micro-Hematocrit Centrifugation Technique
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MSC	Modified Single Centrifugation Technique
NADH	Nicotinamide Adenine Dinucleotide+H
NECT	Nifurtimox–Eflornithine Combination Therapy
NITOR	Nigerian Institute for Trypanosomiasis and Onchocerciasis Research
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy

OECD	Organization for Economic Co-operation and Development
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PPV	Positive Predictive Value
PTLC	Preparative Thin Layer Chromatography
RBC	Red Blood Cell
RDT	Rapid Diagnostic Tests
RF	Radio Frequency
RNA	Ribonucleic Acid
RNAP I	RNA Polymerase I
RNAP II	RNA Polymerase II
rRNA	Ribosomal Ribonucleic Acid
rTAO	Recombinant Trypanosome Alternative Oxidase
SD	Standard Deviation
SHAM	Salicylhydroxamic Acid
SL	Spliced Leader’
Spp	Species (Plural)
TAC	Total Alkaloid Content
TAO	Trypanosome Alternative Oxidase
TbGK	*Trypanosoma brucei* Glycerol Kinase
TFC	Total Flavoniod Content
TL	Immune Trypanolysis

TLC Thin Layer Chromatography

TNF-α Tissue Necrotic Factor

TP Total Protein

TWBC Total White Blood Cell

VSG Variable Surface Glycoprotein

WBCWhite Blood Cell

WHO World Health Organisation

TBARSThiobarbituric Acid Reactive Substances

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

African trypanosomiasis is a disregarded tropical disease that is common in sub-Saharan Africa and impacts both humans and animals. It is caused by a protozoan parasite of the genus *Trypanosoma* (Alsan, 2015). The disease is transmitted by tsetse fly (*Glossina spp*.) during a blood feeding (Geiger *et al*., 2018). There are several *Trypanosoma* species which include *Trypanosomabrucei**brucei, T. congolense* and *T. vivax*that causes the disease known as "nagana" in animals such as cattle, sheep and goats (Ruiz *et al.*, 2015),*T. simiae*that causes disease in pigs (Zweygarth*et al.*, 1992),*T. evansi* and *T. equiperdum*that causes surra and dourine that affect equines and camels (Atawodi*et al*., 2002) and*T.brucei rhodesiense* in East Africa and *T. brucei gambiense* in West and Central Africa (Ponte-sucre, 2016) that causesHuman African Trypanosomiasis(HAT).

African trypanosomiasis is a debilitating parasitic disease of medical, veterinary and socio-economic importance that adversely affects the economic development of sub-Saharan Africa (Sutherland *et al*., 2014; Aksoy *et al*., 2017). Livestock that are affected by the disease experience decreased milk production, weight gain, and reproduction, ultimately leading to their death (Hoet*et al*., 2004). Human trypanosomiasis, on the other hand, presents prominent symptoms such as high fever and anemia, followed by heart and endocrine complications, miscarriage, swelling, and kidney problems (WHO, 2007).In theadvanced stage of the disease in humans, the parasite invades the central nervous system of the host and subsequently triggers behavioural changes including lack of concentration

and indifference to their environment, aggressiveness, loss of appetite, speech disorder, as well as inability to walk, the patient falls into deep coma and finally dies (Franco *et al*., 2014; Buscher*et al*., 2017). In animals, cattle may present swollen lymph nodes and internal organs,anaemia, reproductive failure and wasting. Horses with dourine could present symptoms of ventral and genital oedema and urticaria.

For many decades, treatment of African trypanosomiasis have been through chemotherapy and chemoprophylaxis, but the trypanosome has developed levels of resistance to drugs which are also known to produce side effect as well as been expensive. Thus, there is the need to develop more effective and safer therapy(Horn, 2014). The use of natural products with potent anti-trypanosomal activities would be ideal(Sampath, 2014).

Viscum album (mistletoe) is a parasitic plant which survive on the stem of other plants. The plant obtains water and mineral salts from the host (Werthmann*et al*., 2013). Mistletoes can be observed on numerous economically significant trees in West Africa, such as the shea butter tree (*Vitellaria paradoxaGaertn f*.), neem (*Azadirachta indica*), and sweet orange (*Citrus sinensis*) (Overfield, 1998).

The plant has been used in traditional medicine for the treatment of several illnesses such as epilepsy, diabetes mellitus, cancer, hypertension, headache and arthritis. Currently, there are many *in vitro* and *in vivo* studies that have confirmed the extensive therapeutic potential of *V. album*. There are reports on its anti-inflammatory (Hegde *et al*., 2011), anti-hypertensive (Ofem*et al*., 2007), antidiabetic (Chikezie*et al*., 2015), anticancer (Burger *et al*., 2001), immunomodulatory (Lavastre*et al*., 2004), antioxidant (Orhan, *et al*., 2005), antimicrobial (Orhan, 2014) and antiepileptic (Gupta *et al*., 2012) properties. *Viscum album*

contains several biologically active chemicals such as lectins, viscotoxins (Eremia*et al*., 2008), flavonoids, polysaccharides, alkaloids, terpenoids, saponins, tannins, polysterols and phenolic acids (Orhan, *et al*., 2002;*Vicas et al*., 2011). *V. album*host is an important factor that determine its phyto-contituentsand bioactivity, hence *V. album* from different hostisused for different purposes (Viscas*et al*., 2011; Orhan, 2014). In the *in vitro* and *in vivo* study reported by Wassagwa(2016), the aqueous extract of *Viscum album* leave of*Acassia albida* was found to be potent against*T. b. brucei* infected rats. The effect was comparable to the effect of diminazene. For this reason,the current study aims at isolation and characterization of the antitrypanosomal compoundin*Viscum album* extract.

1.2 Statement of the Problem

African trypanosomiasis poses significant challenges to both livestock production and human well-being in countries across sub-Saharan Africa (Simarro*et al*., 2012; Franco *et al*., 2014; Meyer *et al*., 2016). This disease has experienced a severe and devastating resurgence, particularly in sub-Saharan Africa, making it a top priority for scientific study (Welburn, 2001; Aksoy, 2003). Approximately 60 million humans living in tsetse fly zones are at potential hazards of trypanosomiasis with estimated 5 billion US dollars' annual economic loss to animals (Franco *et al*., 2014; Yaro*et al*., 2016) and about 10 million km^2 fertile land in the tsetse belts is rendered unsuitable for cultivation (Shaw *et al*., 2014). Chemotherapy, the main means of controlling the disease is failing due to parasite resistance and toxicity of the anti-trypanosomal drugs and the poor prospect of vaccine due to antigenic variation of the parasite. The adverse side effects of the current drugs against trypanosomiasis is severe, sometimes life-threatening. For instance, the drug melarsoprol causes encephalopathy in 5-10 % of the cases, half of which are fatal (Atouguia*et al.*,

1995). Other sideeffects include vomitting, abdominal colic and peripheral neuropathy. There are also increasing reports of treatment failure.Therefore, there is a pressing need for safe and effective therapy against the disease

1.3 Justification of the Study

The abundant chemical diversity found in medicinal plants offers limitless possibilities for discovering new drugs, either in the form of pure compounds or standardized extracts. This makes natural products an invaluable resource for drug discovery (Cosa *et al*., 2006).Survey has shown that about 70-80% of Asians and Africans rely on herbal remedies for their health needs (Neergheen-Bhujun, 2013).Due to this factor ethnopharmacology of medicinal plants has garnered growing interest in drug exploration and advancement. *Viscum album* is a parasitic plant that has been used in traditional medicine to treat illnesses such as diabetes mellitus, fever, headache, cancer, epilepsy, hypertension and arthritis (Jadhav *et al*., 2010). The plant hasseveral bioactivities including, antioxidant, antiviral, antibacterial, opoptosis inducing, anticancer and immune modulatory (Onay- Ucar*et al*., 2006). *1n vitro* and *in vivo* findings by Wassagwa(2016) revealed that *Viscum album* leaf and stem aqueous extracts have efficacy against *Trypanosoma b. brucei*. In view of this, the work is designed to isolate and characterize the compound with possible anti-trypanosomalactivitiy.

1.4 Aim and Objectives of the Study

The aim of this study was to isolate and characterize the antitrypanosomal compound from *Viscum album* extracts.

The specific objectives are to:

i. Investigate the *in vitro* anti-trypanosomal activity of *Viscum album* extracts from different host plant (*Azadirachta indica*, *Psidium guajava*, *Acacia albida, Khaya senegalensis* and *Moringa oleifera*)

ii. Fractionate the most active extract and isolate the antitrypanosomal compound

iii. Structurally elucidate the anti-trypanosomal compound

iv. Determine the acute toxicity of the anti-trypanosomal compound isolated

v. Determine the *in vivo* effects of the anti-trypanosomal compound on *Trypanosoma brucei brucei* infected albino mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Trypanosomiasis

Trypanosomiasis is the name of a disease that affects humans and animals caused by a parasitic protozoan of the genus *Trypanosoma* (Alsan,2015). Human African trypanosomiasis (sleeping sickness) is caused by *T. brucei rhodesiense* and *T. b. gambiense*while African animal trypanosomiasis (nagana) is caused by *T. b. brucei, T. congolense, and T. vivax* (Geiger *et al*., 2018). Trypanosomiasis is transmitted primarily by the bite of the blood-sucking tsetse fly of the genus *Glossina.*

2.1.1 The Life Cycle of Trypanosome

Trypanosomes are blood-feeding parasites, which are transmitted from one animal to another during grazing. In the case of *Trypanosoma brucei*, the parasite is injected into the bloodstream by the vector when it bites. Inside the human body, *T. brucei* reproduces through binary fission in various fluids like blood and cerebrospinal fluid. Once *T. brucei* proliferates in humans, it is taken in by the fly while feeding on bood leading to further multiplication in the fly's midgut. As a result, the parasite transforms into an infectious stage that can penetrate the salivary gland. When the fly subsequently bites to feed, it infects its host with the parasite.

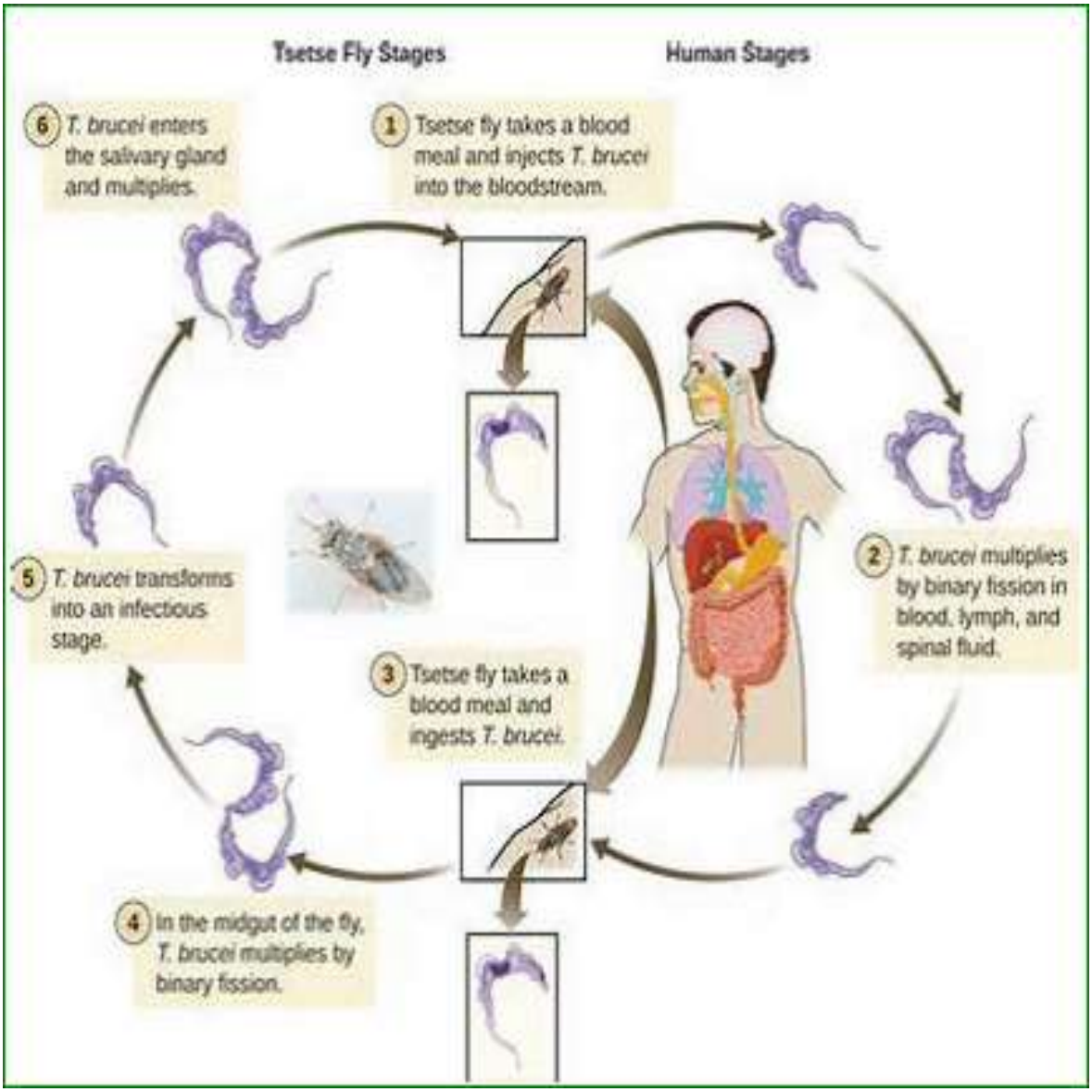


Figure 2.1: Life Cycle of *Trypanosoma brucei* (Kayode *et al*., 2020).

2.1.2 The parasite

Trypanosoma brucei is a member of the *Trypanosomatidae* family, which consists exclusively of parasitic organisms found worldwide in vertebrates and insects (Hoarse, 1972). These single-celled parasites have co-evolved with their hosts to such an extent that most of them have a commensal relationship rather than being pathogens (Steverding, 2008). The species *T. brucei* comprise of three subspecies that are morphologically indistinguishable. *T. b. brucei* causes African animal trypanosomiasis (AAT) but is not infective to humans. *T. b. rhodesiense* and *T. b. gambiense* have developed the ability to infect humans by resisting apolipoprotein A (ApoL1), a serum protein that is lethal to other trypanosomes (Vanhollebeke and Pays, 2010).

Trypanosomabrucei is an extracellular parasite and has significant metabolic adaptations reflected in morphological changes. In the blood and tissues of mammals, trypanosomes take the form of spindle-shaped cells and exhibit an undulating movement. Occasionally, shorter forms can also be observed, which are metabolically adapted for survival in the intestines of tsetse flies. Within the mammalian host, the trypanosome cell membrane is covered by a glycoprotein coat that shields the underlying membrane from innate immune attacks. These highly immunogenic glycoproteins stimulate a specific antibody response, leading to the destruction of all trypanosomes marked with these antibodies. To evade this antibody-mediated immune response, trypanosomes have developed "antigenic variation," whereby the glycoprotein coat on the cell membrane is periodically replaced with a distinct antigenic coat (Horn, 2014). The interplay between the host's immune response and the parasite antigenic variation results in fluctuating parasitemia, accompanied by irregular fever during the elimination of trypanosomes.

Infection with *T. b. brucei* typically triggers polyclonal activation of B-cells, resulting in exceptionally high levels of IgM antibodies and a variety of non-specific antibodies, including auto-antibodies. These antibodies contribute to the pathogenesis of the infection and can cause non-specific reactions in antibody detection assays for other infections (Bisser*et al*., 2000). In mammalian hosts, the infection begins with the injection of metacyclic trypanosomes into the epidermis. After several days of proliferation, the trypanosomes travel through the lymph and circulatory systems to various peripheral organs and tissues. Eventually, the parasites invade the brain parenchyma, leading to local inflammation and neurological damage (Kristenssone*t al*., 2013).

2.1.3 The Vector

Trypanosoma brucei relies on tsetse flies for its cyclical transmission. Both male and female tsetse flies are blood-feeding and capable of transmitting trypanosomes. Tsetse flies exhibit viviparity, where the female deposits a fully developed larva that penetrates the soil, undergoes pupation, and emerges as an adult fly approximately one month later. The 31 species and subspecies of tsetse flies are categorized as forest, riverine, or savannah based on morphological distinctions and habitat preferences (Checchi *et al*., 2008). *Glossina fuscipes* and *Glossina palpalis*, belonging to the *palpalis* (riverine) group, are the primary vectors of *T. brucei gambiense*(WHO, 2013; Grebaut*et al*., 2016). The vectors for *T. brucei rhodesiense* include *G. fuscipes* and species from the savannah group, such as *Glossina morsitans* and *Glossina pallidipes* (Solano *et al*., 2013).

When tsetse flies take in trypanosomes from contaminated blood, they contract *T. brucei*, as shown in experimental inoculation (Capwell*et al*., 2016). Once taken in, the short

stumpy trypanosomes set off on a challenging journey through the tissues of the fly until they reach the salivary glands and transform into the metacyclic forms that can infect humans. These metacyclic forms are free-swimming and resemble the short stumpy form in terms of morphology (Ooi*et al*., 2013). In natural populations of tsetse flies, only a small proportion (approximately 0.01%) carry a mature infection of *T. brucei*, meaning they have metacyclic trypanosomes in their salivary glands (Auty*et al*., 2012; Jamonneau*et al*., 2004).

Tsetse flies rely on human blood as their source of nutrition, potentially infecting multiple individuals during their lifespan of 2 to 3 months. One approach to reducing or interrupting the transmission of *T. brucei* is by eliminating tsetse flies or minimizing contact between tsetse flies and humans (Auty*et al*., 2012; Jamonneau*et al*., 2004).

2.1.4 Antigenic Variation in African Trypanosomes

Antigenic variation is the mechanism employed by the parasite to modify its variant surface glycoprotein (VSG) coat, enabling it to evade antibody responses (Rudenko *et al*., 1998). A trypanosome has the capacity to express hundreds of distinct VSGs, but only one is active at a time. The switching between VSGs is not triggered by antibodies and seems to occur spontaneously. Similar variation strategies are observed in various bacterial and protozoan pathogens, facilitating traits like host cell attachment and evasion of immune responses (Deitsch*et al*., 1997; Koomey, 1997). While there is a general similarity in the rate of variation among these organisms, indicating convergent evolution (Cross, 1996), several features of antigenic variation at the trypanosome population level within the host contribute to prolonged infection.

VSG switching is divergent, and the typical course of infection involves periodic peaks of parasitemia, with each peak comprising a combination of variable antigen types (Van Meirvenne*et al*., 1975). The potential for variation is not infinite, and it is believed that trypanosomes possess a finite repertoire of VSGs (Capbern*et al*., 1977; Nantulya*et al*., 1984). However, this repertoire may be temporarily expanded through the generation of mosaic genes via recombination during infection (Barbet and Kamper, 1993). Another significant aspect contributing to the duration of infection is the hierarchical expression of the VSG repertoire. Some variant antigen types (VATs) are prone to emerge early in an illness, others do not occur until late in infection, and the other variants are produced at intermediate phases(Capbern*et al*., 1977; Barry, 1986). The regulation of VSG expression is critical to these characteristics.

2.1.5 Economic Significance of African Trypanosomiasis

In sub-Saharan Africa, trypanosomiasis poses a serious threat to the wellbeing of livestock and their ability to generate income (Alsan, 2015). Rural households depend on livestock production for different economic and sociocultural reasons, such as provision of food and nourishment, draught power, revenue from employment, soil fertility, and capital accumulation. In all livestock production systems in sub-Saharan Africa, livestock production contributes for 40% of total household income (Staal*et al*., 2009). Additionally, according to Staal*et al*. (2009), the region is home to nearly 309 million livestock owners who make less than $2 per day. Farmers who rely on animals for their livelihood are more susceptible to a number of diseases, including trypanosomiasis. According to estimates, 32% of the livestock population in the sub-Saharan Africa is located in tsetse fly-infested regions, with the severity of the danger varying by location (Kristjanson*et al*., 1999).

According to Kristjanson*et al*. (1999), trypanosomiasis poses a threat to 48%, 76%, 28%, and 8% of the world's cattle populations in Western Africa, Central Africa, Eastern Africa, and Southern Africa, respectively.

Trypanosomiasis lowers the output and revenue of milk and meat (Swallow, 2000). By decreasing the productivity of oxen's draught power, it indirectly influences land utilization. Farmers stay away from productive tsetse-fly-infested areas because of the risk of contracting trypanosomiasis, even though these places may be necessary given the rapid population development (Meyer *et al*., 2016; Shaw *et al*., 2014). According to estimates, it kills 3 million cattle annually, resulting in a direct economic loss of US$ 1-1.2 billion to the cattle industry. Sub-Saharan Africa could lose up to 4.75 billion in gross domestic product (GDP) year after accounting for indirect economic costs (Cecchi and Mattioli, 2009; Shaw *et al*., 2014).

2.1.6 Transmission of African Trypanosomiasis

According to Degneh*et al*. (2018) and Kasozi*et al*. (20221), different species of *Glossina* tsetse flies as well as flies such as*Stomoxys* and *Tabanus* species are the biological carriers of the disease trypanosomiasis. The primary tsetse fly species responsible for transmitting trypanosome parasites are *Glossina morsitans*, which inhabits open savannah areas, *Glossina palpalis*, found in shaded habitats near rivers and lakes (Lehane*et al*., 2016), and *Glossina fusca*which is predominantly found in densely forested regions (Muse *et al*., 2015; Nthiwa*et al*., 2015). The distribution of tsetse flies relies on the presence of suitable habitats, including specific climatic conditions and types of land cover (McCord *et al*., 2012). Various habitats, such as tropical rainforests, semi-arid grass savannahs, wet

mangroves, riparian areas, and woody savannah ecosystems, provide the necessary land cover for tsetse flies,

allowing them to mitigate the impacts of high temperatures or dry conditions (McCord *et al*., 2012).According to historical records, Kenya is home to eight distinct species of tsetse flies, namely *G. Brevipalpis, G. londipennis, G. fuscipleuris, G. pallidipes, G. austeni, G. swynnertoni, G. morsitanssubmorsitans,* and*Fuscipesfuscipes* (Ngari*et al*., 2020).

2.1.7 Wild life as Reservoir Host

Tsetse flies are known to feed on a variety of wild animals, including primates (baboons and monkeys), suids, and a wide range of other mammals and birds (Anosa, 1983). Investigations into the sources of the blood meals of wild caught tsetse flies have shown this. Similar research by Sasaki *et al.* (1995) revealed that *Glossina pallidipes* and *Glossina longipennis*feed on bushbucks, ostriches, elephants, buffalo, warthogs, bush pigs, waterbuck, and roan antelopes as their primary sources of blood meals. Anosa (1988) noted that more research has been done on the role of wild animals as trypanosome reservoirs in East Africa than West Africa. Trypanosomes, principally *Trypanosoma vivax* and *Trypanosoma congolense*, were found in 6.3% of 1242 different game species in East Africa, including waterbuck (33%), reed buck (26%), giraffe (16%), and bush pig (12%). By exposing one *Grimm's duiker* to *Glossina palpalis*, Desowitz (1960) effectively transmitted *Trypanosoma vivax* to the animal. However, he was unable to do the same with *Trypanosoma congolense* or *Trypanosoma brucei*. According to Moloo *et al*. (1972), *Glossina morsitans* may be able to transmit *Trypanosoma brucei* and *Trypanosoma rhodesiense* between domestic ruminants and bushbuck (*Tragelaphus scriptus*). They also mentioned the potential for cross contamination between domestic ruminants and lab

animals. Several kinds of captive wild animals have been identified as reservoirs of pathogenic trypanosomes in the desert region of Nigeria (Mbaya*et al*., 2008). Numerous trypanosome species have also reportedly been found to reside in marine species (Jennings, 1988).

2.1.8 Trypanosome Infection in Laboratory Animals

Trypanosoma vivax infections of rats have completely failed or resulted in temporary parasitaemia (Kalu *et al*., 1986). It is possible to continue serial passage of animals infected with *Trypanosoma congolense* and *Trypanosoma brucei*, despite the fact that attempts to serially passage *Trypanosoma vivax* from rodents have failed after the second passage. There was success in developing an acute *Trypanosoma vivax* infection in 4 rabbits after numerous efforts and the addition of goat serum supplement, keeping the strain in the animal through 8 generations over the course of 2 months. However, using either *Trypanosoma congolense* or *Trypanosoma brucei*, identical infections in the animals were significantly simpler to produce. After multiple passes, it was later shown that some *Trypanosoma vivax* strains could adapt to laboratory rodents without serum supplementation (Hull *et al*., 1971). According to Hull *et al.* (1971), splenectomy may also help *Trypanosoma vivax* strains adapt to laboratory animals. According to Leeflang*et al.* (1976), mice could pass through 75 bovine isolates of *Trypanosoma vivax* without the need for additional serum for an endless amount of time. The same applied to *Trypanosoma congolense*. The parasite became more virulent as a result of this transit with *Trypanosoma brucei*, reducing the incubation period to 2 days or even fewer (Leeflang*et al*., 1976). Furthermore, it was noted that 127 isolates of *Trypanosoma vivax* from naturally infected cattle were used in two experiments and that 50% of these isolates caused mice to develop

parasitaemia to varied degrees (Leeflang*et al*., 1976). Additionally, it was found that blood from early infections in rats and cattle had higher infectivity to mice than blood from later phases of infections (Leeflang*et al*., 1976).

2.1.9Clinical Symptoms of Human African Trypanosomiasis

The clinical manifestation of trypanosomiasis that affect people in Africa is influenced by factors such as the subspecies of the trypanosome, the host's immune response, and the stage of the illness. Variations in virulence and pathogenicity have been observed among different strains of the parasite (Van Marck*et al*., 1981; MacLean *et al*., 2010). While there have been cases of healthy individuals acting as carriers and instances of self-cure reported for *gambiense* HAT, untreated trypanosomiasis typically leads to death (Jamonneau*et al*., 2012). According to Odiit*et al*. (1997), *Rhodesiense* HAT usually starts off acute, progressing to the second stage within a few weeks, and ending in mortality after six months. The chronic, progressive course of gambian HAT is predicted to last for three years on average, while there is significant inter-individual variation. Firstly,hemo-lymphatic stage and secondly meningo-encephalitic stage, in which trypanosomes enter the brain and the spinal cord, are the two stages of the disease. The second stage is characterized by neurological problems, particularly sleep disorders, however the majority of signs and symptoms are similar between the two stages. In between 5 and 26% of native *rhodesiense* HAT patients, a skin reaction at the site of the tsetse bite manifests 2-3 days after the bite, whereas it is uncommon in *gambiense* HAT (MacLean *et al*., 2010; Küpfer*et al*.**,** 2011).

Primarily, long-lasting intermittent fever, headache, pruritus, and lymphadenopathy characterize first stage *gambiense* HAT. Enlarged liver, swelling, and endocrine disruption

(amenorrhea, infertility, miscarriage in female; decreased libido, inability to reproduce in males) are less common symptoms. The second stage adds neurobehavioral abnormalities to the first stage characteristics while decreasing the frequency of fever. The symptoms of the sleep disorder include nocturnal insomnia, overwhelming impulses to sleep, and daytime somnolence (Mpandzou*et al*., 2011; Njamnshi*et al*., 2012). Additional neurological symptoms include hypotonia, tremor in the hands and fingers as well as choreiform, oscillatory motions of the limbs, fasciculation, motor weakness, ataxia, akinesia, and speech problems. Attention deficit disorder, apathy, aggressiveness, stereotypic behavior, dissociative fugue, manic episodes, melancholia, disorientation, and lost of memory are all prevalent mental abnormalities. According to Blum *et al.* (2006), neuropsychiatric problems get worse when an illness gets worse. The disruption of circadian rhythms of hormonal secretion, including prolactin, renin, growth hormone, and cortisol, is caused by infiltration of endocrine organs (mostly thyroid and adrenals) and the hypothalamic-hypophysial axis, but is typically not treated(Bugue*et al*., 2001). The *rhodesiense* HAT clinical signs are comparable to those of the *gambiense* HAT. Children are more likely to experience fever during both disease stages (Kato *et al*., 2015).

2.1.10 The Clinical Signs of African Animal Trypanosomiasis

Animal trypanosomiasis manifests clinically as frequent fever, anorexia, discharge from the eye, photophobia, pale mucous membranes, swelling of visible lymph nodes, oedema of the throat, dullness, lethargy, emaciation, decreased milk production and ability to work in draught animals, infertility, abortion, stillbirth, and deaths. According to Reddy and Hafeez (1996), symptoms of *T. evansi* include ataxia, circling movements, lack of coordination, stumbling gait, head banging and pushing. Pigs infected with the *simiae* parasite may die

within a few hours. According to Losos (1986), Symptoms include fever, increased breathing, dullness, a stiff and unsteady stride, and skin hyperaemia. The clinical indicators of dourine (Radostits*et al*., 1994)typically manifest during reproduction. The male reproductive organ swell, discharge from the penis, and swollen lymph nodes can all be genital symptoms of dourine in male. The female exhibits sign of sexual arousal, including vulvar enlargement, vaginal discharge, hyperaemia, and vaginal mucosal ulcers. In the tertiary stage, anemia, emaciation, and neurological symptoms which include lack of coordination, ataxia, and paralysis occur, occasionally along with hindquarter atrophy.

2.1.11 Pathogenesis of *Trypanosoma brucei* Infections

There are significant resembancesin the injury and illness progression impede by different subspecies of the *T. brucei* parasite, particularly *T. b. brucei, T. b. gambiense*, and *T. b. rhodesiense* (Poltera, 1985; Losos, 1986). In natural circumstances, infection occurs when the host is inoculated subcutaneously with trypanosomes by a vector. Ormerod (1970) reported that a dose of 300 to 450 trypanosomes is required to cause infection in humans. The parasitesreproduce in the skin, leading to the development of a chancre. From the chancre, trypanosomes enter the bloodstream or lymphatic system and disseminated in the body, resulting in parasitemia and lodging in various organs and tissues. The incubation period in humans varies from one to three weeks (Olowe, 1975).

The *T. brucei* group of trypanosomes tends to localize in tissues, particularly the connective tissue stroma of various organs, leading to extensive degenerative changes and anemia (Poltera, 1985). *Trypanosoma brucei* is invasive to tissues, with blood changes being of secondary importance (Losos and Ikede, 1972). The characteristic lesions result from

extensive inflammation in the primary affected tissues, mediated by increased levels of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) (Reincke*et al*., 1988), and infiltration of lymphocytes, plasma cells, macrophages, and neutrophils (Morrison *et al*., 1981).

The role of oxidative stress induced by free radicals in the pathogenesis of trypanosome infections has been described (Akanji*et al*., 2009). Administration of methanol extracts of *Vernonia amygdalina* leaves has been shown to improve the oxidative status of *T. brucei*-infected animals, resulting in a reduction in parasitemia, anemia, and liver damage (Yusuf *et al*., 2012).

The pathogenesis of trypanosome-induced reproductive dysfunction, including reduced levels of circulating luteinizing hormone, testosterone in males, and follicle-stimulating hormone in females, has been discussed (Ikede*et al*., 1988; Raheem, 2014). These factors, along with direct testicular lesions, affect spermatogenesis, resulting in poor semen quality and infertility in males. Ovarian lesions and endometritis cause irregular estrous cycles, infertility, fetal deaths, and abortion in females, as observed in women with advanced cases of Gambian trypanosomiasis (Emeh and Nduka, 1983) and *T. brucei*-infected dogs (Obi *et al*., 2013). Anosa (1988) described factors contributing to anemia in African trypanosomiasis, including hemolysis, hemorrhages, and dyserythropoiesis, while the role of hemodilution remains uncertain. Several studies have identified biologically active substances produced by both living and dead trypanosomes, including hemolytic factors in *T. b. brucei* (Huan *et al*., 1975) and similar factors in *T. congolense, T. vivax, T. b. gambiense,* and *T. b. rhodesiense* (Morrison *et al*., 1978). Trypanosomes are known to

contain macromolecules that are toxic to the host (Tizard, 1985). Heat-labile, non-complement-dependent platelet agglutination factors produced by *T. b. rhodesiense* have also been demonstrated (Davis *et al*., 1974). The roles of various autocoids such as kinins, histamine, and catecholamines in the pathogenesis of African trypanosomiasis have been reviewed (Tizard, 1985). Trypanosome-induced immunosuppression lowers the host's resistance to other antigens, leading to intercurrent infections (Henson and Noel, 1979; WHO, 1998). Macrophages play a central role in immunosuppression during African trypanosomiasis (Tizard, 1985).

2.1.12 Pathology of *Trypanosoma brucei* Infections

Sleeping sickness cases had only a few postmortem reports (WHO, 1998). Ayub*et al*. (2011) reported a necropsy performed on a military officer who died from HAT while serving on a United Nations peace mission in Liberia. The degree of injury is correlated with the number of trypanosomes present in the tissues (Morrison *et al*.,1983, Kaggwa *et al*.,1983), and these discoveries on experimental animal models (Welldee*t al*., 1989) provide the foundation of current knowledge on the pathology of HAT. Swelling of the face and appendages, ascites, and palpable enlargement of cervical lymph nodes are frequent findings in the gross pathology of both Ghodesian and Gambian human trypanosomiasis (Jordan, 1986). According to Poltera (1985), the chancre is an erythematous cutaneous reaction at the site of a tsetse bite. Other changes include progressive myocarditis and pericardial effusion coupled with cardiac enlargement (WHO, 1998). In *T. b. gambiense* infection however, heart injurywas complicated by bacterial infection (Poltera, 1985). The involvement of the brain and spinal is linked to chronicity (WHO, 1998) and may manifest ostensibly as meningeal and cerebral oedema. According to Morrison *et al.* (1981), oedema

and the presence of a large number of trypanosomes mixed with cellular infiltrates of the plexus caused the choroid plexus to swell in *T. brucei* infection. The thalamus, hypothalamus, hippocampus, and basal ganglia are the areas of the brain that are frequently affected (Losos and Ikede, 1972). Skeletal muscles are only sometimes affected by emaciation and muscular loss in men (Poltera, 1985). Skeletal muscle atrophy had previously been documented by Losos and Ikede (1972) and Kaggwa *et al*. (1983). Van den Ingh (1976) reported pale kidneys, severe degeneration of the kidneys in most of the rabbits with chronic *T. b. brucei* infection. Although African trypanosomiasis frequently causes hepato-splenomegaly (Anosa and Kaneko, 1983; Poltera, 1985) and the liver may show atrophy in *T. brucei* infections, the spleen may be hard and dark crimson in color (Losos and Ikede, 1972). The formation of ovarian cysts (Zwart, 1989) and placental disease linked to abortion and oophoritis (Losos and Ikede, 1972; Poltera, 1985) are two examples of lesions that might disrupt reproduction. These have been verified through animal observations by Akpavie*et al*. (1987) and Ikede*et al*. (1988)

2.1.13 Biochemical Changes

The assessment of biochemical parameters, including enzymes, plasma albumin and globulin, and products in plasma and cerebrospinal fluid, can be used to evaluate the status of different organs. Enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and gamma glutamine transaminase (GGT) facilitate the transfer of the amino group from L-alanine to alpha-ketoglutarate, with the liver being the primary site of their presence. These enzymes are commonly employed in liver function tests. They are frequently used for screening liver disorders because the elevated level could indicate the presence of medical issues like liver damage, diabetes, viral hepatitis,

and cholangitis (Ghouri*et al*., 2010). The liver, heart, skeletal muscles, kidney, brain, and red blood cells all contain AST. According to Berg *et al*. (2006), liver parenchyma cells are related to both AST and ALT. AST may also be raised in disorders of other organs, such as the heart, ALT is mostly found in the liver and in small quantities in other organs, and it is more specific for liver inflammation. As indicators for liver health, the AST/ALT ratio is frequently assessed (Nyblom*et al*., 2004).

The break down of the phosphate ester that is in the extracellular space is catalyzed by a group of iso enzymes known as ALP. It is used to identify bone or liver diseases. ALP levels in the blood rise as a result of damaged liver cells. The ALP test is frequently used to find blocked bile ducts (Shipman *et al*., 2013). The blood level of ALP, which serves as a marker for vitamin D deficiency, can be affected by conditions that either slow down bone formation or boost bone cell activity.

The liver has the largest concentration of GGT among all the body's organs. GGT levels may increase if the liver or bile ducts are clogged. Because the GGT levels can be increased with many different liver disorders, including liver cancer and non-hepatic ailments, it is not very specific and cannot be used to distinguish between different sources of liver damage (Kaplan, 1985). Conditions that damage the bone tissues cause a rise in ALP levels. Even a tiny amount of alcohol consumption can raise GGT levels. The biochemical changes induced by natural human African as observed by Awobode (2006) showed that, plasma enzymes (ALT, AST and ALP) levels in trypanosome positive subjects were significantly higher than that of control, while GGT showed no significant differences.The findings point to possible *Trypanosoma brucei gambiense* infiltration of the liver, muscles, and kidney at the time of diagnosis. The modification of membrane permeability brought

on by cell injury leads to the release of enzymes into the extracellular fluid. Alteration in the level of liver enzymes serve as index for measuring the status of the body organs during infections by trypanosome species because the elevated enzyme levels may also result from the effect of trypanosome lyses as a result of the host's defense mechanism (Kennedy, 2004).

During a trypanosome infection, albumin and globulin and their ratio are frequently approximated and measured in (g/dL). Plasma protein levels changed as a result of *Trypanosoma brucei* infection in mice, according to research. Due to hyper gamma globulinemia, albumin levels decreased while globulin levels increased (Anosa, 1988). According to another study (Wassell, 2000), plasma globulin levels increase as a result of tissue degradation caused by *Trypanosoma brucei*. An indicator of the body's functioning status is the unstable level of plasma proteins that occur during infections with trypanosome species.

Urea is a waste product and a major component of human urine. Blood urea nitrogen (BUN) testing is used as an indicator of renal function (Klein *et al*., 2011). Increased urea levels are observed in *T. rhodesiense* infections and can indicate high parasitemia (Anosa (1988). Renal disorders such as nephritis and urinary tract obstruction are common features of trypanosomiasis and can lead to increased BUN levels.

Creatinine is excreted by the kidney. It is the by-product of creatine phosphate metabolism in then muscle. In addition to kidney-specific issues, the BUN-creatinine ratio can point to additional issues. Report by Awobode (2006) show an increased amount of creatinine in *T. b. gambiense* infection, which was consistent with the increased creatinine in infected

monkeys with *T. b. gambiense*and *T. b. rhodesiense* (Abenga and Anosa, 2005). The fluctuation in urea and creatinine readings observed in trypanosome infections is utilized as a metric to assess the body's functioning status.

2.1.14 Treatment for Animal Trypanosomiasis

2.1.14.1 Diminazene aceturate

Diminazene aceturate is a compound belonging to the diamidine group of compounds. It is widely used as a trypanocide in ruminant animals due to its effectiveness on *T. congolense and T. vivax,* as well as its relatively low toxicity. It is also an effective treatment for surra and is commonly used to treat *T. b. evansi* in the Philippines (Reid, 2002). The recommended therapeutic dose varies depending on the type of infection, with a dosage of 3.5 mg kg^{-1} body weight recommended for African trypanosomiasis animal (AAT) caused by *T. congolense*and *T. vivax.* A higher dosage of 7 mg $kg^{-1}$is recommended for resistant isolates and for AAT caused by *T. brucei* (Connor, 1992). The drug is administered through intramuscular or subcutaneous injection and is used solely for curative purposes, not as a preventive measure. After administration, animals primarily eliminate diminazene through urine (Kellner *et al*., 1985). Residual diminazene can persist in the tissues of animals. To ensure the safety of meat from treated animals, a withdrawal period of 21 to 35 days before slaughter is recommended. This allows sufficient time for diminazene residues to decrease below acceptable levels. Similarly, a 3-day milk discard period is advised for dairy animals. These guidelines are provided by (FAO, 990) and supported by studies conducted by Kellner *et al.* (1985), Peregrine and Mamman (1993).

2.1.14.2 Homidium salts

In Africa, homidium is employed to treat infections caused by *T. congolense and T. vivax* in ruminant animals, despite its toxicity as mentioned by Sutcliffe *et al*. (2014). However, due to its toxicity, the use of homidium is not recommended (Sutcliffe *et al*., 2014). While homidium is primarily used as chemotherapeutic drug, it also possesses some chemoprophylactic properties, although they are not as pronounced as those of isometamidium. The recommended dose for both purposes is 1 mg kg^{-1}, administered through intramuscular injection, as specified by Peregrine (1994). Homidium has a wide distribution in the body outside the blood vessels and tends to accumulate mainly in renal and hepatic cells, as noted by Murilla*et al*. (1996). This distribution pattern poses certain risks in terms of products derived from treated animals that are intended for human consumption. Homidium can be used in combination with diminazene for therapeutic purposes, but it cannot be paired with isometamidium due to the presence of a shared phenanthridine core, which leads to cross-resistance as reported by Peregrine *et al*. (1997)

2.1.14.3 Isometamidium chloride

Since its introduction in the 1960s, isometamidium has been the sole available drug against AAT.Isometamidium is primarily used in Africa as medicine against infections caused by *T. congolense*and *T. vivax* in livestock. However, its effectiveness against *T. brucei* species is less pronounced. It can also be employed against certain strains of *T. b. evansi*, except when the infection has reached the brain and spinal cord since isometamidium is unable to cross the blood-brain barrier. The recommended dosage for cattle is a single dose of 0.25-1.0 mg kg^{-1} for treatment and 0.5-1.0 mg kg^{-1} for prevention, as specified by Leach and Roberts (1981). For *T. b. evansi* infections, the typical dosage ranges from 1-2 mg kg^{-1}, but

in horses, it is advised not to exceed 0.5 mg kg^{-1} due to concerns about toxicity, according to Uilenberg (1998) and Desquesnes*et al*. (2013).

2.1.14.4 Quinapyramine sulphate

Until 1976, quinapyramine was used to treat trypanosome-infected cattle. However, its use was discontinued in many regions due to the emergence of wide coverage resistance as reported by Connor (1992). In 1984, the drug was reinstituted specifically for the treatment of *T. b. evansi* infections in camels and horses, as mentioned by Peregrine (1994). Quinapyramine is still utilized today, as noted by Ranjithkumar*et al.* (2014). It is administered through subcutaneous injection at a dose of 5 mg kg^{-1}, but it can cause severe but temporary side effects in animals, according to Auty*et al*. (2008).

2.1.15 Drug Resistance

Suspected drug resistance arises when standard drug dosages fail to achieve successful treatment. However, in the field, interpreting treatment failure solely as an indication of drug resistance can be misleading. Various factors can contribute to treatment failure. For instance, the presence of trypanosomes in treated livestock might be attributed to new infections rather than reappearance, especially in areas with high parasite occurrence, as discussed by Rowlands *et al.* (2001).

Several other factors can also lead to treatment failure. The overall health condition of the livestock, such as starvation, low immunity, coinfections, and the use of poor-quality drugs (resulting from improper storage or counterfeit products), can contribute to treatment inefficacy, as highlighted by Sutcliffe *et al*. (2014). Additional factors include improper drug constitution and weight estimation of animal as mentioned by Van den Bossche*et al*.

(2000) and Grace *et al*. (2009). Further more, under-dosage plays a critical function in the development of drug resistance in the field because sub-curative drug concentrations expose parasites to the drugs without effectively eliminating them, as explained by Leach and Roberts (1981).

2.1.16Trypanotolerance

Trypanotolerance refers to thethe capability of certain animal breeds to endure, multiply, and maintain productivity, in areas at risk of trypanosomiasis without relying on trypanocidal drugs. To promote sustainable livestock production, the utilization of trypanotolerant breeds is practiced in 19 West and Central African countries. In most of these regions resistant cattle, primarily N'Dama breed, have been introduced into high-risk areas. As a result, N'Dama herds can now be found in most West and Central African countries (D'Ieteren, 1994). The trypanotolerance trait has been well-documented in N'Dama and West African shorthorn cattle, while variations in resistance to trypanosomiasis have been observed among different types of zebu (*Bos indicus*) (Dolan *et al*., 1994; Mwangi *et al*., 1994). It is widely acknowledged that trypanotolerance is primarily governed by genetic factors. However, environmental factors such as excessive workload, concurrent illnesses, repeated blood loss, pregnancy, parturition, and nutrition can also influence the expression of trypanotolerance. Research conducted by ILCA (International Livestock Centre for Africa) and Food and Agriculture Organization (FAO), showed that these breeds maintain productivity under trypanosomiasis risk. (ILCA, 1979).

In situations with higher trypanosomiasis risk, where resistant animals are kept without the use of trypanocides, strategic interventions would improve productivity. These interventions include the targeted administration of trypanocide at certain period, tsetse fly

control measures, or selective breeding to increase resistance in animals. However, there are limited reports assessing the effects of trypanocides or tsetse fly control on the output of resistant cattle. Diali*et al*. (1992) highlighted the necessity of supporting N'Dama cattle with trypanocidal drugs under high trypanosomiasis risk in Mali. In central Côte d'Ivoire, a prophylactic trypanocide, isometamidium chloride, has been routinely administered to N'Dama calves as a single dose at weaning, although the long-term effects of this treatment have not been evaluated. ILCA conducted a study in Gabon which showed that the strategic use of preventive drugs before the three-month breeding season improved the calving percentage of lactating cows from 52% to 64% (ILCA, 1990).

2.1.17 Treatment of Human African Trypanosomiasis

Pentamidine and suramin are two of the five medications used in HAT therapy. Melarsoprol, eflornithine, and nifurtimox are used to treat second-stage illness. You can get the medications directly from WHO in Geneva or from a select group of institutions that maintain strategic stocks all across the world. HAT has increased chances of being cured if treated earlier. The disease stage and the etiology influence the treatment option. Due to their toxicity and difficult logistics, second stage medications are not used in first stage medication. In general, first stage medications will not cure a second stage. In reality, pharmaceuticals that pass the blood-brain barrier are necessary for the medication of second stage, and these treaments are frequently hazardous and challenging to use.

2.1.17.1 Pentamidine

It is used for the treatment of first-stage *gambiense* HAT, and is also an alternative for *rhodesiense* HAT, although data on its efficacy against *rhodesiense* HAT are still limited (Urech*et al*., 2011; Simarro*et al*., 2012).Pentamidine is injected deep into the muscles once

daily for seven days, however it can also be administered intravenously as a saline infusion over two hours. To prevent hypoglycemia, administration should be preceded by consuming 10–20 grams of sugar, and to prevent the effects of hypotension, lying down in horizontal position for one to two hours afterward is recommended. In general, pentamidine is well tolerated. Pain and temporary swelling are side effects of the intramuscular injection. According to Pohling*et al*. (2016), other negative effects include hypoglycemia (5–40%), hypotension, stomach pain, and digestive issues.

2.1.17.2 Suramin

Although suramin is efficacious in the early stages of both *gambiense*and *rhodesiense* HAT, it is only utilized in *rhodesiense* HAT since pentamidine administration is less complicated.Suramin is administered intravenously. Due to the possibility of severe hypersensitivity reactions, a trial dosage is given before the start of mediction. Febrility, renal toxicity, agranulocytosis, peripheral neuritiand thrombocytopenia are the negative consequences (Burri*et al*., 2014).

2.1.17.3. Nifurtimox–eflornithine combination therapy

The combination therapy of nifurtimox and eflornithine (NECT) isa drug for second stage *gambiense* HAT. NECT is thought to prevent parasite medication resistance because of its greater efficacy (95–98%), lower mortality rates (1%) and less adverse effects (Alirol*et al*., 2013; Lutje*et al*., 2013). Because nifurtimox is only licensed to treat American trypanosomiasis and not African trypanosomiasis, it can only be used off-label to cure HAT patients with the express permission and admission of responsibility of national authorities. Oral nifurtimox and intravenous eflornithine make up NECT. If vomiting happens within 30 minutes, another dosage of nifurtimox needs to be given. Abdominal discomfort,

vomiting, and headache are the most typical treatment-emergent adverse effects (Franco *et al*., 2012; Schmid *et al*., 2012). Although the frequency and severity of adverse events are lower than with nifurtimox and eflornithine monotherapies, this may be due to the shorter duration of drug exposure. Children tolerate NECT more than mature person (Buscher*et al*., 2017).

2.1.17.4 Eflornithine monotherapy

When nifurtimox is not accessible or is not recommended, eflornithine (also known as DFMO or -difluoromethylornithine) is administered as a monotherapy for *gambiense* HAT. It is a trypanostatic and cytostatic medication. There is proof that a healthy immune system is necessary for recovery (Bitonti, 1986). The most common side effects include myelosuppression (anemia, leucopenia, thrombocytopenia), myelitis, febrility, itching, high blood pressure, nausea, diarrhea, stomach discomfort and headaches(Buscher*et al*., 2017).

2.1.17.5. Melarsoprol

Melarsoprol is only approved for the second stage of *rhodesiense* HAT. The sole remaining indication for *gambiense* HAT is the management of recurrence following NECT or eflornithine monotherapy. Disorder of the brain is the common reaction which affects 5–18% of all cases that are treated and kills 10–70% of those who are affected (Seixas, 2004). *Rhodesiense* HAT has higher incidence and death rates than *gambiense* HAT. Prednisolone co-administration may have a protective effect. Pyrexia, headaches, general malaise,nausea, vomiting, diarrhea, and skin itching are common adverse reactions; exfoliative dermatitis and other serious consequences only occur in around 1% of cases, according to Pepin *et al*. (1994). Cardiac failure is a frequent and potentially fatal condition, but it may also be caused by HAT (Adams *et al*., 1986).

2.1.18 Energy Metabolism of Trypanosome

The blood stream forms (BSFs) of trypanosomes have a suppressed mitochondrion that is deficient in all of the enzymes required for the Krebs cycle and oxidative phosphorylation, therefore all of their energy requirements are met by glycolysis (Hannaert*et al*., 2003). The trypanosome's glycolytic pathway is divided into peroxisome-like organelles termed glycosomes. The seven glycolytic enzymes are located in these organelles, along with two enzymes involved in glycerol metabolism, including glycerol kinase (GK) and glycerol-3-phosphate dehydrogenase (NAD-dependent)(Guerra-Giraldez *et al*., 2002). Two ATP molecules are created from glycolysis in the blood stream forms of the trypanosome for each glucose that is catabolized, and this activity is closely correlated with the function of the trypanosome alternative oxidase located in the mitochondria(TAO). GK participates in glycolysis and produces one ATP per molecule of glucose for the BSFs during anaerobiosis or when TAO is blocked. GK thus becomes crucial for BSFs (Gualdron-Lopez *et al*., 2013; Saimoto*et al*., 2013).

ADP and glycerol 3-phosphate (G3P) are produced when GK catalyzes the phosphate group transfer from ATP to glycerol (forwards reaction) (Dipple *et al*., 2001; Rahib*et al*., 2007). GK of other organismscatalyzes forward reactions. The reverse process, or the transfer of phosphate from glycerol-3-phosphate to adenosine diphosphate to form adenosine triphosphate and glycerol, can be catalyzed by trypanosome GK (Kralova*et al*., 2000; Bringaud*et al*., 2006). In fact, when TAO is inhibited, GK of BSFs uses this reverse process to participate in anaerobic glycolysis to prevent mortality (Minagawa *et al*., 1997; Ohashi-Suzuki *et al*., 2011). It has long been known that trypanosomes may utilise both glucose and glycerol equally well in aerobic conditions. When glucose is not present,

glycerol serves as an energy source (Marshall, 1948). Therefore, it would be important for drug discovery to focus on TAO, trypanosome GK, and glucose metabolism.

2.1.19 Inhibitors of Trypanasome Alternative Oxidase

Few compounds have been identified as TAO inhibitors. These compounds all resemble ubiquinol, the TAO substrate, structurally and are believed to function as competitive inhibitors by attaching to the ubiquinol binding site. These substances are 3,4-Dihydroxybenzoic acid (Grady *et al*., 1993; Ott *et al*., 2006) and salicylhydroxamic acid (SHAM) (Opperdoes*et al*., 1976; Pollakis*et al*., 1995).

2.1.20 Plants Potential for Anti-trypanosomal Activities

In contrast to other protozoal infections like malaria, there are not as many reports on the effects of plant extracts on African trypanosomes (Hoet*et al*., 2004). Only fewer publications have been written about this topic in relation to isolated natural compounds. The majority of the works were completed using active fractions or crude extracts. While *in vitro* tests utilizing axenic cultures of trypanosomes are more commonly reported in Western countries, *in vivo* investigations on laboratory animals are more common in underdeveloped nations. This can be because underdeveloped nations lack the knowledge, tools, and facilities required to carry out *in vitro* cultivation techniques. In the same way, *in vitro* assays are more practical in industrialized nations due to challenges associated with working with experimental animals, such as expensive animal costs and stringent adherence to ethics and animal rights. *In vivo* research should ideally be facilitated by *in vitro* assays. By doing this, inactive plants can be discarded, and attention can be focused on plants that shows*in vitro*bioactivity for *in vivo* research. A few extracts that exhibit activity *in vitro* exhibit*in vivo*activity. Considering the fact that *in vitro* experiments can be

performed without the host animal. This avoids any potential issues that could arise from the host's metabolic and absorption processes. The capacity of a test medicine to diffuse into an isolate*in vitro*, however, may differ from the pattern *in vivo* for a variety of reasons. Unpredictable metabolic and pharmacokinetic changes are brought on by the host-parasite relationship.

Numerous plants have been studied for their potential anti-trypanosomal properties. Among them are the leaves of *Morinda lucida* (*Rubiaceae*) (Asuzu and Chineme, 1990). Locally known as "nfia," *Morinda lucida* is an extremely bitter herb (Anaga, 2003). The indigenous use it to treat malaria and to induce emesis, diuresis, and purgation. In a dose-dependent manner, the extract of *M. lucida* caused purgation in mice and dramatically reduced the amount of parasite in mice infected with *T. b. brucei* (Asuzu and Chineme, 1990). Northern Nigeria traditionally treats sleeping sickness using *Annona senegalensis Pers*. (*Annonaceae*) (Igwe and Onabanjo, 1989). Additionally, it is utilized in folk medicine to cure cancer (Gbile and Adesina, 1986). At a dose of 200 mg/kg, the aqueous extract of the leaves of *A. senegalensis* treated experimentally infected mice with *T. b. brucei* (Ogbadoyi*et al*., 2007).

Landolphiauniflora is a plant traditionally used in the treatment of fever by the Fulani herdsmen of Northern Nigeria. It is also used specifically in the treatment of trypanosomiasis (Atawodi*et al*., 2002). The stem bark aqueous extract was used traditionally. Experimentally, extracts of different parts of the plant were tested using mice infected with *T. brucei brucei*. Extracts from the leaves and stem barks suppressed the multiplication of the trypanosomes while the methanolic extract of the roots was able to

clear parasitaemia at 200 and 300 mg/kg body weights when given intramuscularly for five consecutive days. There was no relapse observed for up to ten days' post treatment.

In addition, it was discovered that the ethanolic extracts of *Alchorniacordifolia's* leaves and fruits prevented the growth of *T. b. brucei* both *in vitro* and *in vivo* in rats (Agbe and Oguntimein, 1987). The result was similar to that of suramin. On the other hand, it was shown that the active fractions were hazardous to rats after further separation.

Traditional healers in Uganda use the plant *Entada abyssinica* (*Leguminosae*) to treat sleeping sickness. A diastereo isomer of the clerodane type diterpene kolavenol was isolated using bioassay-guided fractionation of the dichloromethane extract of the root bark (Freiburghaus*et al*., 1998). It demonstrated trypanocidaleffect on *T. b. rhodesiense* (IC_{50} 2.5 g/mL)

In Southern and East Africa, *Holarrhenaafricana* preparations are used as a febrifuge and tonic (Nwodo*et al*., 2007). In Eastern Nigeria, *H. africana*is a component of steam baths used to cure trypanosomiasis, malaria, and other feverish diseases. Oliver-Bever (1986) conducted earlier experiments that shown the plant might lessen intestinal and hepatic amoebiasis in rats and hamsters. Fresh leaf aqueous extract shown strong anti-*T. bruceispp.* action. *In vitro*effect on *T. b. rhodesiense* was demonstrated by the active fraction following fractionation, with an IC_{50} of 0.785 g/mg without cytotoxicity on L-6 cells (Nwodo*et al*., 2007). *In vivo* testing of the antitrypanosomal activity was also done on mice that had been inoculated with *T. b. brucei*. The active portion was administered to the infected mice for five days in a row at doses of 40 and 80 mg/kg. The active fraction

eliminated parasites from the blood at both doses. Infection returned after three days'treatment was discontinued.

In rats infected with *T. brucei*, the aqueous extract of the stem bark of *Khaya senegalensis* was similarly found to significantly lower parasitaemia (Ibrahim *et al*., 2008). The potential of *Brassica* plants, such as cabbage, cauliflower, and *Brussels sprouts*, as dietary sources of anti-cancer chemicals is gaining attention (Wattenberg, 1983). Drugs used to treat cancer are tested for potential trypanocidal action. This may be because protozoan parasites like those that cause trypanosomiasis, leishmaniasis, and malaria share a lot of characteristics with malignant cells that proliferate and some types of cardiac disease (Hide, 1989). In fact, it is known that all four medications used to treat sleeping sickness in clinical settings have some anti-cancer properties (Barrett and Barrett, 2000). Even the anti-cancer medication di-fluoromethylornithine (DMFO) was initially created. The activity of *Brassica oleracea L*. aqueous extract was investigated *in vitro*on *T. b. brucei* (Lafia strain) (Igweh*et al*., 2002). Within 3 hours of incubation, the extract immobilized the trypanosomes and rendered them incapable of infecting mice.

2.2 Mistletoe

Mistletoe is a parasitic flowering plant that survive on other plants and is a member of the sandalwood order (*Santalales*). Although *Santalales* contains all mistletoe, this behavior evolved five times independently, making them not monophyletic. *Loranthaceae* is the mistletoe family with the most genera. Three genera cannot be referred to as mistletoes since they exclusively cling to roots. Finally, genera that adhere to both stems and roots, like *Tripodanthus*, go beyond the definition and reveal nature's aversion to human

classification. The concepts of which members of the S*antalales* are mistletoes and how they are related to one another have been substantially elucidated by recent molecular phylogenetic study (Mathiasen and Daugherty, 2008).

2.2.1. Biology of Mistletoe

All mistletoes are thought to have a similar host infection, however certain groups have had more in-depth descriptions of it than others (Kolb, 2002). Seeds develop a holdfast that clings securely to the host branch after germination. After germination, the mistletoe seeds of the family *Viscaceae*can produce simple sugars as an energy source because they have a chlorophyllous endosperm and embryo (Lamont, 1983). On the holdfast's bottom surface, a penetration peg forms that mechanically pierces the epidermis or bark before making contact with the phloem and xylem of the host. Since there hasn't been any evidence of host tissue breaking down chemically, host tissue penetration is only possible mechanically. The mistletoe forms its haustorium and then aerial branches after penetrating the host tissue.

2.2.2. Pollination

In addition to wind, birds and insects also pollinate mistletoe. The bright flowers of many mistletoe plants in the *Loranthaceae* family generate sugar-rich nectar that draws avian pollinators. In several of these *loranth*species, complex pollination and seed dissemination methods involving birds have evolved (Montgomery *et al*., 2003; Kelly *et al*., 2004). In order to access the fused corollas, for instance, birds must pry them apart. When they do, pollen is forcefully releasedonto the head of the bird (Ladley*et al*., 1997). Because mistletoe is dichogamous (protandrous), pollination occurs when birds visit flowers in both the male and female phases after they have visited blooms in the male phase. Mistletoes and their bird pollinators have co-evolved, and this relationship is so intricately entwined

that breaking up this association could have long-term harmful effects for both interacting organisms and possibly the entire ecosystem (Watson, 2001). No bird may be regarded as a mistletoe pollen specialist because a wide variety of species provide services for various mistletoes that are pollinated by birds (Watson, 2001). Hummingbirds are important pollinators of mistletoes with big, spectacular red or yellow flowers in Mexico, Central America, and South America (Tadey and Aizen, 2001; Azperitia and Lara, 2006). The primary pollinators of mistletoe in the families *Viscaceae* and *Loranthaceae* are a variety of insects, while bats are the most likely pollinators among mammals.

2.2.3 Toxicological Studies on Mistletoe Leaf Extracts

Mistletoe plants contain certain constituents, such as lectins, which can be toxic to specific organisms. The level of toxicity in mistletoe varies among different species. African mistletoe fruits have been described as potentially harmful berries (Adodo, 2004) that can cause vomiting, hypotension, brain dysfunction, and even death from cardiac attack if consumed in substantial quantities (Dibong*et al*., 2009). However, the fruits of *D. Falcata* were reported to have a pleasant flavor and were consumed as food (Mallavadhani*et al*., 2006).

Studies on mistletoe leaf extracts have not shown toxicity in laboratory animals. Okpuzor*et al*. (2009) examined the toxicity of G*lobimetulabraunii* leaf extracts in rats and determined potential liver and haematological effects. The rats were fed various plant extracts at a dose of 200 mg/kg body weight for 14 days. The were rats given the $CHCl_3$, EtOAc, and Water fractions.There were significant increases in packed cell volume (PCV) and hemoglobin (Hb). The red blood cell (RBC) and white blood cell (WBC) counts also increased after

administration of the $CHCl_3$ and EtOAc fractions, except for the BuOH fraction. The liver enzymes were also assessed, and the activity of AST and ALT was significantly reduced in rats treated with the $CHCl_3$ fraction, while ALT activity was elevated in the BuOH group. The administration of other fractions resulted in increased enzyme activities, except for the MeOH fraction. The EtOAc and hexane fractions led to increases in both total and direct bilirubin, while the crude fraction only increased direct bilirubin. The $CHCl_3$ fraction of *G. braunii* was found to affect the haematological indices and hepatic enzyme concentrations in rats.

Another study investigated the hepatoprotective effect of crude MeOH extracts of *Tapinanthusbangwensis* and its different fractions against CCl_4-induced hepatotoxicity in Wistar albino rats (Patrick-Iwuanyanwu*et al*., 2010). Rats treated with CCl_4 alone showed the highest levels of activity for the marker enzymes ALT, AST, and ALP, as well as bilirubin. However, the activity of these enzymes and bilirubin significantly decreased after receiving the extract (400 mg/kg bw) for seven days. The concentration of total protein increased significantly. The extracts also reduced the concentration of thiobarbituric acid reactive substances (TBARS), indicating a decrease in lipid peroxidation. Histopathological analysis revealed that rats given the extracts had hepatocytes with normal architecture, while rats given CCl_4 alone showed liver necrosis. Moreover, even at a dosage of 2000 mg/kg bw, the crude MeOH extract did not cause any mortality. The research demonstrated that mistletoe exhibits strong antioxidant effects and has the ability to protect the liver from CCl_4-induced damage in rats.

2.2.4 Ethnopharmacology of Mistletoe

The ethnomedical applications of mistletoe had, for a very long time, been in the hands of a very small number of herbalists who asserted a general usage to counter sorcery and magical powers, to treat mental disorders, infertility, and health issues related with the urino-genital system, rheumatism, and pain. According to Adodo (2004) and Jadhav *et al*. (2010), mistletoe of the *Loranthaceae* and *Viscaceae*is used in many cultures as a diuretic or to cure a variety of illnesses, such as diabetes, cancer, and hypertension. The *Loranthaceae spp*. used to make tea is thought to treat body pain and bone fractures (Ken'ichi*et al*., 2006). According to reports, South-Western Ethiopians treat tumorby chopping fresh *Tapinanthusglobiferus* leaves and combining them with cold water for oral administration (Yineger and Yewhalaw, 2007). Additionally, to treat syphilis in the Ebolowa region of Cameroon, one handful of fresh *Tapinanthusglobiferus* leaves is typically combined with one handful of *Boswella odorata* root bark, and the mixture is macerated in five liters of local beer (Noumi and Eloumou, 2011). Fresh stems of the plant *Tapinanthusglobiferus* are administered orally to various kinds of livestock in Saudi Arabia to treat fever and remove the placenta following delivery (Sher and Alyemeni, 2011). According to Urech*et al*. (2005), a variety of *Viscum album* preparations are available in Europe under the brands Helixor, Iscador, and Abnobaviscum and are used to treat epilepsy, infertility, hypertension, and arthritis. More than twenty different health issues have been successfully treated with mistletoe, which is now referred to as the "cure all" (Adodo, 2004).

For the less well-known African mistletoes in particular, the connections between *Loranthaceae*and their hosts have been and will continue to be a topic of study. The sucker

allows water and nutrients to be diverted from the plant to the parasite while ensuring the continuity of the key elements, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} (Bannister *et al*., 2002). According to research by Mallavadhani*et al*. (2006), *Dendrophthoe falcata's* therapeutic characteristics may have varying effects according to the different hosts it interacts with. According to Wang *et al*. (2008), *Taxillus spp*. *Loranthaceae* showed strong inhibition of fatty acid synthase, and the differences in host plants had little effect on the parasite's ability to block this enzyme.

It was the goal of a recent ethnopharmacological study (Dibong*et al.,* 2009) on hemi-parasites growing in Cameroon to learn more about the biology, ecology, and therapeutic applications of the *Loranthaceae* family. Approximately 22 different disorders, including hypertension, hypotension, irregular menstruation, menopause, convulsions, diabetes, rheumatism-related pains, rheumatism-related pain, epilepsy, kidney and chronic muscular pains, were advised for therapy by 82% of the healers, according to Dibong*et al*. (2009). The dosage is typically not standardized, and fresh leaves are typically utilized in an infusion or macerated form.

According to Okpuzu*et al*. (2009), the ethyl acetate fraction of *Globimetulabraunii* leaf exhibited antioxidant properties. It significantly increased the levels of superoxide dismutase, catalase, glutathione peroxidase, and malonyldialdehyde (MDA), while decreasing the triacylglycerol level compared to the control group.Meng *et al*. (2002), Cebović*et al*. (2008), Kienle and Kiene (2010), and Edem (2009) reported that *Viscum album* leaf displayed anti-cancer activity and a hypoglycemic effect. Le and Zam (2008) found that *Globimetulabraunii* leaf extract had oxytocic properties similar to oxytocin, a

standard uterine stimulant, while showing antagonistic effects on uterine smooth muscle compared to atropine and salbutamol. Talha *et al.* (2011) demonstrated that *G. cupulata* leaf extract had anti-hypertensive and hypoglycemic properties. Wang *et al*. (2008) found that *Taxillus chinensis* leaf exhibited high inhibitory activity against fatty acid synthase.

Akinmoladun*et al*. (2010) investigated the phytochemical composition and antioxidant properties of *Globimetulacupulatum* leaf extracts, which exhibited strong antioxidant properties and potent inhibition of lipid peroxidation, indicating its potential in preventing oxidative damage. Ojewole and Adewole (2007) evaluated the hypoglycemic properties of *Globimetulacupulata* leaf and found that it was comparable to reference drugs for diabetes mellitus in reducing blood glucose levels, suggesting its potential as a natural treatment for diabetes mellitus. Patrick-Iwuanyanwu*et al*. (2010) reported the anti-inflammatory properties of *Tapinanthusbagwensis* leaf extract, while Ekhaise*et al.* (2011) demonstrated the anti-diabetic properties of the leaf extract of *Tapinanthusbagwensis.*

Okpako and Ajaiyeoba (2004) found that the methanol leaf extracts of *Tapinanthussessifolius* had antiplasmodialeffect *in vitro* but demonstrated intrinsic antimalarial properties *in vivo*. Deeni and Sadiq (2002) concluded that *Tapinanthusdodoneifolius* leaf extract exhibited antimicrobial effect against drug-resistant bacteria and fungi isolated from farm animals. *Tapinanthusglobiferus* leaf extract showed inhibitory effects on the growth of several bacteria but had no effect on *Salmonella typhi*, *Aspergillus niger*, and *Candida albicans*. The extract showed both bacteriostatic and bactericidal activity, comparable to standard drugs according to Ndukwe*et al*. (2001). The findings of Erukainure*et al*. (2010) demostratedthat *G. braunii* could lower lipid levels and

reduce cholesterol, which may havepotential benefits for cardiovascular health in both normal and hypercholesterolemic conditions.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1Chemicals and Reagents

Methanol,phosphate buffer saline glucose (PBSG),diminazeneaceuturate,0.1% ferric chloride, 1% HCl, Mayer's reagent, Dragendorffs reagent, glacial acetic acid, ferric chloride, concentrated sulphuric acid, magnesium ribbon, concentrated hydrochloric acid, chloroform, acetic anhydride, diethyl ether, sodium hydroxide, Molisch's reagent, Barfoed's reagent, Fehling's solution I and II, Aluminum TLC plate coated with silica gel, gallic acid, Folin-Ciocalteu reagent, deionized water, sodium carbonate, rutin, sodium dioxonitrate (iii), diosgenin, n-hexane, ethyl acetate, n-butanoldeuterated solvent, 10% DMSO, EDTA, Turk's diluting fluid, pyruvate, 2,4-dinitrophenyl hydrazine, P-nitrophenyl phosphate, magnesium chloride, di-ethanol amine, 5% sodium tungstate, picric acid, Diacetyl monoxime, Tris-HCl buffer, ubiquinol oxidase, 5 mM $MgSO_4$, 0.5 mM $NADP^+$, 50 mM glucose, 2 mM ADP, 10

mM glycerol-3-phosphate, one unit of hexokinase and glucose-6-phosphate dehydrogenase.

3.1.2Equipmement

Light microscope (MCS-BIO7T), microtitre plates, spectrophotometer, rotary evaporator(REV-3000 series)**,** NMR instruments**(**Bruker AV-600), microhaematocrit reader (SKU:1050-00), evaporating dish, microhaematocrit centrifuge (DSC-100MH-3), micro pipette, weighing balance (BA-E series),P^H meter (PH-P11OB), water bath, refrigerator, centrifuging machine (CFG-16D) and waterdistiller.

3.2 Methods

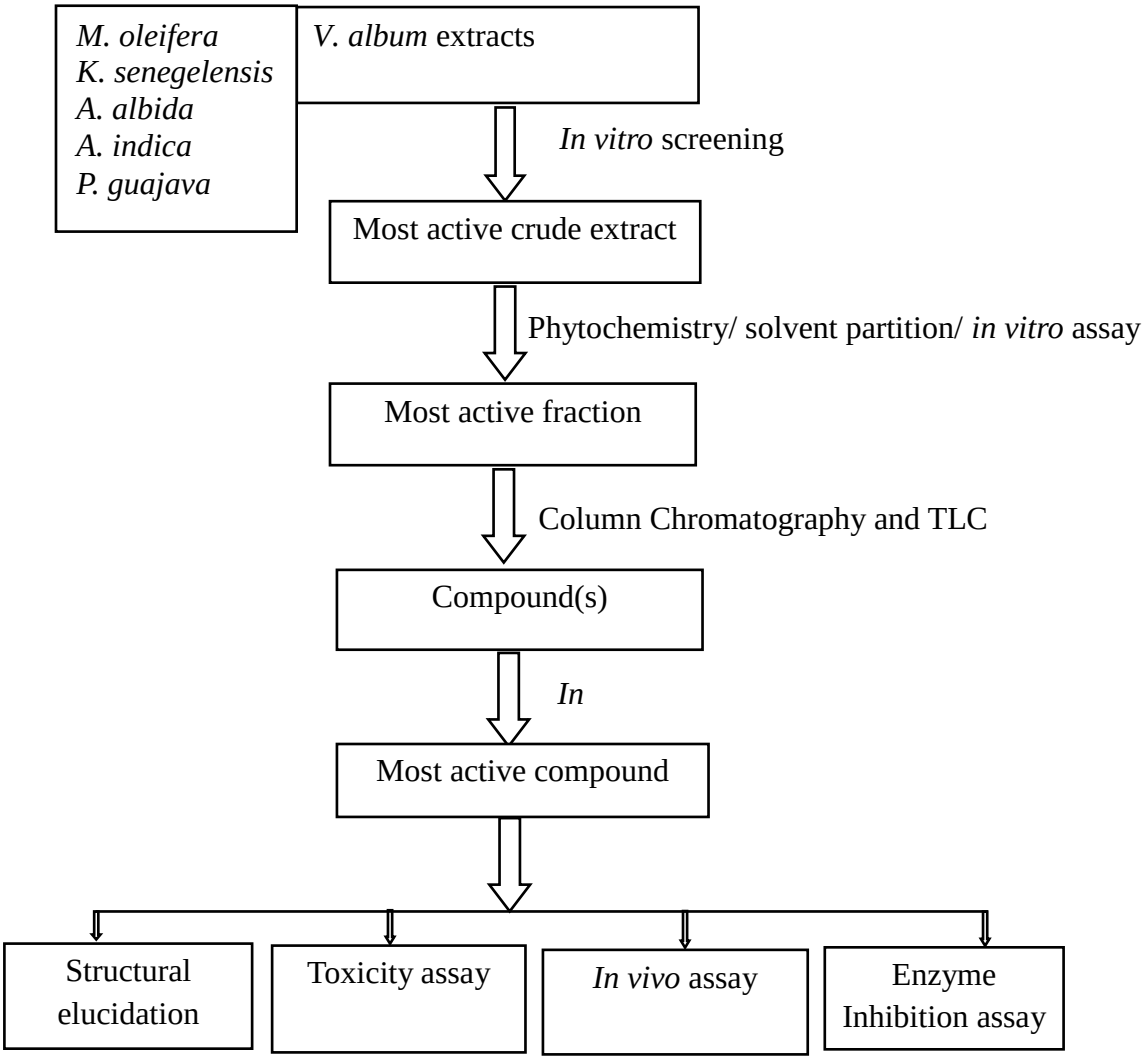


Figure 3.1 Research Design

3.2.1 PlantsCollection, Identification and Processing

In December 2018, leaves and stems of *Viscum album* were collected from the host plants: *A. indica* (neem), *P. guajava* (guava), *A. albida, K. senegalensis*, and *M. oleifera*. The collection site was Federal Government College Maiduguri in Jere Local Government Area, Borno State. The identification of *Viscum album* and the host plants was done by Namadi Sanusi at the Department of Botany, Ahmadu Bello University Zaria, Nigeria. Voucher specimens were preserved in the Herbarium of the Department. The parts collected were washed with water and dried in the shade for one week. After drying, the leaves and stems were ground using a mortar and pestle to obtain a fine powder for each sample.

Table 3.1:Identification of *Viscum album* and its hosts

Plants	Family	Voucher number
Viscum album	Loranthaceae	6986
(Neem) *Azadirachta indica*	Meliaceae	0418
Acacia albida	Fabaceae	01380
(guava) *Psidium guajava*	Myrfaceae	03253
Khaya senegelensis	Meliaceae	090081
Moringa oleifera	Moringaceae	0517





V. album on *A. indica**V. album* on *M. oleifera*

V. album on *A. albida**V. album* on *P. guajava*

V. album on *K.senegelensis*

Plate I:*Viscum album*on different hosts



Plate II: Pulverized Samples of *V. album* Leaves and Stems from *Azadirachta indica, Psidium guajava, Acacia albida, Khaya senegalensis* and *Moringa oleifera*.

3.2.2 Extraction of the Plant Materials

The extraction procedure was conducted at the Department of Veterinary Pharmacology and Toxicology Laboratory, Ahmadu Bello University, Zaria. Each crushed plant sample weighing 100 g was used for the activity screening. The samples were immersed in methanol at a ratio of 1:3 and shaken occasionally for 72 hours. During the filtration process, a filter paper was employed to separate the extracts from the plant residue (marc). To ensure maximum extraction, fresh methanol was added to the marc at a ratio of 1:2 and allow to stay for 24 hours. The filtrates were collected to obtain a dry crude extract, which was achieved by allowing them to evaporate in the open air. For the most active plant (*Viscum album* leaves from *A.albida*), exactly 3 kg of the plant material was macerated to obtain the crude extract. The yield of the extracts was determined using the formula bellow

$$\text{Yield} = \frac{\text{weight of extract}}{\text{weight of sample}} \times 100$$



PlateIII: Samples During Extraction

3.2.3Parasite collection and Determination of Parasitaemia Load

The Nigerian Institute for Trypanosomiasis and Onchocerciasis Research (NITOR), in Vom, Plateau State, provided the *T. b. brucei* (Federe strain) specimen. The organism was kept alive by constant passage in donor albino mice kept in the Department of Veterinary Pharmacology's animal facility at Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The intraperitoneal inoculation contained1×10^6 parasites in 0.1 mL of blood/normal saline. Blood collected from the tail was used each day to check the parasitaemia level. Using a light microscope (Olympus, Japan) equipped with a wet mount technique, the presence of *T. b. brucei* in the blood of an albino mouse that had been infected with the parasite was determined after the tail had been sterilized with methylated spirit **(**Murray *et al.*1983), parasitaemia was determined using the rapid matching technique of Herbert and Lumsden (1976).

3.2.4 *In vitro* Screening forAnti-TrypanosomalActivity

To assess the *in vitro*trypanocidal activity, 10 mg of each sample was dissolved in 1 mL of distilled water. Serial dilutions were made from this stock solution to concentrations ranging from 0.63 to 10 mg/mL. The evaluation was conducted in triplicates using 96-well microtitre plates. 20 µL of blood containing approximately 50-70 *T. b. brucei*per field, obtained from donor mice, was mixed with 5 µL of extract solution at final concentrations of 0.8, 0.4, 0.2, 0.1, and 0.05 mg/mL.A control group was included, which contained parasites suspended in phosphate buffer saline glucose only. As a reference test, the same concentration of diminazeneaceuturate, a commercial trypanocidal drug, was used. After a 10-minute incubation at 37°C in the microtitre plates, 2 µL of the test mixtures was dropped on a microscopic slide, covered with a cover slip, and the parasites were observed

every 10 minutes for a total duration of 180 minutes. A reduction or cessation of parasite motility in the blood treated with the extracts, compared to the control blood without extracts, was considered as an indication of trypanocidal activity. This process was conducted based on the study by Atawodi*et al*. (2003). For each extract, the concentration which inhibit parasite population by 50% ($1C_{50}$) was determined (Scory and Steverding, 1997).

3.2.5 Qualitative Phytochemical Evaluation of the Methanol Extract of *Viscum album* Leaves of*A. albida*

Standard procedures were used to determine qualitative phytochemical constituents of the most active extract of *Viscum album* leaves of*Acacia albida*. Tannins and saponins (Ejikeme*et al*., 2014); glycosides (Parekh and Chanda, 2007); flavonoids,alkaloids anthraquinones, and terpenes (Kumar*et al*., 2007);phlabotannins, steroids, coumarins, phenolics,general test for carbohydrate,generaltest for monosaccharide andtest for reducing sugar (Evans, 2000).

3.2.6 Quantitative Phytochemical Analysis

Quantitative phytochemical constituents of the crude methanol extract of *Viscum album* leaves of*A. albida*for total saponins, taninns, alkaloid and flavonoids were carried out usingspectrophotometric method.Total flavoniod content(Zhishen*et al*., 1999), total alkaloid content (Shamsa*et al*., 2008), tannin content (AfifyAel*et al*., 2012) andtotal saponin content (Rajeev *et al*., 2012).

3.2.7 Thin-Layer Chromatography of Methanol Extract *V. Album*Leaves of *A. Albida*

To determine the phytochemical components, thin layer chromatography (TLC) of the most potent plant extract was carried out. A 10 cm by 5 cm aluminum TLC plate coated with silica gel was trimmed to size, and the origin was marked by making a pencil mark 1.5 cm from the lower edge of the end of the plate. The dissolved extracts were spotted onto the TLC plate in a single spot, with a capillary tube on the line separating each spot by about 0.5 to 1 cm. The distance prevented the spots from overlapping as they separated.

Various solvent systems with different n-hexane: ethyl acetate ratios (8:2, 6:4, 4:6, 5:5, 2:8, and 1:9) were prepared for the purpose of selecting the most suitable solvent system that would provide optimal resolution. The extracts were applied to TLC plates using capillary tubes, and these plates were subsequently developed in a TLC chamber using the appropriate mobile phase. After the development process, the TLC plates were taken out, allowed to air dry, and examined under ultraviolet light (UV) at 254 nm to visualize the formation of distinct bands. Some of the plates were also sprayed with various spraying reagents, while others were subjected to a hot air oven for two minutes. The movement of the analyte on the TLC plates was quantified as the retention factor (Rf). Rf values were calculated for different samples to determine the relative migration distances of the compounds.

$$\text{Rf} = \frac{\text{distance moved by solute}}{\text{distance moved by solvent}}$$

3.2.8 Partitioning of Extracts into Fractions

According to Ebada*et al.* (2008), 180 g of the methanol extract of *Viscum album* leaves from *A. albida* that showed the best activity was partitioned into various fractions using n-

hexane, ethyl acetate, and n-butanol.This was performed initially with n-hexane: water (1:2). About 10-20 mL of water was placed into a separating funnel containing 30 g of the sample to form a crude suspension. 500 mL of n-hexane was then poured into the separating funnel after which the mixture was agitated three to four times at intervals of two to three minutes. The funnel was left standing for one hour. The mixture was seperated into layers, and the top layer (n-hexane) was carefully removed and placed in a beaker. To make sure that all n-hexane soluble molecules were removed, this was done several times. Rotary evaporator was used to concentrate the fractions, before being stored at -20°C.

500 mL of ethyl acetate was added to the separating funnel containing the residual crude suspension, then the mixture was agitated 3-4 times for 2-3 minutes and let to stand undisturbed for 1 hour. To get rid of all ethyl acetate-soluble compounds, the lower layer was collected, and the procedure was repeated. The fraction was concentrated using a rotary evaporator before being stored at -20°C.

500 mL of n-butanol was added to the separating funnel containing the residual crude suspension, the mixture was shaken 3-4 times for 2-3 minutes and left to stand undisturbed for 1 hour. The bottom layer was removed. To get rid of all n-butanol soluble molecules, this procedure was repeated. The fraction was concentrated on a water bath before being stored at -20°C. Percentage yield of fractions in various solvents were calculated**.**

3.2.9 Column Chromatography

Glass column chromatography with silica gel (70-230 mesh) was used to separate the most active portion (n-butanol fraction). Silica gel (500 g) was mixed with n-hexane to make a homogeneous slurry. A glass column was then filled with the silica gel slurry. The sample

was prepared by mixing 30 g of silica with 20 g of butanol extract in 100 mL of a 9:1 mixture of n-hexane and ethyl acetate, then stirring the mixture and was allowed to air dry for a while. The column was covered with a coating of the dried silica extract mixture. The column was initially eluted using n-hexane and ethyl acetate, whose polarity was increased by 10% increments. The polarity was further elevated by adding 10% increments of methanol after 100% ethyl acetate. The fractions for each eluent system were collected in vials. The fractions were concentrated under a fan until dry (Harvey, 2000).

Thin layer chromatography (TLC) of the fractions obtained from the column were carried out on TLC plate covered with silica gel. Each sample was applied with capillary tube and was allowed dry. The components in the spot movedupward by capillary action after about five minutes when the plate was submerged in different solvents with varied n-hexane: ethyl acetate ratio (8:2, 6:4, 5:5, 2:8, and 1:9). After that, the plate was taken out of the solvent and allowed to dry. The position of different components was examined under UV-light followed by using spray reagent for the presence of specific class of compound. Similar fractions were pooled (Mbaveng*et al*., 2008).

3.2.10Bioguided-Assay

The bioassay-guided isolation method combines the outcomes of biological testing with the processes of separating substances in a mixture utilizing a variety of analytical techniques. Methanol was used to extract plant material, and the crude methanol extract was then tested for *in vitro*antitrypanosomal activity followed by phytochemical analyses. N-Hexane, ethylacetate, and n-butanol were used to fractionate the most active crude extract in order of increasing polarity. Anti-trypanosomal activity of the fractions were evaluated. Column chromatography was used to further fractionate the fraction that showed the best activity.

Thin layer chromatography was employed to monitor the purity and number of compounds, and the inactive fractions were either set aside or discarded. Biological tests and fractionation were performed until pure compound wasobained.

3.2.11 Structural Determination of the Bioactive Compound

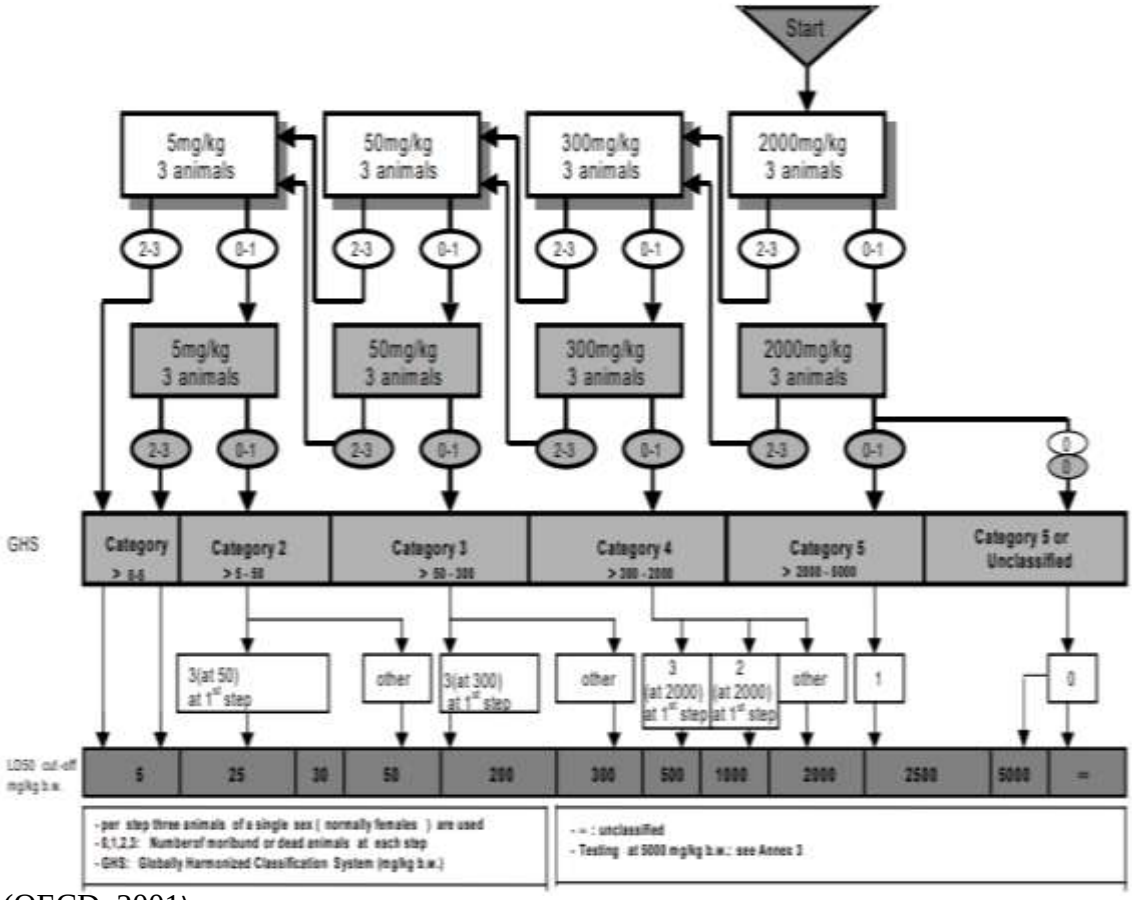
Nuclear magnetic resonance (NMR) spectroscopy was used to obtain the structure of the bioactive molecule. Using chloroform ($CDCl_{3)}$as solvent, NMR spectra were obtained on a spectrophotometer (Bruker AV-600), and chemical shifts are reported in ppm in relation to TMS. MestReNova software was used to process the NMR spectra.One dimensional (1D) and two dimensional (2D) proton (^{1}H) and carbon (^{13}C) NMR such as COSY, HMBC and HSQC were used. A glass wool plug-equipped pasteur pipette was used to filter the pure substance, which was diluted in 1.0 mL of a deuterated chloroform and then discharged into a 23 5 mm NMR tube (Aldrich Z412848) that was clearly marked with a concentric label. Bruker AV-600 instruments were used to capture the 1D and 2D spectra. The equipment is located in the University of Strathclyde's Chemistry Department in Glasgow, UK. To determine the structures of the compound, the spectra were analyzed and compared with data published in the literature (McMurry,2000).

3.2.12 Acute Toxicity Testing

Toxicity test was carried out following the guidelines outlined by OECD (2008) using a fixed-dose approach. As per OECD recommendations, testing in one gender (usually females) is considered adequate (OECD, 2001). This method provides valuable data on the potential harmful characteristics of the substance and enables its classification and ranking based on the Globally Harmonized System (GHS) for acute toxicity grouping of chemicals. The animals employed in this experiment were female albino mice that were 8 to 10 weeks

old, nulliparous, and not pregnant, with minimal body weights of 20 to 30 grams. The mice were acclimatized for seven days in plastic cages with adequate ventilation. Overnight fasting was followed by body weight measurements on the mice. According to OECD recommendations, a limit test dose of 2000 mg/kg b.w was used (Reinhart *et al*., 2008). 10% DMSO in phosphate buffer saline was used to prepare the drug for delivery. At 48-hour intervals, three female mice were dosed for each stage. According to the recommendation (OECD, 2001), mice in the control group were given 10% DMSO in phosphate buffer saline without the substance to make a comparative negative control group.

Acute toxicity symptoms and behavioral abnormalities in each mouse was assessed 1 hour after administration and at least once per day for 14 days. Changes in behavior, dyspnea, weakness, apetite, arching back, sleep, coma, and mortality were observed. the weights of the animals as measured just prior to the testing substance's administration and at the conclusion of each week for two (2) weeks. All the dosed animals were still alive, and the therapy was continued until the three mice limit dose of 5000 mg/kg b.w was reached. According to the Globally Harmonized System (GHS) for the classification of compounds that induce acute toxicity (OECD, 2001), the substance was graded and categorized.



(OECD, 2001)

Figure 3.2: Procedure for Acute Toxicity Test using Starting Dose of 2000 mg/kgb.w

3.2.13*In vivo* Assay

To perform this assay, thirty-five adult albino mice with male and female weight ranging from 20 to 30 g for each group of 5 mice were used.

Group I: uninfected and untreated

Group II: uninfected and treated with the compound at 1000 mg/kg b.w

Group III: infected with *T. brucei brucei* and untreated

Group IV: infected with *T. brucei brucei*and treated with diminazene at 3.5 mg/kg b.w

Group Va: infected with *T. brucei brucei*andtreated with the compound 500 mg/kg b.w

Group Vb: infected with *T. brucei brucei*andtreated with the compound 1000 mg/kg b.w

Group Vc: infected with *T. brucei brucei*andtreated with the compound 2000 mg/kg b.w

3.2.14Haematological Studies

At the conclusion of the experiment, a blood sample of precisely 1 mL was collected from humanely sacrificed albino mice and placed in a serum bottle containing an anticoagulant. The blood sample was then analyzed using the Mindray Bc-5000 Automatic Hematological Analyzer to determine various haematological parameters including packed cell volume (PCV), red blood cell count (RBC), hemoglobin concentration (Hb), and white blood cell count (WBC).

3.2.15 Biochemical Studies

Exactly 1 mL blood sample was introduced into serum bottles without anticoagulant from humanely sacrificed albino mice at the end of the experiment and allowed to separate without centrifugation at 4^{o}C and the sera was used for the biochemical analysis stated below:

3.2.15.1 Aspartate amino transferase (AST IU/L)

According to Reitman and Frankel (1957), serum aspartate amino transferase activity was measured using a colorimetric approach and commercial kits. It is known that aspartate amino transferase affects the amino group transfer that transforms alpha-ketoglutarate into glutamate and oxaloacetate, respectively. With the help of 2, 4-dinitrophenylhydrazine, the oxaloacetate that has thus been formed is combined to form a colorful complex, the absorbance is measured at 505 nm. Two test tubes were used for each serum or control sample (one for the "test" and one for a sample blank), one for the reagent, one for the blank, and one for the standard, in order to measure the activity of aspartate amino transferase. Each tube was filled with 0.5 mL buffered substrate (29.2 mg of alpha-ketoglutaric acid and 2.66 mg of aspartic acid) in 100 mL of phosphate buffer using a pipette, followed by 20 mL of NaOH for 5 minutes in a water bath at 37 °C. The "test" tubes were filled with 100 microliters of test or control serum samples, while the "standard" tubes were filled with 100 microliters of pyruvate standard (4 mmol/L), 100 microliters of water, and 100 microliters of reagent blank. The "test" tubes were mixed and kept at 37^{o}C. Each tube received 0.5 mL of (2, 4-dinitrophenylhydrazine) at 1 mmol/L, was mixed, and then taken out of the water bath after exactly 60 minutes. The sample blank tubes were then filled with 100 L of test or control serum and was kept at room temperature for 20 minutes. Then, 5 mL of NaOH solution (400 mmol/L) was added, and everything was properly mixed. The absorbance was measured at 505 nm after the tubes were kept at 37^{o}Cfor 5 minutes. For the reagent blank, the spectrometer was calibrated to zero, and the amount of pyruvate produced was determined as follows:

Amount of pyruvate formed (µmol/minutes/litre)

$$\frac{(\text{A test} - \text{A sample blank} - \text{A reagent blank})}{\text{A standard} - \text{A reagent blank}} \times \frac{4 \times 1 \times 1000}{60}$$

$$\frac{(\text{A test} - \text{A sample blank})}{\text{A standard}} \times 66.7$$

where A_{test} = Absorbance of the test

$A_{sample\ blank}$ = Absorbance of the sample blank

$A_{standard}$= Absorbance of the standard

3.2.15.2 Alanine amino transferase (ALT IU/L)

Using commercial kits (Randox Laboratories Limited, U.K.) and the colorimetric technique described by Reitman and Frankel (1957), the level of serum alanine amino transferase was determined. Alpha keto acid and L-alanine were hydrolyzed by an enzyme called alanine amino transferase to produce L-glutamate and pyruvate. The enzyme's activity was determined by the amount of pyruvate hydrazone that was produced when 2,4-dinitrophenyl-hydrazine was added. The enzyme activity was tested against the reagent blank and the sample blank, respectively, in two (2) test tubes. Two test tubes were marked "reagent blank" and "sample blank" in order to compare enzyme activity against them. The reagent blank tube received 0.2 mL of distilled water and 1 mL of solution 1 while the sample test tube received 0.2 mL of blood sample and 1 mL of solution 1 (2 mmol/L oxoglutarate and 200 mmol/L L-alanine). After shaking the contents of the tubes and incubating them for 30 minutes at 37 °C, 1 mL of solution 2 (1 mmol/L 2,4-dinitrophenyl hydrazine) was added to the reagent blank test tube and the sample blank test tube, respectively. Each tube's contents were combined, then let to stand at 25 ^{o}C for two minutes. After that, 10 mL of sodium hydroxide solution was added to the test tubes

holding the reagent and the sample. A spectrophotometer (Boerhringer 4070, W. Germany) was used to measure the sample's absorbance at 520 nm using the reagent blank as a reference after the contents of each tube had been combined and let to stand for 5 minutes. The other two tubes were labeled "sample test" and "sample blank" correspondingly for the purpose of measuring enzyme activity against a sample blank, and the same steps as for the reagent blank mentioned above were then carried out.

3.2.15.3 Alkaline phosphatases (ALP IU/L)

P-nitrophenyl phosphate is hydrolyzed by alkaline phosphatase to produce phosphate and P-nitrophenol, which is be measured using commercial kits from Randox Laboratories, Ltd., Aardmore, and the United Kingdom. Rec (1972) first reported this process. P-nitrophenol causes a rise in absorbance that is detected by a spectrometer at 405 nm in wavelength. Using the proper pipettes, 0.05, 0.02, and 0.01 milliliter of the samples were dropped in macro, semi-micro, and micro cuvettes, respectively, and maintained at various temperatures to measure the serum alkaline phosphatase activity. The micro and semi-micro cuvettes were maintained at 37 ^{o}Cand 30^{o}C, respectively, while the macro cuvettes were held at 25^{o}C. The semi-cuvette received 3 mL of the reagent, which contained 10 mmol/L of P-nitrophenyl phosphate, 0.5 mmol/L of magnesium chloride, and 1 mol/L of di-ethanol amine buffer pH 9.8, while the microcuvette received 0.5 mL of the reagent. Initial absorbance values were simultaneously taken while the cuvettes' contents were being mixed, using a timer. After 1, 2, and 3 minutes, the absorbance was once more measured with a spectrophotometer (Boerhinger 4010, West Germany) at 405 nm. The alkaline phosphatase activity was obtained using the formula:

µmol/L = 3300 x A 405 nm/min -: macro

µmol/L = 2760 x A 405 nm/min -: semi-micro

µmol/L = 2760 x A 405 nm/min -: micro

3.2.15.4 Creatinine (mg/dL)

The Jaffe's reaction method of Seaton and Ali (1984) was used to evaluate the level of serum creatinine. In theory, creatinine creates a yellow-red complex, whose absorbance is measured at 490 nm, when combined with an alkaline picrate reagent. Since it is known that the complex needs to be acidified in order to compensate for non-creatinine chromogens in the sample, four test tubes were set and labeled "blank," "standard," "test," and "control," and 500 mL of distilled water and 500 mL of 5% sodium tungstate were poured into each of them. Then, 500 mL of the serum sample was added to the "test" tube, 500 mL of control serum was added to the "standard" tube, and 500 mL of distilled water was added to the "blank" tube. 500 mL of 0.33 M sulphuric acid (H_2SO_4) was added to each of these tubes (blank, standard, test, and control) in order to precipitate protein. After shaking and centrifuging the contents of the tubes for five minutes, 1 mL of the supernatant from each tube was taken and placed in the corresponding tubes labeled "blank," "standard," "test," and "control." Next, 1 mL of distilled water and 1 mL of 0.04 M picric acid were added to each tube. The tubes were then filled with 1 mL of 0.75 M NaOH and incubated at 37^{o}Cfor 10 minutes, and the absorbance measured using a spectrometer (Boerhringer 4010, West Germany) at 490 nm. Utilizing the following formula, the creatinine concentration was determined:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of Standard}} \text{ x Concentration of standard}$$

3.2.15.5 Determination of serum urea (mg/dL)

The diacetyl monoxime technique was used for this (Afonja, 1997). Since diacetyl is unstable, it is often produced through the reaction between diacetyl monoxime and an acid, and is detected at 540 nm, the technique involves condensation of diacetyl monoxime with urea to form chromogen diazine. To stabilize and improve the color, ferric ions and thiosemicarbazide are added to the mixture. Three test tubes with the labels "blank," "standard," and "test" were used for the urea estimation. The blank tube was then filled with two milliliters (2 mL) of distilled water, and the tubes marked "standard" and "test" were each filled with 2 mL of the "standard" solution (50 mol/L) and "test" solution, respectively. After that, each test tube received 2 mL of the color reagent (Diacetyl monoxime 5 g/L) and was thoroughly mixed. The three test tubes were then filled with 2 mL of the acid reagent (5 g of ferric chloride hexahydrate and 85% phosphoric acid in 250 mL of distilled water), well mixed, and incubated at 100 ^{o}Cfor 20 minutes. After the test tube contents had cooled, a spectrophotometer set to 540 nm was used to measure their absorbance. The following formula was used to get the urea concentration:

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \frac{\text{Conc .of Standard}}{1}$$

The standard solution was made by combining 20 mL of distilled water with 0.1 mL of the standard, whereas the test sample was made by combining 0.1 mL of the serum sample with 20 mL of distilled water.

3.2.16 Enzyme Inhibition Assay

The recombinant trypanosome alternative oxidase (rTAO) and *Trypanosoma bruceigambiense*glycerol kinase(TbgGK) used in this experiment was provided by Dr.

Emmanuel O. Balogun, Department of BiochemistryAhmadu Bello University, Zaria.Inhibitory effects of HDN-one on trypanosome alternative oxidase and trypanosome glycerol kinase was carried out as described below.

3.2.16.1 Trypanosome alternative oxidase

The compound's inhibitory effect on trypanosome alternative oxidase was evaluated using the ubiquinol oxidase assay, as described by Kido *et al*. (2010). In a 1 mL quartz cuvette, 10 g of the compound was incubated with 75 ng of rTAO and 50 mM Tris-HCl at pH 7.4 for 2 minutes. Subsequently, a 150 µM solution of ubiquinol-1 was added to initiate the reaction. The control group consisted of Tris-HCl, rTAO, and ubiquinol-1 alone. The inhibitory effect of the compound on the enzyme was measured in triplicate by monitoring the absorbance change at 278 nm using a UV-Vis spectrophotometer (Cary 300 UV-Vis Agilent Technologies) at a temperature of 25°C. The activity of trypanosome alternative oxidase was quantified in units, representing the amount of enzyme in mol/minute/mg that could cause a change in absorbance of one mol of ubiquinol-1 per minute under the specified conditions

3.2.16.2 Trypanosome glycerol kinase

According to Ford *et al*. (1996), the glycerol kinase activity of *Trypanosoma bruciegambiense* was assessed using the bioluminescence test technique. (pNPP + ADP → pNP + ATP) reaction, which is thought to be TbgGK-catalyzed, was first set up using 20 mM pNPP and 2 mM Ultrapure ADP in a buffer system made up of 10 mM $MgSO_4$, 100 mM NaCl, and 100 mM acetate buffer, pH 5.0. For the test reaction, TbgGK was diluted to a final concentration of 40 g/mL, whereas the blank control received an identical volume of

buffer but no TbgGK. The phosphatase activity of TbgGK was initially evaluated using 4-NPP as a substrate. The product, 4-NP was measured at 405 nm. One unit of *Trypanosoma brucei gambienseglycerole* kinase is the enzyme activity measured in mol/minute/mg, which, under the mentioned conditions, can convert one mol of pNPP to one mol of pNP per minute.

3.2.17Statistical Analysis

Data obtained from the experiments were expressed as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA) to determine the extent of variation and "p" values equal to or less than $p \leq 0.05$ were regarded as statistically significant.The statistical software used for theanalysis is Statistical Package for Social Sciences (SPSS) version 20, Chicago, Illinois, USA.

CHAPTER FOUR

4.0 RESULTS

4.1 Yield of theMethanol Extracts of *Viscum album*

The methanol extracts yield of *V. album* leaves and stems is presented in Figure 4.1. *Viscum album* leaves from *A. indica*, *A. albida*,*P. guajava*, *M. oleifera* and *K. senegelensis* yielded 9.68, 18.77, 14.78, 8.89, and 13.54% respectively. *Viscum album* stem from *A. indica*, *A. albida*, *M. oleifera*, *P. guajava* and *K. senegelensis* yielded 4.35, 5.83, 5.95, 3.02 and 5.96% respectively.

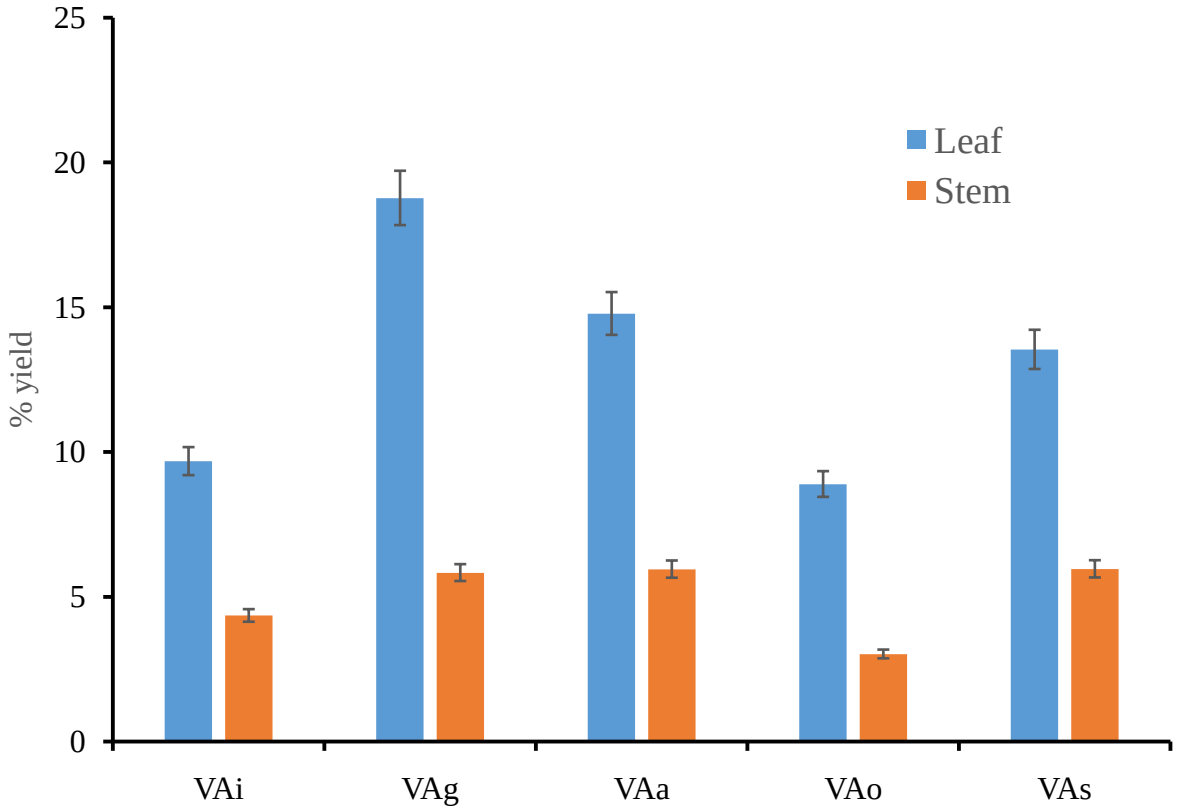


Figure 4.1: Yield of the methanol Leaves and Stems Extract of *V. album* from *A. albida*, *A. indica*, *M. oleifera*, *P. guajava* and *K. senegelensis*

VAi, VAg, VAa, VAo and VAs are *V. album* leaves and stems from *A. albida*, *A. indica*, *M. oleifera*, *P. guajava* and *K. senegelensis* respectively.

4.2 *In vitro* Anti-trypanosomal Activity of the Methanol Extract of *Viscum album*Leaves and Stems

The *in vitro*antitrypanosomal effect of the methanol extract of *V. album*leaves and stems from five hosts plants (*A. albida, A. indica, M. oleifera, P. guajava* and *K. senegelensis*) is presented in Table 4.1. *Viscum album* leaves and stems from*A. albida* and *A. indica* had *in vitro*activity on *T. brucei brucei* while *V. album* leaves and stems from *M. oleifera, P. guajava* and *K. senegelensis* had no activity on *T. brucei brucei*. The methanol extract of *V. album* leaves and stems from *A. albida* had IC_{50} of 0.30 and 0.77 mg/mL respectively while those from *A. indica* leaves and stems had 0.51 and 0.73 mg/mL respectively. The methanol extract of *Viscum album* leaves from *A. albida* was the most active with the lowest ($IC_{50 =}$ 0.30mg/mL). The IC_{50} of Diminazene aceturate was 0.12 mg/mL.

Table 4.1:*In vitro* Anti-trypanosomal Activity of *Viscum album* Leaves and Stems

V. album extract	Host	Activity	IC_{50} (mg/mL)
Leaves	*A. indica*	+	0.51
Stems	*A. indica*	+	0.72
Leaves	*P. guajava*	-	-
Stems	*P. guajava*	-	-
Leaves	*K. senegelensis*	-	-
Stems	*K. senegelensis*	-	-
Leaves	*M. oleifera*	-	-
Stems	*M. oleifera*	-	-
Leaves	*A. albida*	+	0.30
Stems	*A. albida*	+	0.77
Dminazene aceturate		+	0.12

Keys: (+) Activity present

(─) Activity absent

4.3 Qualitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leavesof *A. albida*

The results for qualitative phytochemical profile of the most active methanol extract of *V. album*leaves of*A. albida* is presented in Table 4.2. The phytochemical constituents detected included alkaloids, tannins, saponins, anthraquinone, phenols, steroids, terpenes, courmarins, flavonoids, and glycosides.

Table 4.2: Qualitative Phytochemical Profile of the Methanol Extract of *Viscum album*Leaves of *A.albida*

Phytochemicals	Tests	Results
Tannin	Ferric chloride	+
Saponin	Frothing	+
Alkaloid	Dragendorffs	+
Anthraquinone	Borntrager's	+
Phenols	Ferric chloride	+
Steriods	Acetic anhydride	+
Terpenes	Leiberman-Buchard's	+
Coumarins	Sodium hydroxide	+
Flavanoids	Pew's	+
Carbohydrate	Molisc's	+
Glycoside	Keller-Killian's	+
Reducing sugar	Fehling's	+

Key: (+)Present

4.4 Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leaves of *A. albida*

The quantitative phytochemical profile of the most active extract of *V. album* leaves from *A. albida* is presented in Figure 4.2. The percentage composition of phenolics, flavonoids, alkaloids, and saponins evaluated were 0.82, 0.04, 1.91, and 2.02% respectively. Flavonoids had the lowest percentage of 0.04% while saponins had the highest percentage of 2.02%.

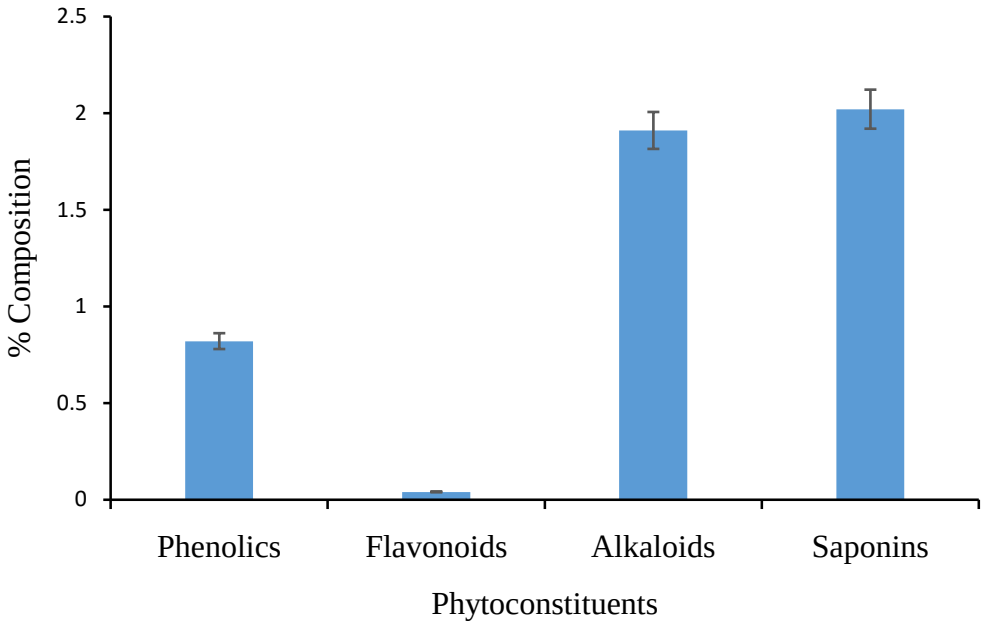


Figure 4.2: Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album*Leaves of *A. albida*

4.5 Thin Layer Chromatogram ofthe Methanol Extract of *Viscumalbum*Leaves of*A. albida*

The TLC of the methanol extract of *Viscum album*leaves from *A. albida*on precoated silica gel plate developed in chloroform: ethyl acetate: methanol: water in the ratio 4:8:4:1is presented in plate IV. The plateswere sprayed with different reagents to identify the phytochemicals. **A** was observed under UV light and showed 2 sports with Rf value of 0.75 and 0.88, **B** was sprayed withP-anisaldehyde (general spray) showed seven spots (R_f values: 0.20, 0.32, 0.48, 0.64, 0.75, 0.85, 0.95), **C** was sprayed with ferric chloride (test for tannins) showed four spots ($R_f$values: 0.37, 0.61, 0.78, 0.87), plate **D** was sprayed withBontragger's reagents (test for anthraquinones) showed four spots (R_f values: 0.19, 0.43, 0.71, 0.82) and **E**was sprayedDragendorff's reagents (test for alkaoids) showed two spots (R_f values:0.76, 0.87).

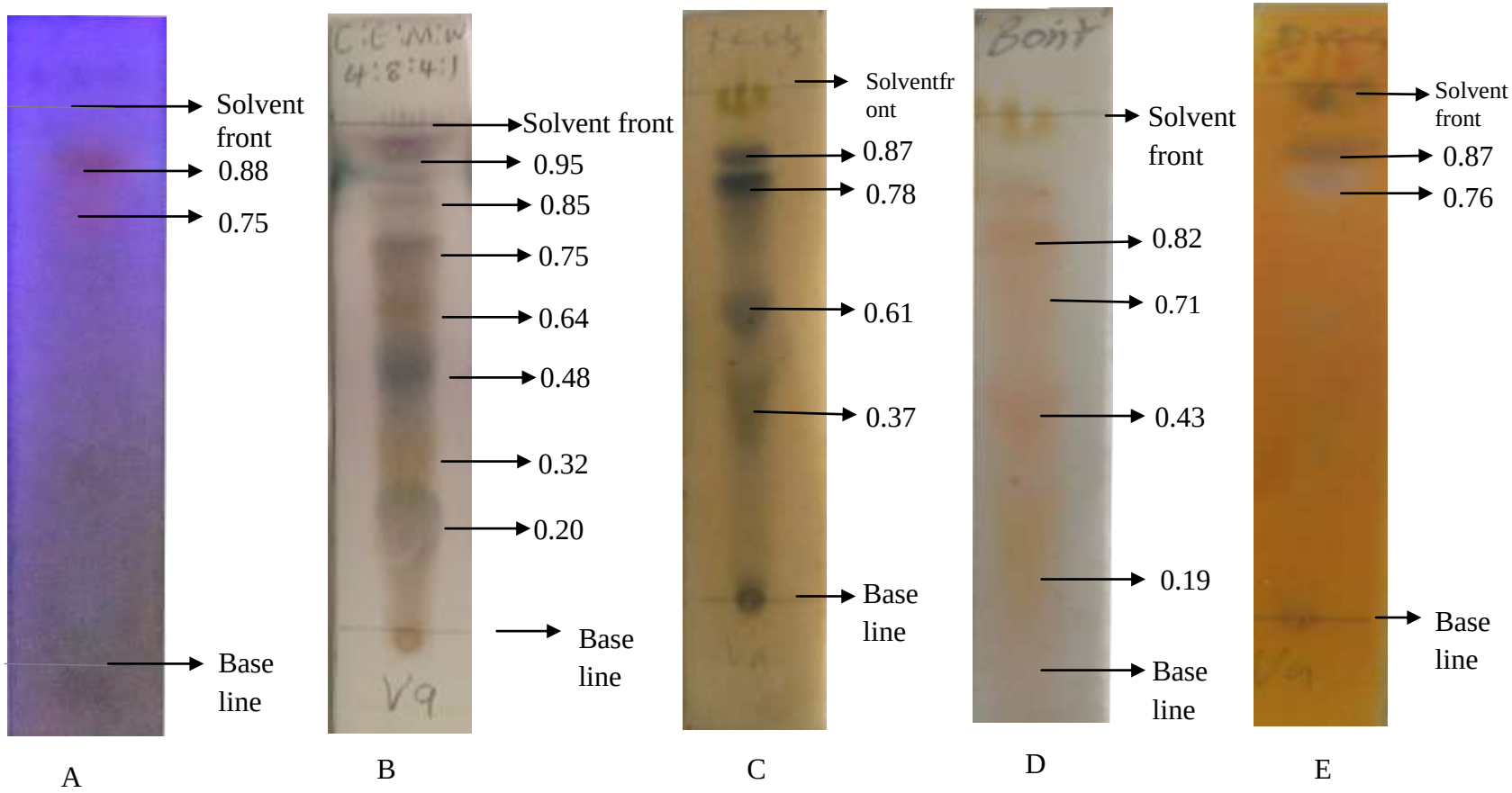


Plate IV: Thin Layer Chromatogram of the Methanol Extract of *Viscum album* Leaves from *A. albida*

A wasobserved under UV light, **B, C, D** and **E** were sprayed with P – anisaldehyde, Ferric chloride, Bontrager's reagent, Dragendorff's reagent respectively.

,

4.6 Solvent Partition Yield of theMethanol Extract of *V. album* Leaves of *A. albida*

The solvent partition yield of the methanol extract of *V. album* leavesfrom*A. albida* is presented in Figure 4.3. Different solvents were used. N-hexane, ethyl acetate, n-butanol and water yielded 0.5, 2.42, 45.26 and 29.30% respectively. The partitioned fractions were dark- brown and sticky.

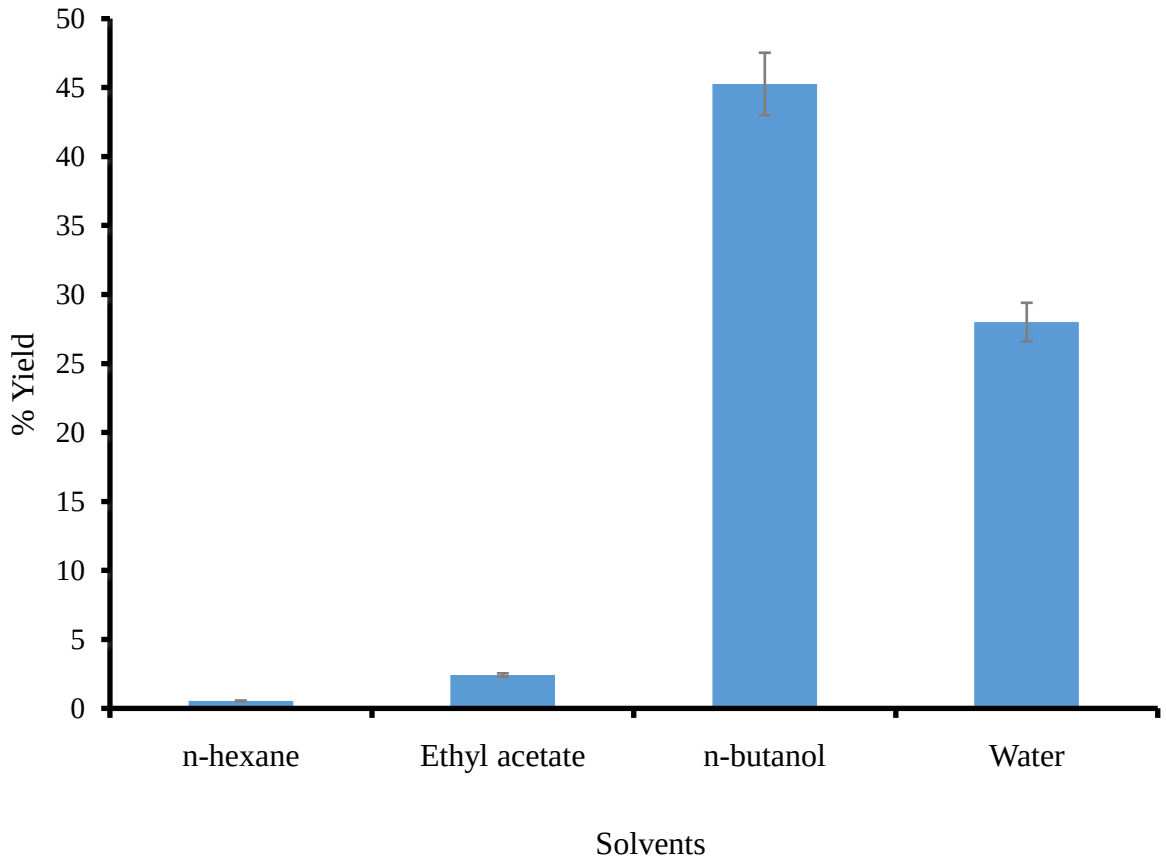


Figure 4.3: Solvent Partition Yield of the Methanol Extract of *V. album* Leaves of *A. albida*

4.7*In vitro* Antitrypanosomal Activity of the Partitioned Fractions of the Methanol Extract of *Viscum album* Leaves of *A. albida*

The *in vitro* antitrypanosomal activity of the partitioned fractions of the methanol extract of *Viscum album*leaves of *A. albida*is presented in Table 4.3. n-butanol fraction had activity against *T. brucei brucei*with 55.40 percent inhibition while diminazeneaceutrate had 72.09 percent inhibition.Hover, N-hexane, ethyl acetate and water fractions had no activity against *T. brucei brucei*, their percentage inhibition were 5.83, 3.54, and 4.27 respectively.

Table 4.3: *In vitro* Antitrypanosomal Activity of the Partitioned Fractionsof the Methanol Extract of *Viscum album* Leaves of *A. albida*

Fraction	activity	% Inhibition
n-hexane	-	5.83
Ethyl acetate	-	3.54
n-butanol	+	55.40
water	-	4.27
Diminazene aceturate	+	72.09
PBSG	-	3.46

PBSG (phosphate buffer saline glucose)

4.8Thin Layer Chromatogram of n – Butanol Fractionof the Methanol Extract of*Viscum album* Leaves of*A. albida*

The thin layer chromatogram of n-butanol fraction which is the most active against *Trypanosoma brucei brucei*developed in 100% ethyl acetate t is presented in plate V.**A**was sprayed with P-anisaldehyde (general spray) showed four (4) spots (R_f value: 0.21, 0.34, 0.63, 0.79), **B** was sprayed with aluminium chloride (test for tannins) showed two (2) spots (R_f values: 0.29, 0.78), **C**was sprayed with Leibermann-Buchard reagents (test for terpenes) showed two (2) spots (R_f values: 0.26, 0.58),**D** was sprayed withBontragger's reagentsshowed two (2) spots (R_f values:0.27, 0.59) and **E**was sprayed with ferric chlorideshowed two spots (Rf values: 0.27, 0.53).

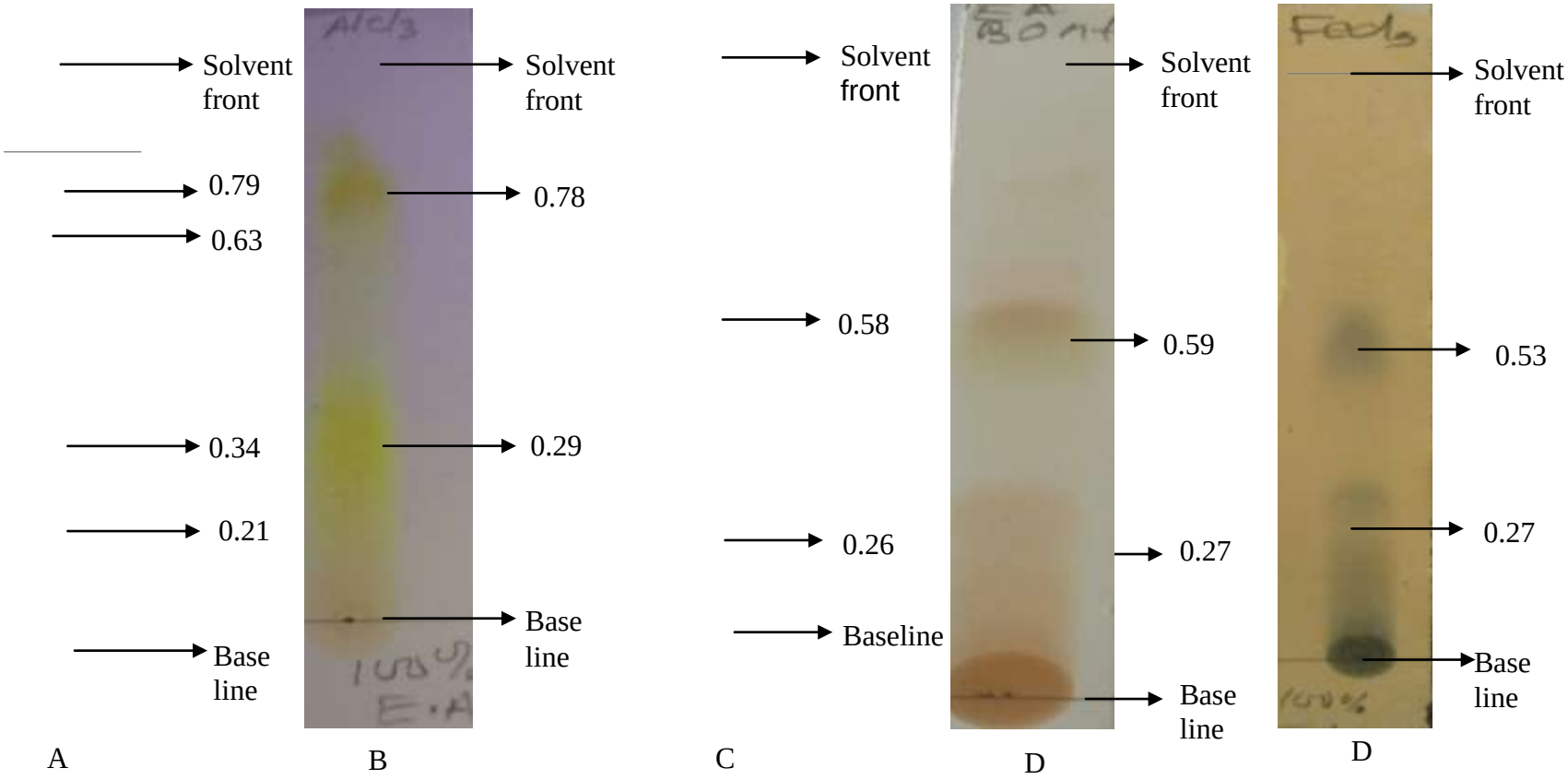


Plate V: Thin Layer Chromatogram of n – Butanol Fraction of the Methanol Extract of *Viscum album* Leaves of *A. albida*

A, B, C, D and **E** were sprayed with p-anisalehyde, aluminium chloride, Libermann-Buchard reagents, Bontragers reagents and ferric chloride respectively.

4.9 *In vitro* Anti-trypanosomalActivity of the n-ButanolFractionsof the Methanol Extract of *Viscum album* Leaves of*A. albida*

Table 4.4 displays the results of the evaluation of the combined fractions collected from column chromatography of the n-butanol fraction for their anti-trypanosomal activity. A total of eighty-seven (87) fractions were collected, and those exhibiting similar characteristics based on thin layer chromatography analysis were combined. Among these, fraction 47-54 demonstrated anti-trypanosomal activity against *T. bruceibrucei* and was chosen for subsequent investigations.

Table 4.4: *In vitro* Anti-trypanosomal Activity of n-Butanol Fractions of the Methanol Extract of *Viscum album* Leaves of *A. albida*

Fractions	Solvent system	Yield (g)	Activity
1-12	H : EA 90 : 10	2.26	-
13-14	80 : 20	1.65	-
15-16	70 : 30	1.60	-
17-25	60 : 40	1.04	-
26-46	50 : 50	1.20	-
47-54	40 : 60	4.25	+
55-65	30 : 70	1.34	-
66-71	20 : 80	0.50	-
72-76	10 : 90	2.91	-
77-82	0 : 100	1.94	-
83-87	M : EA 10 : 90	1.11	-

Keys: (H) n-hexane, (M) Methanol, (EA) ethyl acetate (+) activity present, (─) no activity,

4.10 *In vitro* Anti-trypanosomal Activity of the sub-Fractions(47-54)

The column chromatography of the active n-butanol (fraction 47-54) was carried out to obtain sub-fractions presented in Table 4.5. Thirty-five sub fractions were eluted using n-hexane: ethyl acetate in different solvent ratio (1:1, 2:3, 3:7and 1:4). Similar fractions werepooled. Fractions 16-23 and 24-35 showed *in vitro* anti-trypanosomal activity against *T. brucei brucei* with the IC_{50}0.43 and 0.26 mg/mL respectively.

Table 4.5: *In vitro* Anti-trypanosomal Activity of sub fractions of Fraction (47 – 54)

sub-fractions	Solvent system H : EA	Activity	IC_{50} (mg/mL)	R^2
1 – 8	50 : 50	-	-	-
9 – 15	40 : 60	-	-	-
16 – 23	30 : 70	+	0.43	0.9247
24 – 35	20 : 80	+	0.26	0.8862

Keys: (H) n-hexane, (EA) Ethyl acetate, (+) activity present, (—) no activity

4.11 Thin Layer Chromatogram of the sub-Fraction(24 – 35)

The thin layer chromatogram of the most active sub-fractionon (24 – 35) on precoated silica gel plate developed in n – hexane: ethyl acetate ratio 2:8 is presented in Plate VI.Spots3,4,5,6,7 and 8 on the TLC plate represent fraction (24-25), (26-27) (28-29), (30-31), (32-33) and (34-35) respectively. The fractions showed a a single spot with R_f of 0.7.

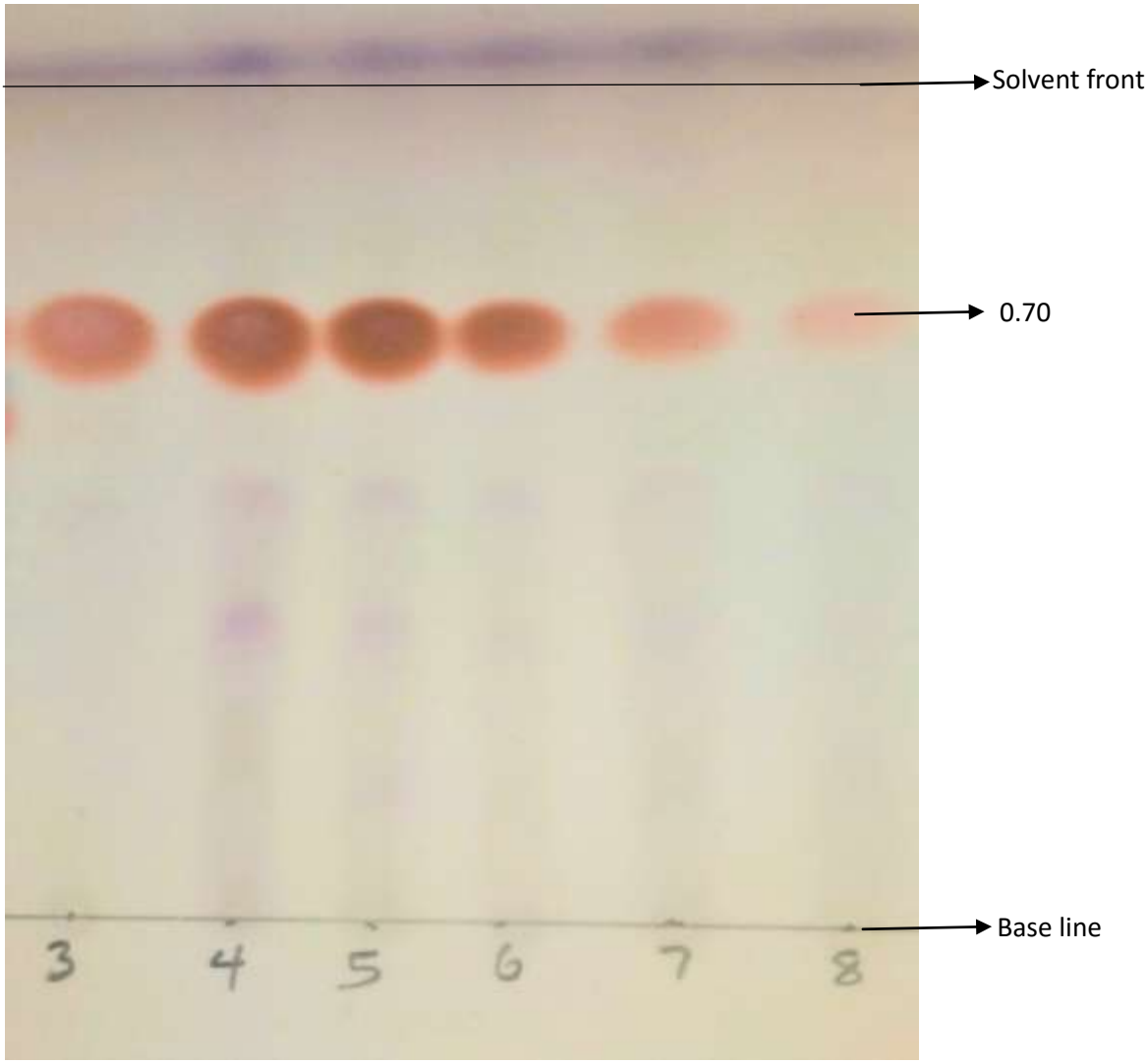


Plate VI: TLC of the Purified Compound Developed in n-Hexane: Ethyl Acetate in the ratio 2:8 with the Code HDN-one

4.12 1D-Proton NMR (^{1}H-NMR) Spectra

The proton NMR of the compoundcode-named HDN-oneisolated from the methanol extract of*Viscum album* leaves of *A. albida* is presented Figure 4.4.The ^{1}H-NMR spectrum for the compound showed signals for two symmetrical aromatic protons at δ_H 6.75 (2H, dd, *J* = 8.5, 2.5 Hz) and δ_H7.03 (H, d, *J* = 8.5 Hz) indicating a para-substituted phenyl ring. Signals between δ_H 1.50 to δ 4.90 ppm indicated the presence of aliphatic protons considering their attachment to carbons (C-1′ C-3′, C-4′, C-3, C-5 and the carbonyl protons at C-2′C-4, C-6). Also, a signals at δ_H 2.17 ppmwas observed for hydroxyl group.

4.13 1D-Carbon NMR (^{13}C-NMR) Spectra

Figure 4.5 displays the results of^{13}C-NMR analysis conducted on HDN-one, which was extracted from the leaves of *V. album* of *A. albida* using methanol. The spectrum revealed various chemical signals including a lactone signal at δC 170.2 ppm (C-2), two aldehyde signals at δC66.0 and 73.0 ppm (C-4 and C-6, respectively), two signals indicating aromatic carbon at δC 129.6 (C-2″ and C-6″) and 115.5 ppm (C-3″ and C-5″), as well as two quaternary aromatic carbon signals at δC 133.8 and 154.0 ppm for C-1″ and C-4″, respectively. Additionally, the ^{13}C-NMR spectrum showed six aliphatic carbon signals in the range of δC 30 to 77 ppm, corresponding to C-2′, C-3′, C-4′, C-3, C-4, and C-6.

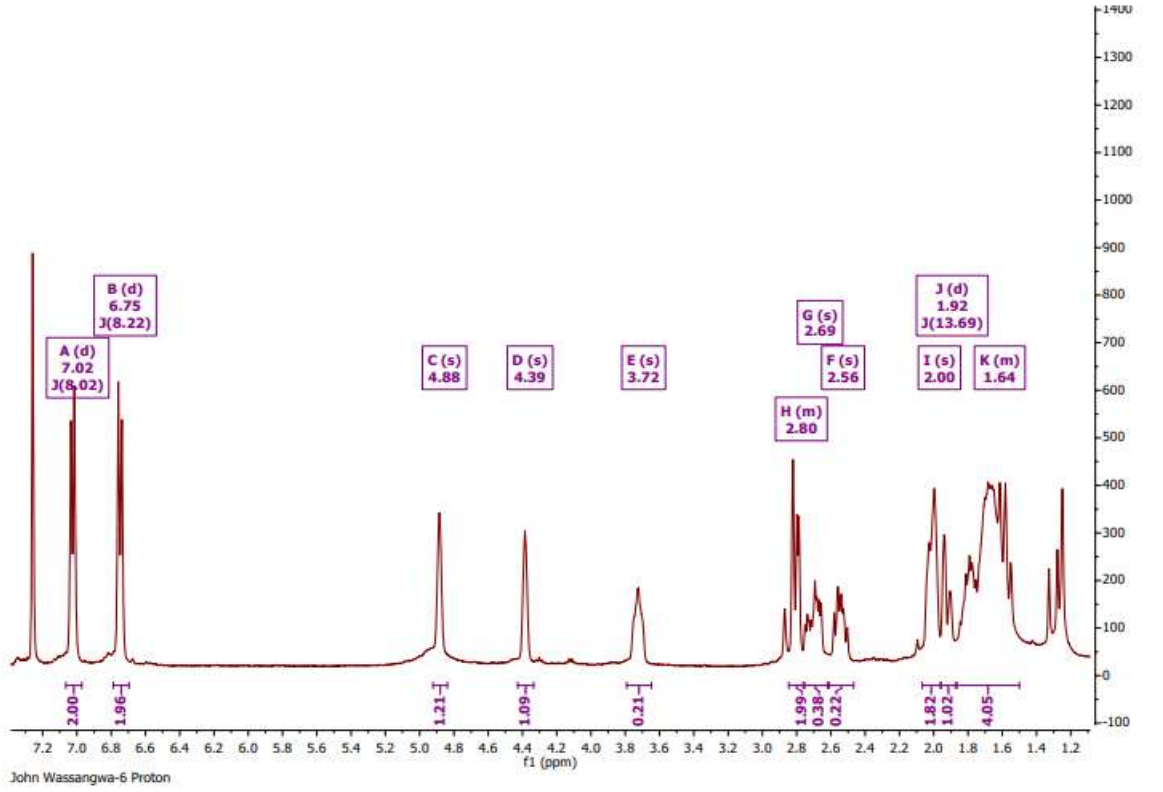


Figure 4.4:1D-Proton NMR (^{1}H-NMR) Spectra of HDN-oneIsolated from the Methanol Extract of *Viscum album* Leaves of*A. abida*

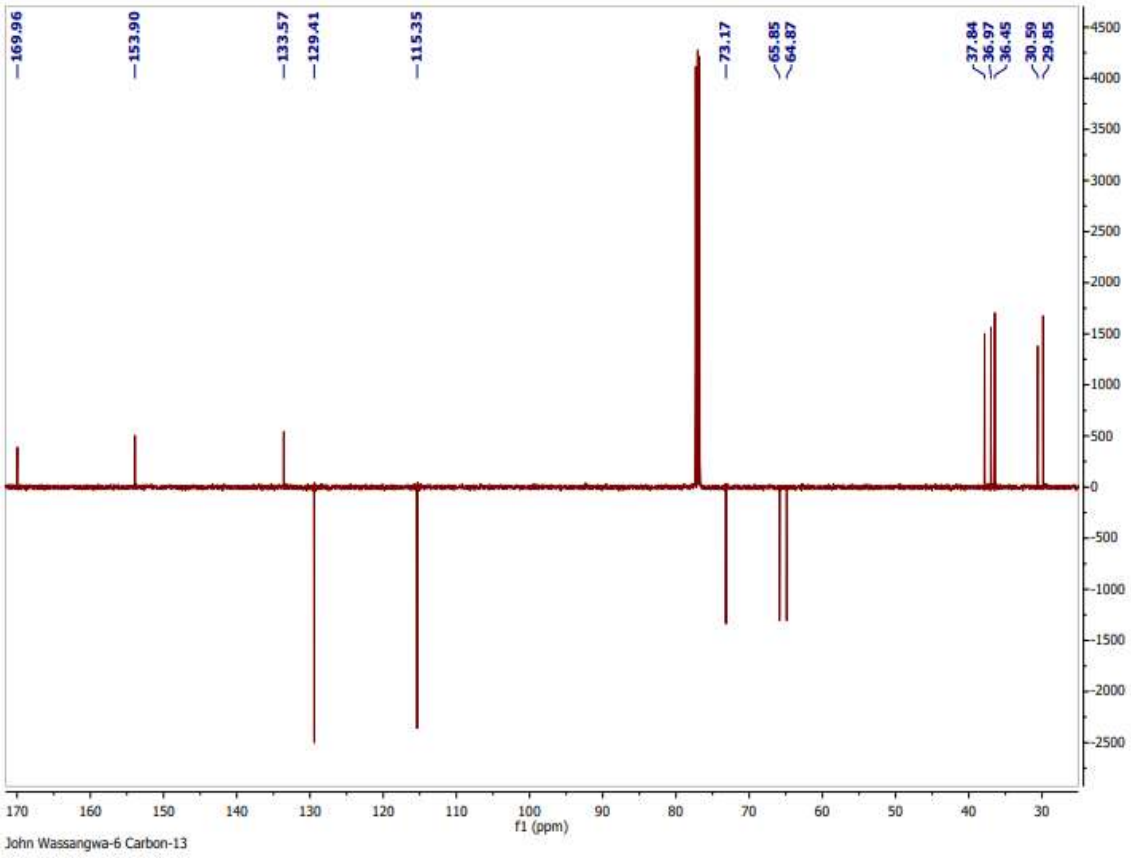


Figure 4.5: 1D-Carbon NMR (^{13}C-NMR) Spectra ofHDN-one Isolated from the Methanol Extract of *Viscum album* Leaves of *A. abida*

4.14 2D ^{1}H, ^{1}H-COSY Correlation Spectroscopy

The results of ^{1}H,^{1}H Correlation Spectroscopy (COSY) of HDN-one isolated from the methanol extract of *Viscum album* leaves of *A. albida* is presented in Figure 4.6. On both axes are shown the ^{1}H-NMR of the compound. A straight line from any of the dark spots to each axis shows which protons is coupled with one another, and which is attached to neighboring carbons. The cross peaks that were symmetric to the diagonal showed the COSY correlations. Correlations in its COSY spectrum (^{1}H-^{1}H through bond) identified the connectivity between H-3 and H-4, H-1′ to H-4′ and the aromatic coupled protons (H-2″/6″ and H3″/5″) protons.

4.15 ^{1}H-^{13}C Heteronuclear Single Quantum Coherence Spectroscopy (HSQC)

^{1}H-^{13}C HSQC of HDN-one isolated from the methanol extract of *Viscum album* leaves of *A. abida* is presented in Figure 4.7correlations in its HSQC identified all the carbons and their attached protons.

4.16 Heteronuclear Multiple Bond Correlation (HMBC) spectra

The (HMBC) spectrum of HDN-one isolated from the methanol extract of *Viscum album* leaves of *A. abida* is presented in Figure 4.8. The long range (^{2}J and ^{3}J) correlations in its HMBC spectrum showed correlations from H-3 and H-4 to the carbonyl carbon at C-2 as well as the saturated carbons at C-5 and C-6) confirming the unsaturated lactone ring. Correlations from H-1′ and H-2′ to the lactone carbons showed the attachment of the butyl chain to the lactone ring while correlations from H-3′ and H-4′ to the phenyl ring also confirmed the linkage to the phenol moiety. Finally, correlations from the aromatic protons confirmed the linkage to the ethyl chain and the presence of hydroxy substituent on the phenyl ring.

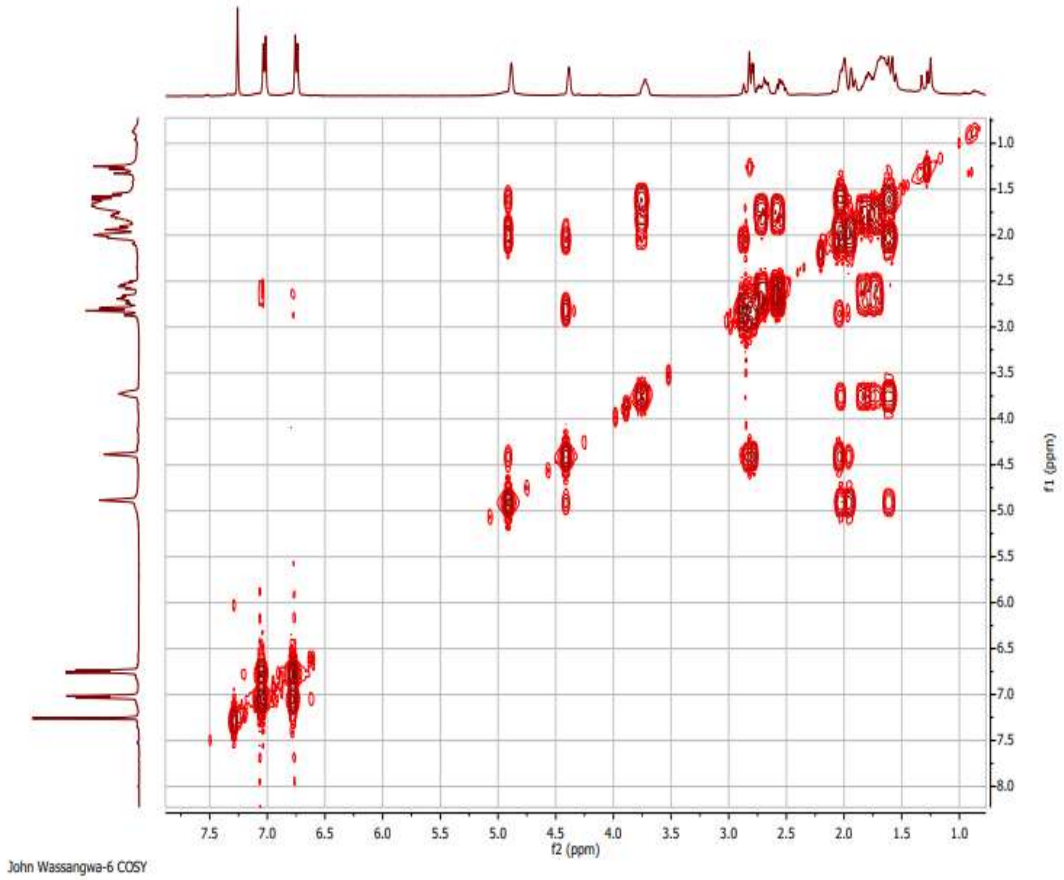


Figure 4.6: 2D $^{1}H,^{1}H$-COSY (Correlated Spectroscopy) of HDN-one Isolated from the Methanol Extract of *Viscum album* Leavesof *A. abida*

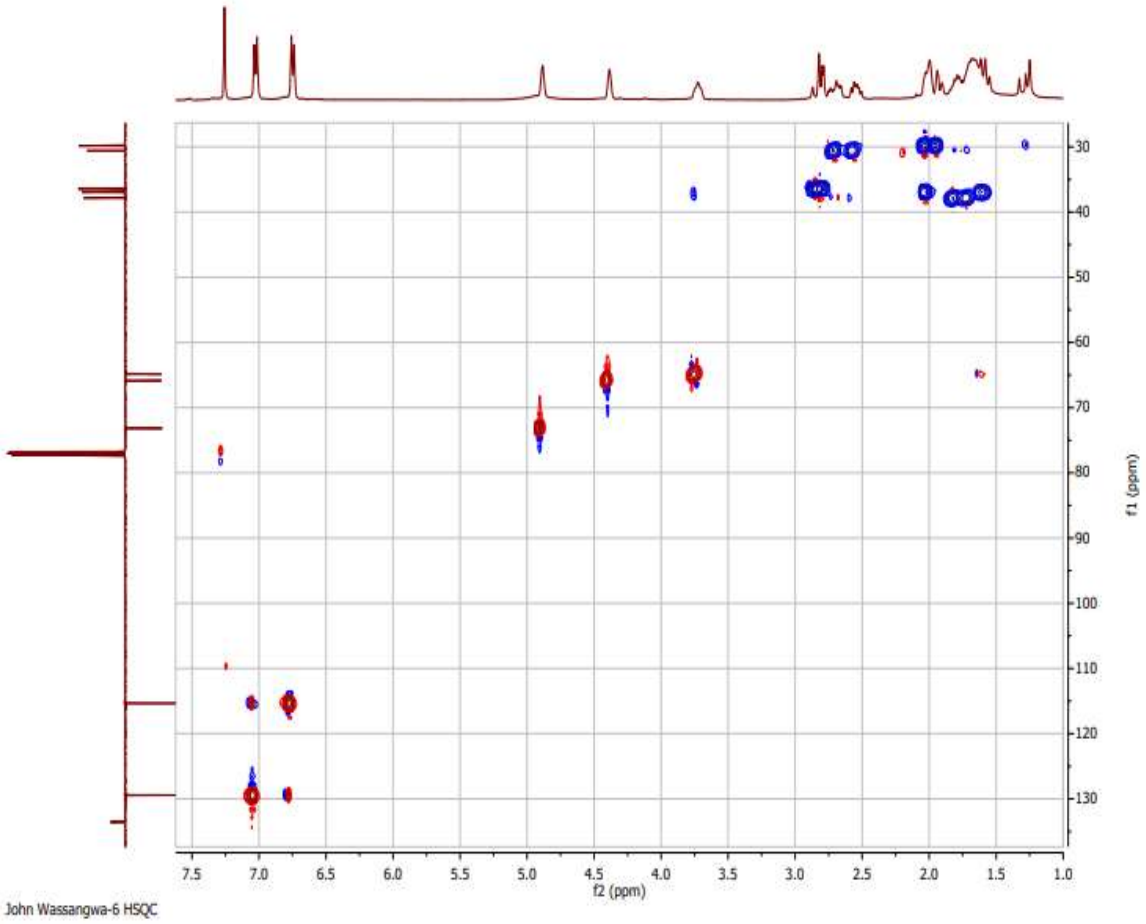


Figure 4.7: HSQC (Heteronuclear Single Quantum Correlation) Spectrum of HND-one Isolated from the Methanol Extract of *Viscum album* Leaves of*A. abida*

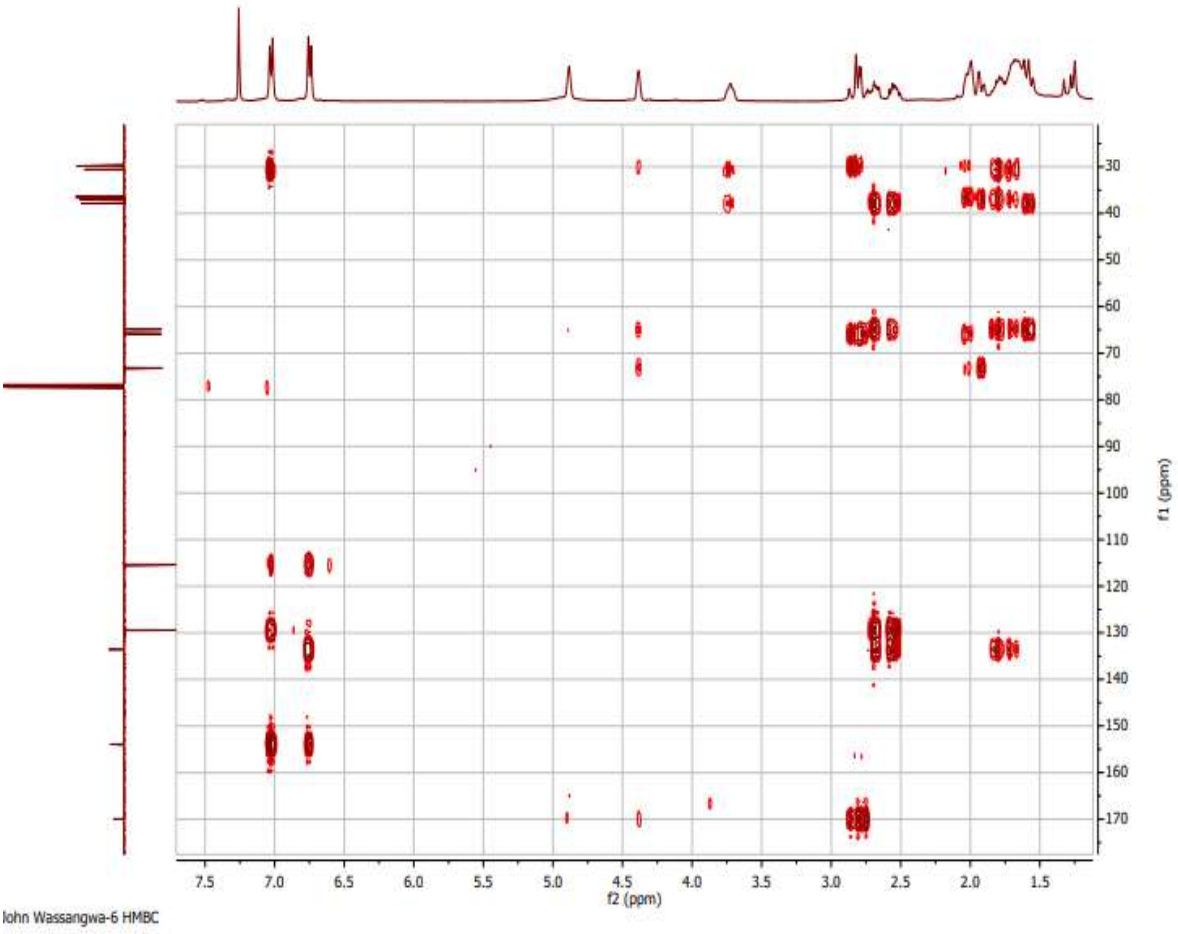


Figure 4.8:HMBC (Heteronuclear Multiple Quantum Correlation) Spectra ofHDN-one Isolated from the Methanol Extract of *Viscum album* Leaves of*A. abida*

4.17 Chemical Shift Values of ^{1}H and ^{13}C NMR

The chemical shift of ^{1}H and ^{13}C NMR of HDN-one isolated from the Methanol extract of *Viscum album* leaves of *A. abida* is presented in Table 4.6. Chemical shift refers to the frequency at which a nucleus resonates compared to a standard in the presence of a magnetic field. The position and quantity of chemical shifts are frequently indicative of a molecule's structure, providing valuable diagnostic information. The experimental and literature values of ^{1}H and ^{13}C NMR are presented.

Table4.6: ^{1}H And ^{13}C NMR Chemical Shift of HDN-one Isolated from the Methanol Extract of *Viscum album* Leaves of*A. abida*

Position	Experimental		Literature (ref)*	
	^{1}H (δ ppm)	^{13}C (δ ppm)	^{1}H (δ ppm)	^{13}C (δ ppm)
1	-	-		-
2	-	170.2		170.3
3	2.72, 2.84	36.6		36.5
4	4.38	66.1	4.34 (br s)	66.0
5	1.92, 2.02	30.0	1.92 (m)	29.9
6	4.89	73.3	4.87 (m)	73.3
1′	1.58, 1.99	37.1	1.98 (m)	37.1
2′	3.73	65.0	3.65 (m)	65.0
3′	1.73, 1.83	38.0	1.7 (m)	38.0
4′	2.57, 2.71	30.6	2.6 (m)	30.7
1″	-	133.8	-	133.6
2″	7.03	129.6	7.02 (d, *J* = 8.3 Hz)	129.5
3″	6.75	115.5	6.75 (d, *J* = 8.4 Hz)	115.5
4″	-	154.0	-	154.1
5″	6.75	115.5	6.75 (d, *J* = 8.4 Hz)	115.5
6″	7.03	129.6	7.02 (d, *J* = 8.3 Hz)	129.5
-OH	2.17 (s)	-		

4.18 The structure ofHDN-one

The Chemical Structure of HDN-one is presented in Figure 4.9.Thecompound was isolated by a combination of chromatographic techniques. It was eluted using n-hexane: ethyl acetate 2:8 from columnchromatography and was obtained as a white crystalline solid soluble in ethyl acetate.Using ^{1}H-NMR,^{12}C-NMR and correlations from 2D (COSY, HSQC and HMBC) spectra the compound was identified as [2-(4-hydroxyphenyl)ethyl]-2,6-dioxabicyclo[3.3.1]nonan-3-one.

Figure 4.9: Chemical Structure of [2-(4-hydroxyphenyl) ethyl]-2,6-dioxabicyclo [3.3.1] nonan-3-one (HDN-one).

4.19 Acute Toxicity Testing for HDN-one

The intraperitoneal acute toxicity test of HDN-one on miceis presented in Table 4.7. Administration of 2000 mg/kgb.w in the first and second step did not cause mortality in mice, however administration of 5000 mg/kgb.w caused mortality within 24 hours. The compound was ranked and classified as Category 5.

Table 4.7: Acute Toxicity Testof HDN-one on Mice

Group	Number of mice	Mortality
2000 mg/kgb.w(step 1)	3	-
2000 mg/kgb.w(step 2)	3	-
5000 mg/kgb.w	3	3

The compound was placed into Category 5 according to the guidelines OECD, (2008) using a fixed-dose procedure.

4.20 Clinical Signs of Acute Toxicity Testof HDN-one in Mice

Table 4.8 lists the clinical symptoms of a mouse HDN-one acute toxicity test. Following a 24-hour treatment of HDN-one at a dose of 2000 mg/kg b.w, no clinical symptoms of toxicity were noticed in the mice. On the other hand, clinical indications such as weakness, dizziness, dyspnea, arched backs, bulging eyes, unsteady gait, lack of appetite, drowsiness, coma, and death within 24 hours were noted after giving 5000 mg/kg b.w.

Table 4.8: The Clinical Signs of ofAcute Toxicity Test of HDN-one on Mice

Clinical Signs	2000mg/kg	5000 mg/kg	Control
Weakness	-	+	-
Dizziness	-	+	-
Dyspnea	-	+	-
Arched back	-	+	-
Bulging eye	-	+	-
Unsteady movement	-	+	-
Lack of appetite	-	+	-
Sleep	-	+	-
Coma	-	+	-
Death	-	+	-

Keys: present (+)

Absent (—)

4.21Mean Body Weight of Mice in Acute Toxicity Test of HDN-one

Figure 4.10 displays the average body weight of albino mice during the toxicity test of HDN-one. The results showed that there was no significant ($p \geq 0.05$) increase in body weight of the mice in the treated groups compared to the control group.

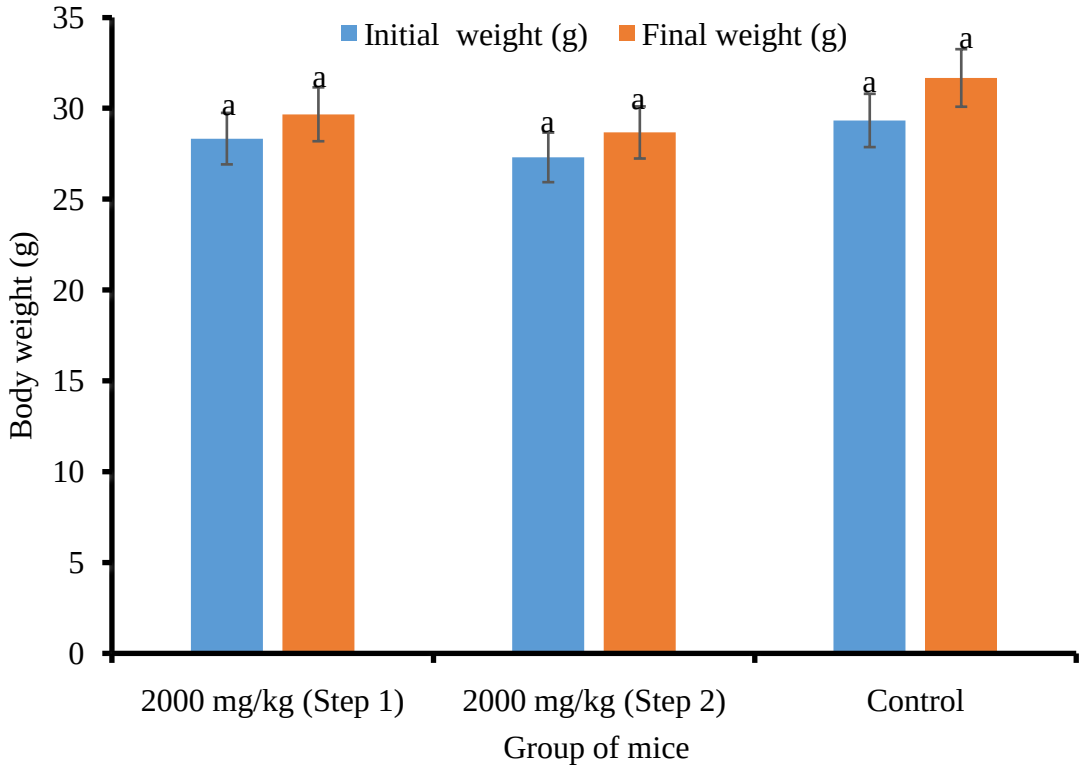


Figure 4.10: Mean Body Weight Changes of Mice in Acute ToxicityTest of HDN-one

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them.

4.22The Relative Organ Weight of Mice in Acute Toxicity Test of HDN-one

Figure 4.11 illustrates the relative organ weight of albino mice during the acute toxicity test involving HDN-one. The findings showed that there wereno significant differences ($p \geq 0.05$) in the average relative weights of the liver, kidney, heart, lungs, and spleen of the mice in the treated groups and those in the control group.

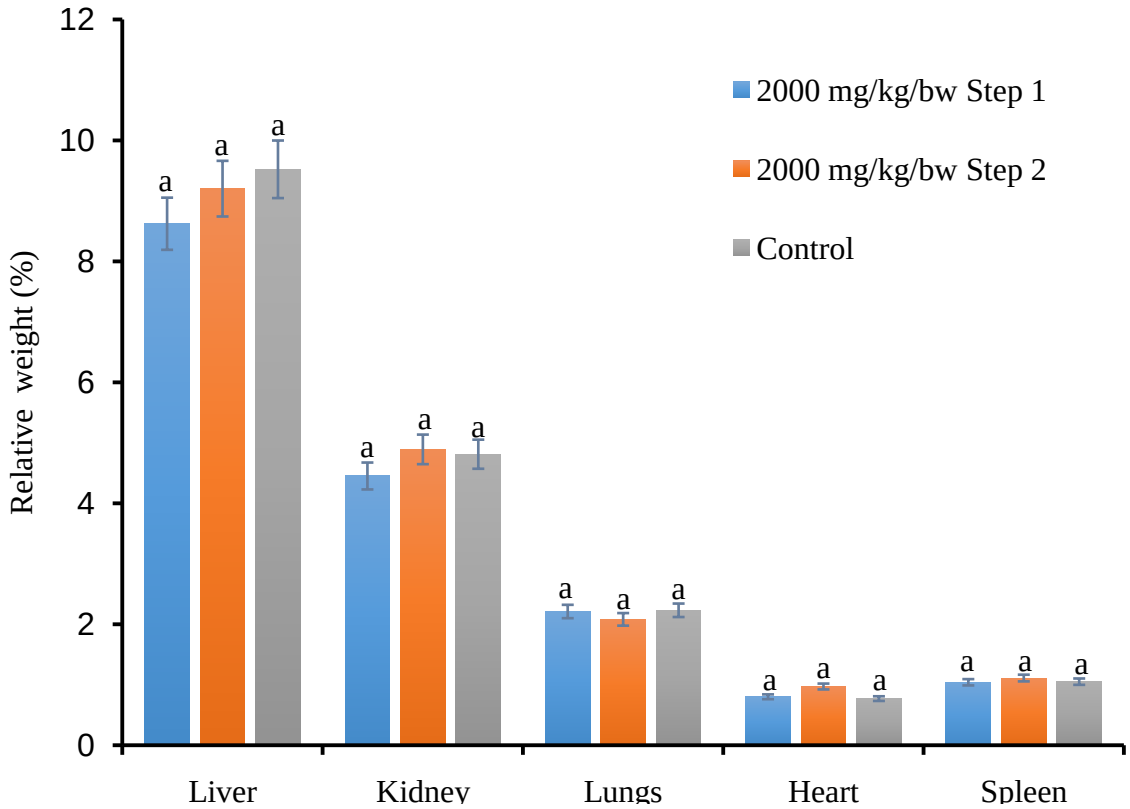


Figure 4.11: Mean Relative Organ Weight of Mice in Acute Toxicity Test of HDN-one

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them.

4.23Haematological Parameters of Mice in Acute Toxicity Test of HDN-one

Figure 4.12 demonstrates the average values of haematological parameters in mice during the acute toxicity test involving HDN-one. Mice were administered 2000 mg/kgb.w of the compound and examined for 14 days. The results indicate that there were no significant differences ($p \geq 0.05$) in PCV, Hb, RBC and total WBC values between the mice treated with HDN-one and the control group.

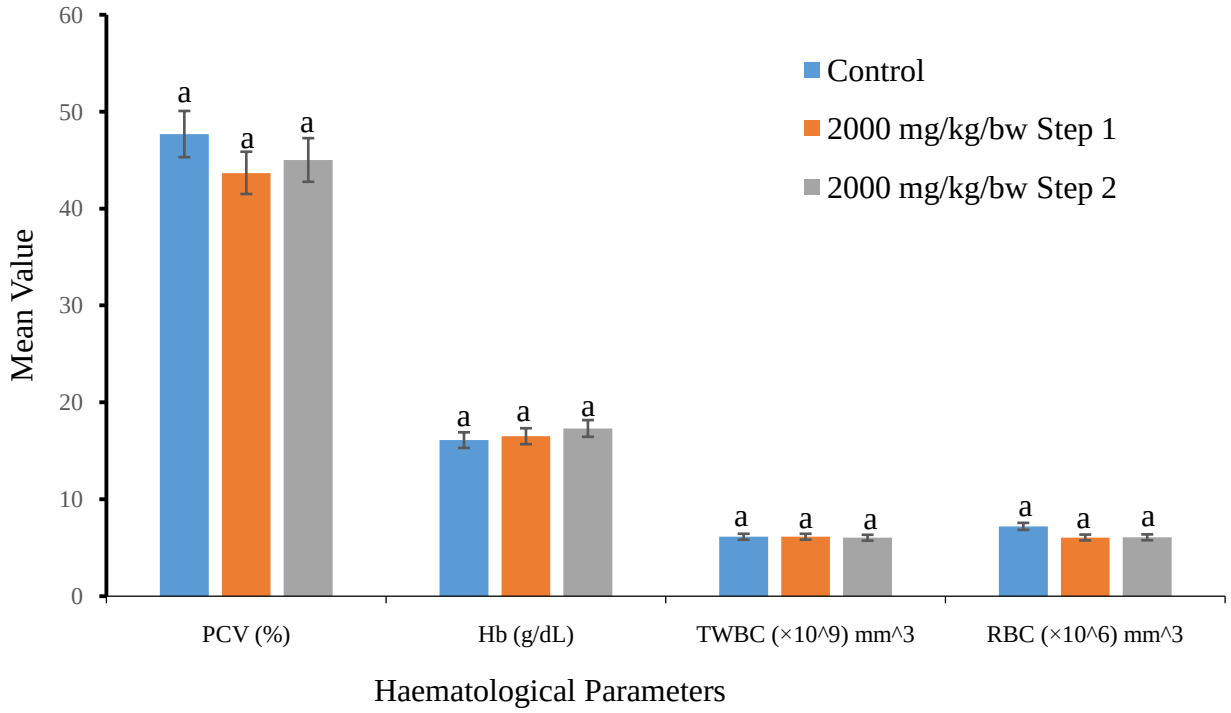


Figure 4.12: Mean Values of Haematological Parameters of Mice in Acute Toxicity Test of HDN-one

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them.

4.24Biochemical Parameters of Mice in Acute Toxicity Test of HDN-one

Figure 4.13 displays the results of the average values of biochemical parameters in albino mice during the acute toxicity test involving HDN-one. Mice in step 1 and step 2 treated groups were administered 2000 mg/kgb.w ofHDN-one and examined for 14 days. Therewereno significant changes ($p \geq 0.05$) in the mean values of BUN, creatinine, albumin, ALP, ALT, and AST compared to the control group.

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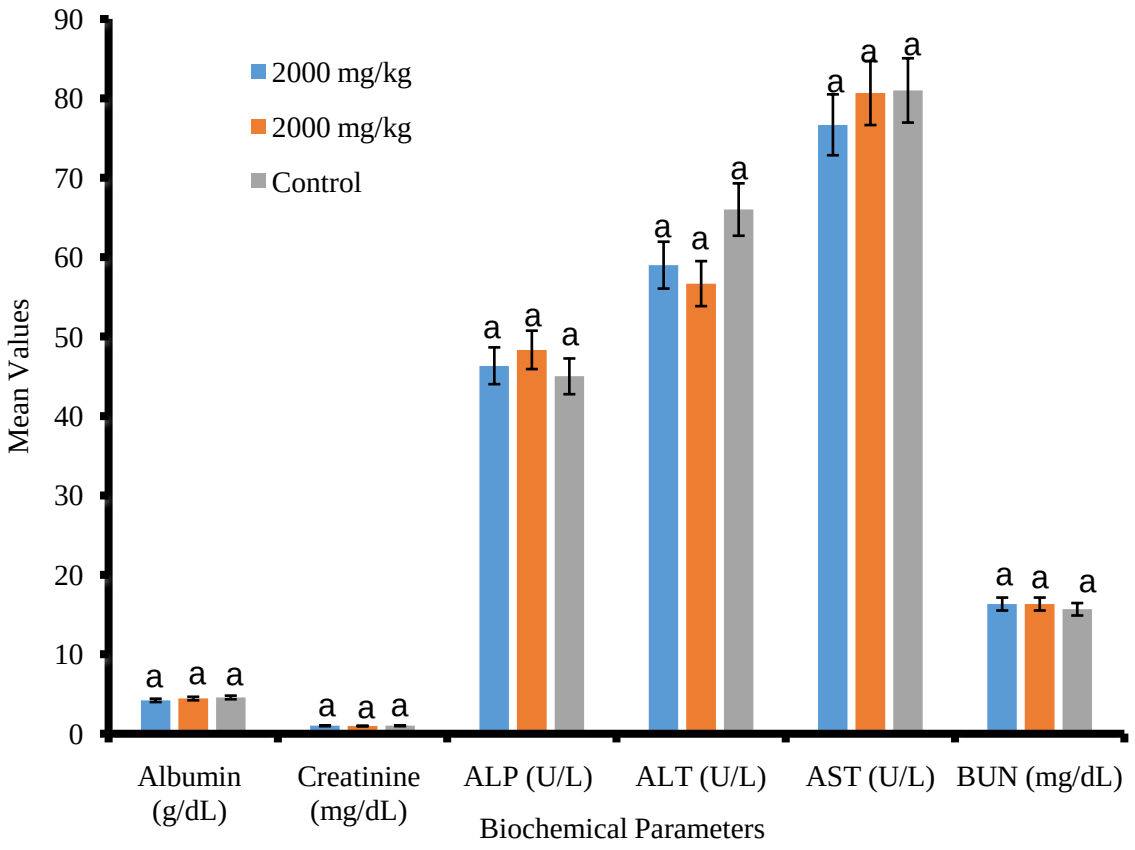


Figure 4.13: Mean Values of Biochemical Parameter of Mice in Acute Toxicity Test of HDN-one

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them.

4.25 Mean Parasite Count Following Treatment withHDN-one and Diminazene Aceturate

Figure 4.14 illustrates the average daily parasite count in *T. b. brucei*-infected albino mice treated with HDN-one. Parasitemia was established in all infected groups on the fourth day after infection, and treatment commenced on the same day. The untreated infected group showed a rapid increase in parasitemia levels until the eleventh day after infection, resulting in the death of all mice. However, the group treated with diminazene aceturate showed a decline in parasitemia, and by the seventh day after inoculation, the parasites were completely cleared. Notably, a significant difference ($P \leq 0.05$) in parasitemia on the eighth day after inoculation for the groups treated with HDN-one at a dose of 1000 mg/kg b.w and on the sixth day after infection for the groups treated with at 2000 mg/kgb.w compared to the untreated infected group. The mice treated at 1000 mg/kgb.w and 2000 mg/kgb.w survived to the fifteenth and fourteenth day after infection, respectively. However, there was no significant difference ($P \geq 0.05$) observed between the group treated with HDN-one at a dosage of 500 mg/kg and the untreated infected group.

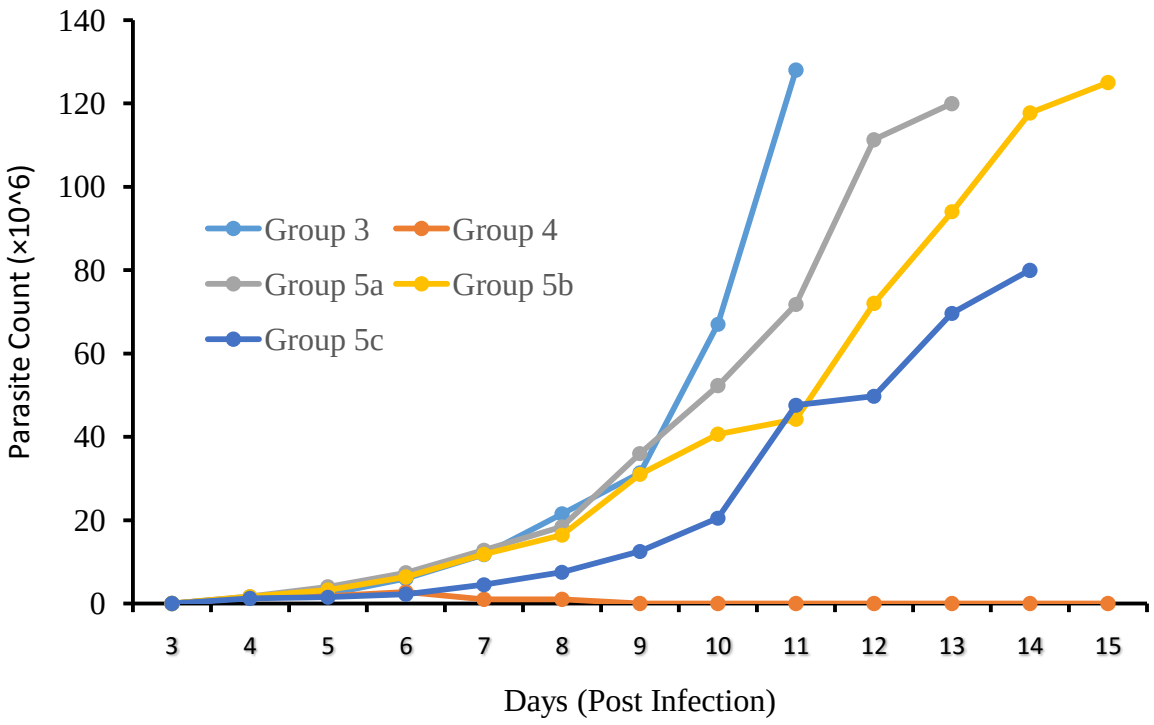


Figure 4.14: The Mean Parasite Count of *Trypanosoma brucei brucei* Infected Mice Treated with HDN-oneand Diminazene Aceturate

Keys:

Group 3 = infected with *T. brucei brucei*and untreated

Group 4 = infected with *T. brucei brucei*and treated with diminazene at 3.5 mg/kg b.w

Group 5a = infected with *T. brucei brucei*andtreated with the compound 500 mg/kg b.w

Group 5b = infected with *T. brucei brucei*andtreated with the compound 1000 mg/kg b.w

Group 5c = infected with *T. brucei brucei*andtreated with the compound 2000 mg/kg b.w

4.26 Kaplan Meier Plot of Percentage Survival Days Post Infection of *T. b. brucei* Infected Mice

Figure 4.15 displays the Kaplan-Meier plot depicting the percentage of survival in *T. b. brucei*-infected mice treated with HDN-one and diminazene aceturate. All the mice infected with *T. bruceibrucei* survived until the tenth day post infection. In contrast, the mice treated with diminazene aceturate all survived until the end of the experiment. The survival rate was higher among the mice treated with the compound compared to the untreated infected groups. Specifically, twenty percent (20%) of the mice treated with HDN-one at dosages of 2000 mg/kg b.w and 1000 mg/kg b.w survived until the fourteenth and fifteenth day post infection, respectively, while the untreated infected group had no survivors by the twelfth day post infection.

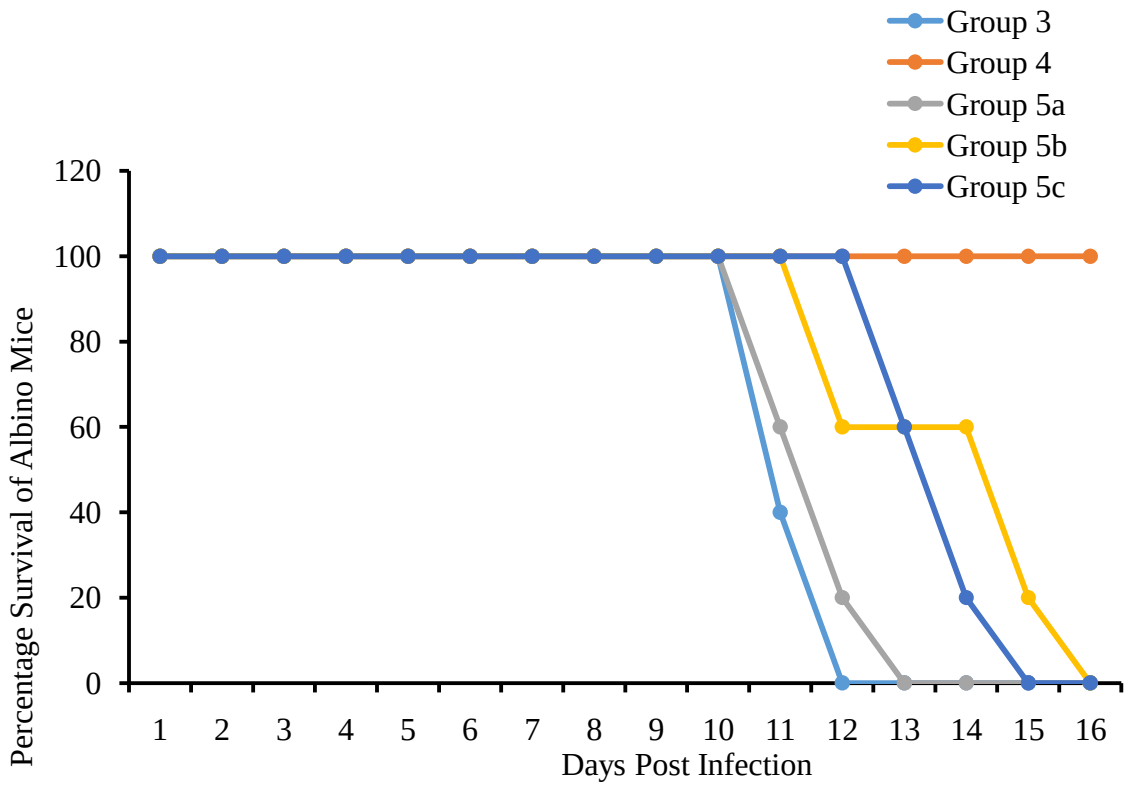


Figure 4.15: Kaplan Meier Plot of Survival Time of *T. b. brucei* Infected Albino Mice treatedwithHDN-oneand Diminazene Aceturate

Keys:

Group 3 = infected with *T. brucei brucei* and untreated

Group 4 = infected with *T. brucei brucei* and treated with diminazene at 3.5 mg/kg b.w

Group 5a = infected with *T. brucei brucei*andtreated with the compound 500 mg/kg b.w

Group 5b = infected with *T. brucei brucei*andtreated with the compound 1000 mg/kg b.w

Group 5c = infected with *T. brucei brucei*andtreated with the compound 2000 mg/kg b.w

4.27Mean Body Weight of *T. b. brucei* Infected Mice treated with HDN-oneand Diminazene Aceturate

The body weight of mice inoculated with *T. b. brucei* treated with HDN-oneand diminazene aceturate is presented in Figure 4.16. There was no significant ($P \geq 0.05$) difference in body weight of the mice treated with diminazeneaceturate and the control group. The weight of mice in infected untreated group andinfectedtreated with HDN-one at different doses decreased significantly ($P \leq 0.05$).

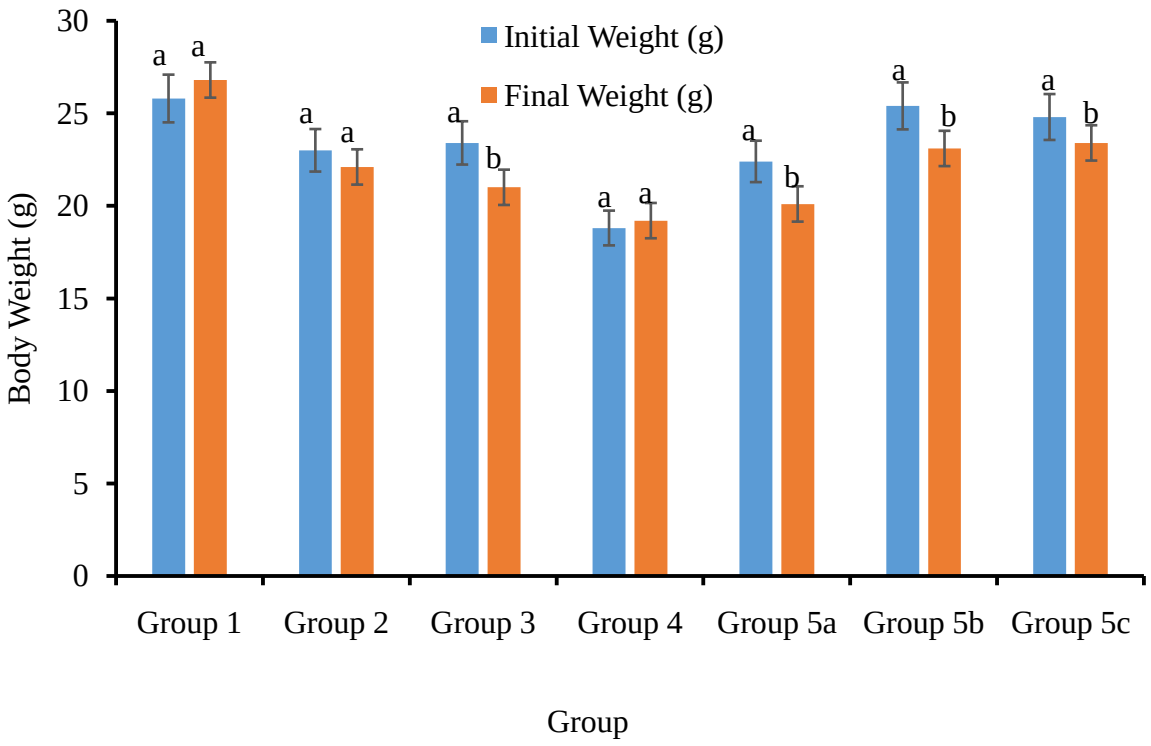


Figure 4.16: Mean Body Weight of *T. b. brucei* Infected Albino Mice Treated with HDN-oneand Diminazene Aceturate

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them.

Keys:

Group 1= uninfected and untreated

Group 2 = uninfected and treated with the compound at 1000 mg/kg b.w

Group 3 = infected with *T. brucei brucei*and untreated

Group 4 = infected with *T. brucei brucei* and treated with diminazene at 3.5 mg/kg b.w

Group 5a = infected with *T. brucei brucei*andtreated with the compound 500 mg/kg b.w

Group 5b = infected with *T. brucei brucei*andtreated with the compound 1000 mg/kg b.w

Group 5c = infected with *T. brucei brucei*andtreated with the compound 2000 mg/kg b.w

4.28 Inhibitory effect of 50 µM of HND-one on Trypanosome Alternative Oxidase

The inhibitory effect of 50 µM of HDN-oneon trypanosome alternative oxidase is presented in Figure 4.17.There wasanincease in the activity of the enzyme in the presence of HDN-one as the time increases.

4.29 Inhibitory effect of 50 µM of HND-one on *T. b. gambiense*glycerol kinase

The inhibitory effect of 50 µM of HDN-oneon *Trypanosoma b. gambiense*glycerol kinase is presented in Figure 4.18. There was an increase in the activity of the enzyme in the presence of HDN-one as the time increases.

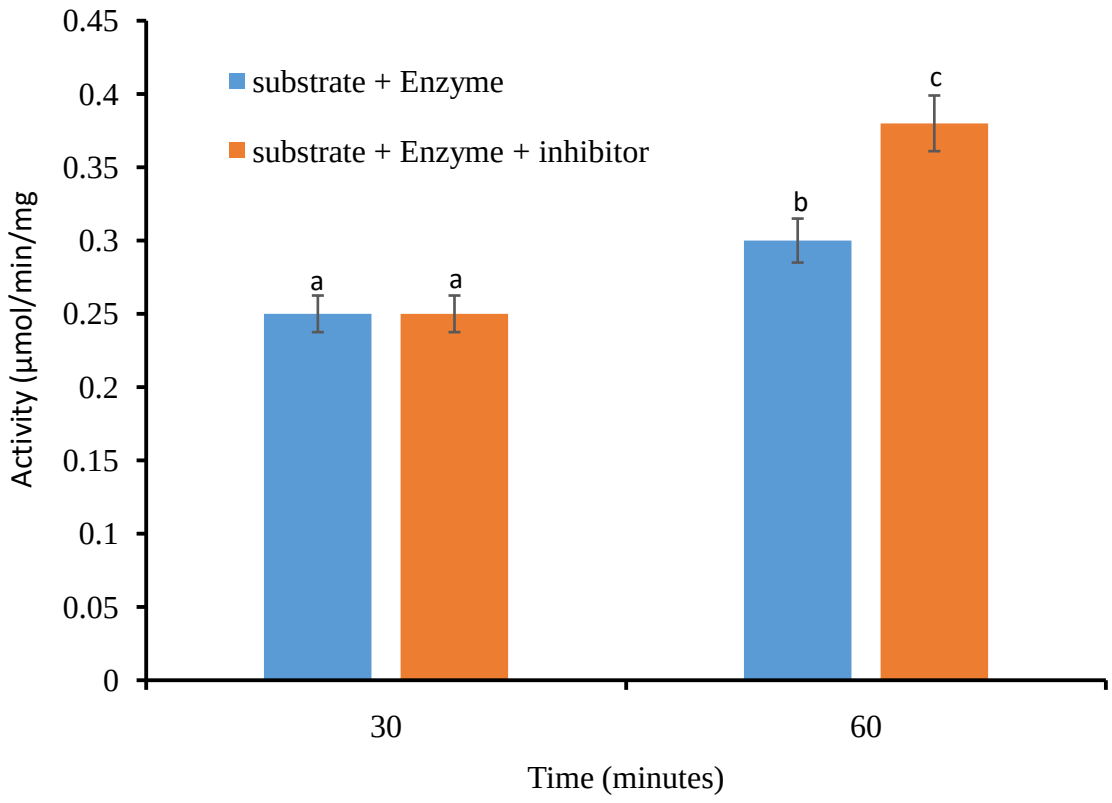


Figure 4.17: Inhibitory effect of 50 µM HDN-oneon Trypanosome Alternative Oxidase

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them

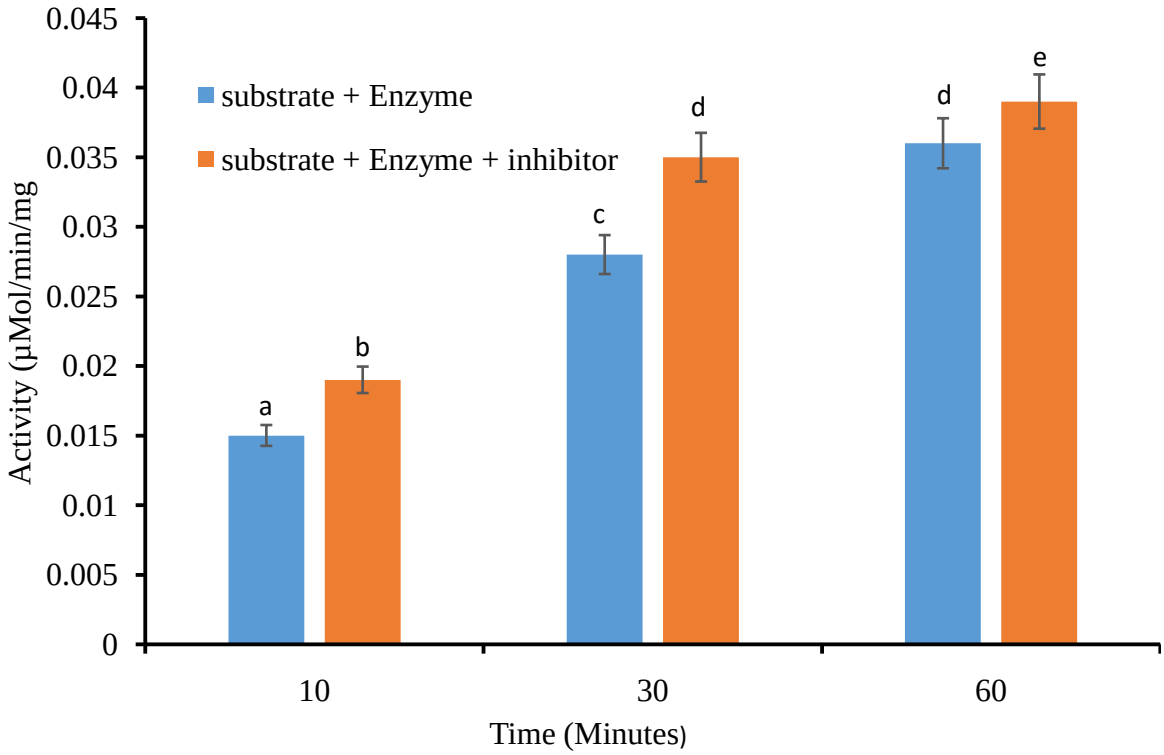


Figure 4.18: Inhibitory effect of 50 µM of HDN-oneon*T. b. gambiense*glycerol kinase

In relation to each parameter, bars labeled with distinct alphabets represent significant differences (P≥0.05) among them

CHAPTER FIVE

5.0 DISCUSSION

In this study the bioassay guided isolation of anti-trypanosomal compound from *V. album* leaves and stems obtained from five hosts were reported.The results of the methanol extraction of *V. album* leaves and stemsfrom the five hostsshowed that *Viscum album* leaves from *P. guajava* had the highest yield while *M. oleifera* stem had the lowest yield. The yield of the leaves extracts was higher than the stem extracts for all the samples. All the crude plant extracts were evaluated *in vitro*for antitrypanosomal activity. The extracts were constituted indistiled water.

The *in vitro*antitrypanosomal activity of the methanol crude extract of *V. album* leaves and stem from *A. albida* and *A. indica* showed activity against *T. brucei brucei*. *Viscum album* leaves from *A. albida* had the best activity with the IC_{50} of 0.30 mg/mL and the stem had the lowest activity with the $IC_{50}$of 0.77 mg/mL.Ndidi*et al*.(2014)andEnyanwa*et al*. (2018) reported the antitrypanosomal activity of the hosts: *Acacia albida* and *Azadirachta indica* respectively. The results of this study agree with the findings of Luczkiewiez*et al*. (2001) and Vicsa*et al*. (2011) that the bioactivity of *Viscum album* depends on the host plants.Phytochemical evaluation of the methanol extract of Leavesof *V. album* from *A. albida* showed the presence of alkaloids, tannins, saponins, anthraquinone, phenols, steroids, terpenes, courmarins, flavonoids, and glycosides which is in agreement with the reports by Wassagwa*et al*., 2018).Further more, these phytochemicals were reported from *Viscum album* species of apple, guava, kolanut, orange, pear and cocoa trees (Hass *et al*., 2003;

Oguntoye,*et al*., 2008 and Taiga, 2013). However, previous studies by Yusuf *et al*. (2013) have also identified the presence of terpenoids, phytates, oxalates, and cardiac glycosides in *Viscum album* obtained from cocoa and cola trees in Nigeria. Similarly, Ilesanm and Olawoye (2011) reported the occurrence of saponins, tannins, anthraquinones, and cardenolides in the stem and leaves of *Viscum album* obtained from orange and guava trees, but noted that phlobatannins were only found in guava. Furthermore, several reports (Luczkiewicz*et al*., 2001; Ekhaise, 2010; Vica*et al*., 2011) have highlighted that the chemical composition of *Viscum album* species can vary depending on factors such as the time of harvest and the specific host trees on which the plant grows.

The variation in percentage yield of the partitioned fraction of the methanol leaves extract of *V. album* from *A. albida* was dependent on the polarity of the solvent used. The yields of n-hexane, ethyl acetate, n-butanol and aqueous solvents reflects the presence of different proportions of constituents of different polarity. N-butanol-soluble constituents had the highest percentage yield being polar while n-hexane had the lowest percentage yield being the non- polar.

Column chromatography of the n-butanol fraction that showed the best *in vitro* activity against *T. b. brucei* was followed by thin-layer chromatography and bioassay guided activity that lead to the isolation of [2-(4-hydroxyphenyl) ethyl]-2,6-dioxabicyclo [3.3.1] nonan-3-one (HDN-one). The compound was obtained as white crystalline solid soluble in ethyl acetate at room temperature. Thin layer chromatogram of the isolated compound using the solvent system n-hexane: ethyl acetate (2:8) showed a single reddish brown spots with Rf value of 0.70.

The structure of HDN-one was established by extensive NMR analysis. A similar compound to HDN-one was isolated by (Ouedragogo*et al*., 2007) from *Tapinanthusdodoneinfolius* which belongs to same family with *V. album* but different species.

In the acute toxicity study of HDN-one in mice, the administration of 2,000 mg/kg b.w did not cause mortality among the experimental mice in both the first and second trials, each consisting of three mice. However, when mice were administered with 5,000 mg/kg b.w,dealth occurred within 24 hours. According to OECD (2001) guidelines, the use of 5,000 mg/kgb.w should only be considered if there is a strong likelihood that the test outcomes is directly relevant to protecting people and animal health.In cases where the main study conducted at a dose of 2,000 mg/kgb.w shows no signs of toxicity, the chemical can be classified as category 5, indicating substances with low acute toxicity hazard. This classification represents the lowest level of toxicity according to OECD (2001) guidelines. Therefore, it can be concluded that HDN-one is well-tolerated up to a dose of 2,000 mg/kg b.w when given as a single dose.

There were no clinical signs observed when 2000 mg/kg b.wof HDN-one was given to mice, howeverclinical signs associated with the administration of 5000 mg/kg b.w of HDN-onewere weakness, arched back, bulging eyes, unsteady movements, lack of appetite, dizziness, dispnoea, sleep, coma and death occurred in 24 hrs. However, administration of 2000 mg/kg b.w did not cause dealthwithin 24 hrs' and during the two weeks' observation

Body weight changesare an indicator of adverse side effects (CCAC, 1998**)**, and loss of more than 20% of the animal body weight is regarded as critical and has been defined as

one of the humane endpoints in several international guidelines (Chitra *et al*., 2015; CCAC, 1998**)**. In this study, there were no significant changes in body weight of mice in the treated groups compared to control.

Another index of the potential toxicity of a substance is the effect it might have on relative organ weights (Kulkarni and Veeranjaneyulu, 2010). The heart, liver, kidneys, spleen, and lungs are the first organs to display metabolic responses to poisonous substances (Ara and Usmani, 2015) Thus, relative organ weight could serve to diagnose any possible injury to organs exposed to toxic materials (Teo *et al*., 2002). Important differences in organ weight between treated and control animals may occur without any morphological changes in response to toxicant exposures (Rong *et al*., 2009) In the acute toxicity study of HDN-one, no significant differences ($p \leq 0.05$) were detected between the treatment and control mice in terms of relative weight of heart spleen, liver, kidney, or lung weights which implies HDN-one was not toxic to the organs.

Hematological parameters play major role in determining the toxicity induced by drugs (Petterino and Argentino-Storino, 2006) Blood parameters analysis is appropriate to risk evaluation as the haematological system has a higher prognostic value for toxicity (Olson *et al*., 2000). Blood serves as the main medium of transport for many drugs and xenobiotics in the body and for that reason components of the blood are exposed to substantial concentrations of toxic compounds. In the present acute toxicity study of HDN-onein mice, the haematological indices indicated that the compound did not affect packed cell volume (PCV), haemoglobin (Hb), red blood cell (RBC) and total white blood cell count (WBC) production as there was no significant difference between the control that received the

vehicle and groups treated with the compound. The results suggest that HDN-one was safe as the compound did cause changes in the constituents of the blood.

Evaluation of kidney and liver function is important in the assessment toxicity of plant extracts as both of them are necessary for the survival of an organism (Olorunnisola*et al*., 2012). In animal model toxicity studies, the serum level of creatinine remains the most widely used laboratory test to estimate renal function. It is kept within a relatively stable range as daily production and renal excretion are continuous in healthy mammals (Yilmaz *et al*., 2007). In this study, for kidney function test, two serum renal biochemical parameters, namely urea and creatinine were analyzed. There were no significant changes observed in urea and creatinine levels between the control and the groups treated with HDN-onein acute toxicity tests. The liver function parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were studied to evaluate effect of HDN-one liver function. Liver enzymes levels are usually raised in acute hepatoxicity but tend to decrease with prolonged intoxication due to damage to the liver (Imafidon and Okunrobo, 2012). In this acute study, there was no significant difference in the levels of TSP, ALP, AST and ALT compared to that of control which implies that renal and hepatic function were not compromised during the assay.

The *in vivo* study revealed that parasitemia was established in the inoculated groups of mice 4^{th} day post infection. The infected untreated group showed the highest parasitaemia levels. The parasitaemia of the infected group treated with diminazene was cleared 7^{th} day post infection. There was an increase in the parasitemia levels of theinfected groups treated with HDN-oneat 1000 mg/kg and 2000 mg/kg, however the increase was significantly lower compared to the infected untreated control. This is consitent with Biryomumaisho

and Katunguka-Rwakishaya (2007) and Alli *et al.* (2011) who proffered that parasitaemia correlates with the severity of infection and a 50% reduction in parasitaemia is an indication of significant activity of a trypanocide. Similarly, the compound significantly reduced weight loss in the test group compared to the infected untreated group.

Enzymes currently make up a significant portion, typically one-third or more, of the distinct drug targets included in the portfolios of major pharmaceutical companies. TAO and GK are critical enzymes involved in the energy metabolism survivalof trypanosomes. TAO and GK were selected because they were available, from our report of *in vitro* and *in vivo*antitrypanosomal effect of HND-one, the inhibitory effect of the compound was evaluated on TAO and GK. The results revealed that 50µM of the compound had no effect on both TAO and GK activityas there was a significant increase in the activity of the enzyme in the presence of the HDN-one as the time increases.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENATIONS

6.1 Summary

The methanol extracts of *V. album* leave and stems harvested from *A. albida* and *A. indica* showed *in vitro* antitrypanosomal activity against *T. b. brucei.* The leaves extract of *V. album* from *A. albida* was the most effective (IC_{50} = 0.30 mg/mL). Phytochemistry of the methanol extract of *V. album* leaf from *A. albida* showed the presence of alkaloids, tannins, saponins, anthraquinones, phenol, steroids, terpenes, courmarins, flavonoids, carbohydrate and glycosides. Bioassay guided fractionation of the methanol extract of *V. album* leaves from *A. albida* and subsequentpurification of the resulting bioactive compound led to the isolation of HDN-onewith *in vitro* activity against *T. b. brucei* (IC_{50} = 0.26 mg/ mL).The intraperitoneal acute toxicity study of HDN-onein mice did not show signs of toxicity at a dose level of 2000 mg/kgb.w and had no effect on body and organ weight change of mice.The *in vivo* assay revealed that HDN-onereduced parasitemia levels and body weight loss in *T. b. brucei* inoculated mice. The compound had no inhibitory effect on trypanosome alternative oxidase and trypanosome glycerol kinase.

6.2 Conclusion

The findings of this study revealed that HDN-one, derived from *Viscum album*, serves as the active compound against *T. b. brucei*.The compound did not show toxicity in albino mice at a dose of 2000 mg/kg b.w and could be explored as a lead compound towards identification of more efficient anti-trypanosomal drug.

6.3 Recommendations

1. Detailed study on subacute and chronic toxicity assay of the compound should be carried out to characterize its potential hazards
2. The compound should be tried against other species of trypanosomes

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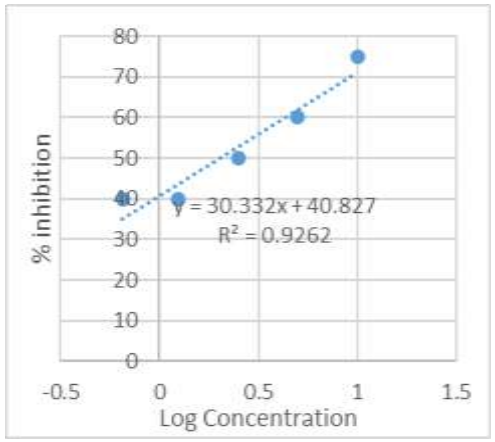
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APPENDICES

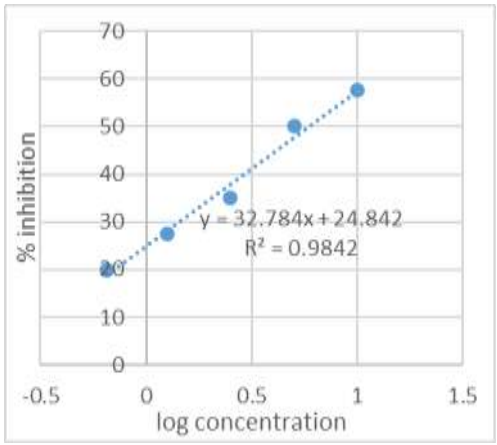
APPENNDIX I: Yield of the Methanol Leaves and Stems Extract of *Viscum album* from Five Host Trees

V. album parts	Host	Sample (g)	Yield (g)	% Yield
Leaf	*A. indica*	100	9.68	9.68
Stem	*A. indica*	100	4.35	4.35
Leaf	*A. albida*	100	18.77	18.77
Stem	*A. albida*	100	5.83	5.83
Leaf	*P. guajava*	100	14.78	14.78
Stem	*P. guajava*	100	5.95	5.95
Leaf	*M. oleifera*	100	8.89	8.89
Stem	*M. oleifera*	100	3.02	3.02
Leaf	*K. senegelensis*	100	13.54	13.54
Stem	*K. senegelensis*	100	5.96	5.96

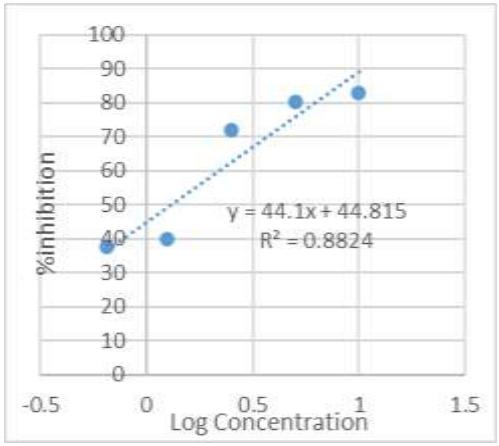
APPENDIX II: IC_{50} Graphs of the Active Methanol Extracts of *Viscum album* Leaf and Stem of *A. albida* and *A. indica*



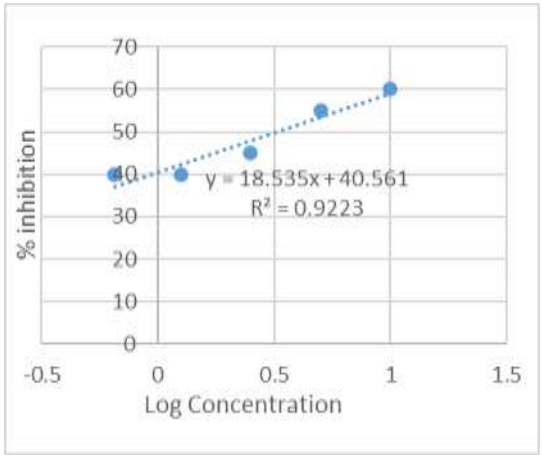
The IC_{50} graph ofV. *album* Leaves of *A. albida*



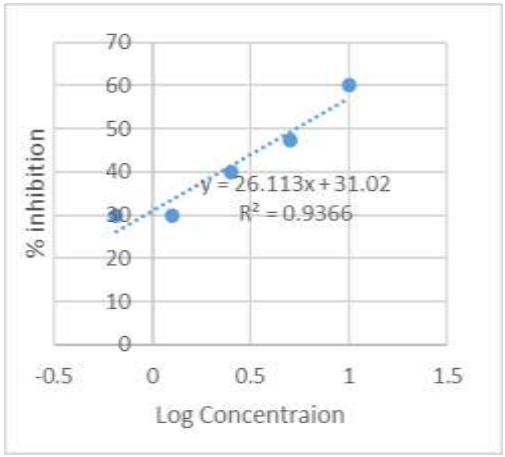
The IC_{50} graph ofV. *album* stems of *A. albida*



The IC_{50} graph ofDiminazene



The IC_{50} graph ofV. *album* Leaves of *A. indica*



The IC_{50} graph ofV. *album* stems of *A. indica*

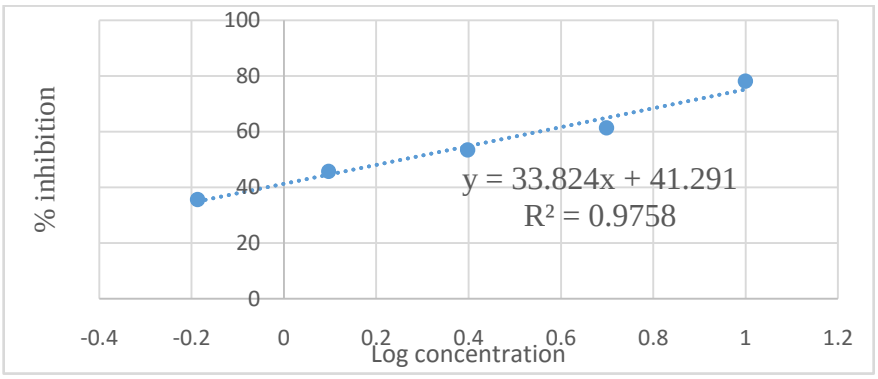
APPENDIX III: Quantitative Phytochemical Profile of the Methanol Extract of *Viscum album* Leaves of *A. albida*

Phytoconstituent	Standard compound	extract (mg)	constituent (mg)	(%)
Phenolics	Gallic acid	10	0.082	0.82
Flavanoids	Quercetin	10	0.004	0.04
Alkaloids	Atropine sulphate	10	0.191	1.91
Saponins	Saponin	10	0.202	2.02

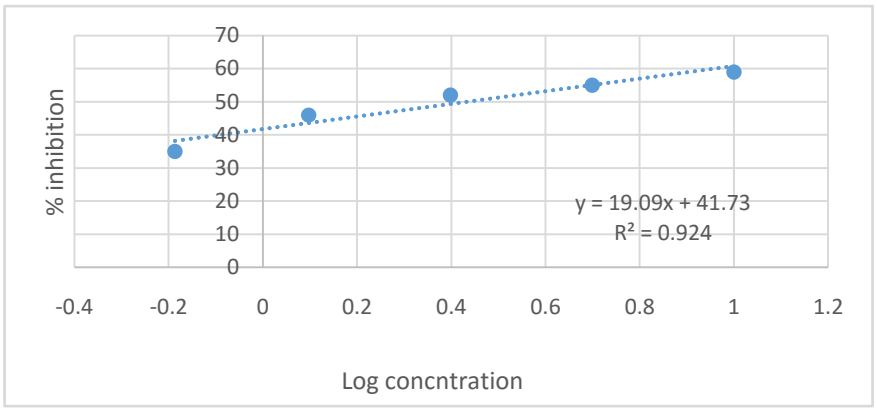
APPENDIX IV: Solvent Partition Yield of the Methanol Extract of *Viscum album* Leaves of *A. albida*

Solvent	Yield (g)	(%) Yield	Activity	Colour
n-hexane	0.8	0.54	-	Dark brown
Ethyl acetate	3.6	2.42	-	Dark brown
n-butanol	67.21	45.26	+	Dark brown
Water	43.56	28.00	-	Dark brown

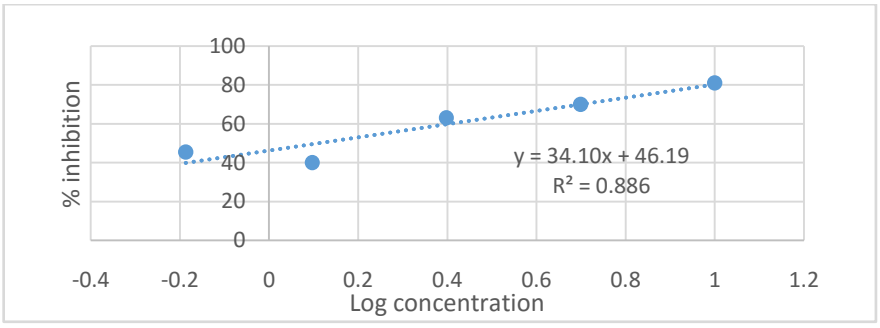
APPENDIX V: The IC_{50} Graph of the Active Fractions



The IC_{50} graph of fraction (24 – 35)



The IC_{50} graph of fraction (16 – 23)



The $1C_{50}$ graphof Diminazene

APPENDIX VI: Mean Body Weight of Mice in Acute Toxicity Test of HDN-one

Dose mg/kg	Initial Body weight (g)	Final Body weight (g)	Weight change (g)
2000(Step 1)	28.33 ± 1.53[a]	29.67 ± 1.53[a]	1.33 ± 0.58[a]
2000 (Step 2)	27.3 3± 1.53[a]	28.67 ± 1.53[a]	1.33 ± 0.58[a]
Control	29.33 ± 1.53[a]	31.6 7± 1.15[a]	2.33 ± 0.58[a]
p-value	0.343	0.098	0.125

APPENDIX VII: Mean Relative Organ Weight of Mice in Acute Toxicity Test of HDN-one

Organ (g)	2000 mg/kg (Step 1)	2000 mg/kg (Step 2)	Control	*p*-value
Liver	8.62 ± 0.16^{a}	9.20 ± 0.16^{a}	9.52 ± 0.16^{a}	0.359
Kidney	4.45 ± 0.04^{a}	4.49 ± 0.14^{a}	4.81 ± 0.03^{a}	0.680
Lungs	2.21 ± 0.04^{a}	2.08 ± 0.05^{a}	2.23 ± 0.05^{a}	0.168
Heart	0.80 ± 0.05^{a}	0.97 ± 0.02^{a}	0.77 ± 0.02^{a}	0.098
Spleen	1.04 ± 0.10^{a}	0.11 ± 0.04^{a}	1.05 ± 0.03^{a}	0.787

APPENDIX VIII: Mean Values of Haematological Parameters of albino Mice in Acute Toxicity Test of HDN-one

Parameter	Unit	Control	2000 mg/kg (Step 1)	2000 mg/kg (Step 2)	*p*-value
PCV	%	47.67±2.52[a]	43.67 ± 4.04[a]	45.00 ± 1.00[a]	0.282
Hb	g/dL	17.33±8.08[a]	16.10± 10.46[a]	16.50 ± 15.27[a]	0.975
WBC	$(\times 10^9)$ mm^3	6.13±0.65[a]	6.13 ± 0.95[a]	6.03 ± 0.31[a]	0.979
RBC	$(\times 10^6)$ mm^3	7.20±0.75[a]	6.05 ± 0.21[a]	6.0 7± 0.60[a]	0.083
TP	g/dL	5.30±0.78[a]	4.83 ± 0.87[a]	4.70 ± 0.57[a]	0.634

APPENDIX IX: Mean Values of Biochemical Parameter of Mice in Acute Toxicity Test of HDN-one

Parameter	Unit	2000 mg/kg (Step 1)	2000 mg/kg (Step 2)	Control	*p*-value
Albumin	g/dL	$4.20 \pm {}^{0.78a}$	4.43 ± 0.50^{a}	4.57 ± 0.74^{a}	0.808
Creatinine	mg/dL	1.00 ± 0.02^{a}	0.97 ± 0.31^{a}	1.00 ± 0.02^{a}	0.981
ALP	U/L	46.33 ± 6.07^{a}	48.33 ± 4.16^{a}	45.00 ± 4.40^{a}	0.892
ALT	U/L	59.00 ± 9.53^{a}	56.67 ± 9.71^{a}	66.00 ± 5.57^{a}	0.428
AST	U/L	76.67 ± 10.02^{a}	80.67 ± 11.15^{a}	81.00 ± 9.00^{a}	0.847
BUN	mg/dL	16.33 ± 2.30^{a}	16.33 ± 4.04^{a}	15.67 ± 2.51^{a}	0.954

APPENDIX X: The Mean Parasite Count of *Trypanosoma brucei brucei* Infected Mice Treated with HDN-oneand Diminazene Aceturate

Post infection Days	Group 3 infected untreated	Group 4 3.5 mg/kg Diminazene	Group 5a 500 mg/kg	Group 5b 1000 mg/kg	Group 5c 2000 mg/kg	*p*-value
3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NA
4	1.50 ± 0.71^{a}	1.50 ± 0.71^{a}	1.67 ± 0.58^{a}	1.67 ± 0.58^{a}	1.25 ± 0.50^{a}	0.234
5	2.25 ± 1.26^{a}	1.80 ± .45^{a}	4.00 ± 1.87^{a}	3.25 ± 1.92^{a}	1.50 ± 0.58^{a}	0.085
6	6.00 ± 2.45^{ab}	2.80 ± 1.30^{bc}	7.40 ± 3.85^{a}	6.40 ± 1.82^{a}	2.25 ± 0.50c	0.011
7	11.75 ± 3.30^{a}	1.00 ± 0.00^{b}	12.80 ± 3.56^{a}	11.80 ± 2.28^{a}	4.50 ± 0.58^{b}	0.000
8	21.50 ± 3.87^{a}	1.00 0.00^{d}	18.40 ± 2.70^{b}	16.40 ± 1.82^{c}	7.25 ± 3.59^{d}	0.000
9	31.35 ± 1.00^{a}	0.00 ± 0.00^{c}	36.00 ± 5.43^{a}	31.00 ± 1.41^{a}	12.50 ± 6.60^{b}	0.000
11	128.00 ± 0.00^{a}	0.00 ± 0.00^{e}	71.80 ±12.69^{b}	62.60 ± 8.11^{c}	35.00 ± 7.07^{d}	0.001
12	-	0.00 ± 0.00^{c}	111.33 ± 16.00^{a}	72.00 ± 8.18^{b}	49.7 5± 10.34^{c}	0.000
13	-	0.00 ± 0.00^{c}	120.00 ± 0.00^{a}	94.00 ± 6.92^{b}	69.66 ± 10.69^{c}	0.001
14	-	0.00 ± 0.00^{c}	-	117.67 ± 7.50^{a}	80.00 ± 0.00^{b}	0.017
15	-	0.00 ± 0.00^{b}	-	125.00 ± 4.24^{a}	-	0.049

APPENDIX XI: Mean Body Weight of *T. b. brucei* Infected Mice Treated with HDN-oneand Diminazene Aceturate

Days of experiment	Group 1 Uninfected Untreated	Group 2 Uninfected 1000 mg/kg	Group 3 Uninfected untreated	Group 4 3.5 mg/kg Diminazene	Group 5A 500 mg/kg	Group 5B 1000 mg/kg	Group 5C 2000 mg/kg	*p*-value
1	5.80±1.64	23.00±5.4	23.40±2.61	18.80±2.39	22.40±3.36	25.40±3.65	24.80±2.47	0.000
4	26.00±1.22	22.80±4.44	23.20±1.48	12.59±8.00	22.60±2.96	25.20±2.68	25.00±1.58	0.000
7	26.40±1.82	21.60±3.21	22.20±2.6	19.60±2.40	22.40±3.20	25.80±2.94	25.60±2.96	0.000
9	26.80±2.05	22.40±2.13	21.00±2.64	19.60±2.30	21.75±3.20	24.80±1.00	22.33±0.58	0.004
12	27.60±2.07	19.80±2.38	-	20.60±2.07	-	24.00±1.00	22.33±0.58	0.002

APPENDIX XII: Inhibitory Effect of 50 µM HDN-oneon Trypanosome Alternative Oxidase

Test	Enzyme activity ((µMol/min/mg	
	30 minutes	60 minutes
Substrate + Enzyme	0.247 ± 0.11	0.3 ± 0.10
Substrate + Enzyme + inhibitor	0.253 ± 0.07	0.38 ± 0.03

APPENDIX XIII: Inhibitory Effect of 50 µM of HDN-oneon *T. b. gambiense* Glycerol Kinase

Test	Enzyme activity (µMol/min/mg)		
	10 minutes	30 minutes	60 minutes
Substrate + Enzyme	0.015 ± 0.06	0.028 ± 0.20	0.036 ± 002
Substrate + Enzyme + inhibitor	0.019 ± 0.030	0.035 ± 0.04	0.039 ± 0.05

APPENDIX XIV: Certificate for Paper Presentation at a Conference 1



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KWAME NKRUMAH WAY, UNIVERSITY OF NIGERIA, NSUKKA CAMPUS, ENUGU STATE.

CERTIFICATE FOR PAPER PRESENTION

TITLED

Toxicity studies and in vivo antitrypanosomal evaluation of HND-ONE isolated from Viscum album leave extract

TO

John Wassagwa

AT THE INTERNATIONAL CONFERENCE ON BIOTECHNOLOGY FOR FOOD SECURITY AND HUMAN HEALTH

16 – 17 NOVEMBER, 2022

DISTINGUISHED PROF
UMEZURUIKE LINUS OPARA
Convener & Conference Chair

PROF BENJAMIN UBI
CO-Chair & Coordinator
of Scientific Programme

APPENDIX XV: Certificate for Paper Presentation at a Conference 2



Nov22PP16

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KWAME NKRUMAH WAY, UNIVERSITY OF NIGERIA, NSUKKA CAMPUS, ENUGU STATE.

CERTIFICATE FOR PAPER PRESENTION

TITLED

Bioassay guided isolation and characterization of trypanocide(s) from mistletoe (Viscum album) extract

TO

John Wassagwa

AT THE INTERNATIONAL CONFERENCE ON BIOTECHNOLOGY FOR FOOD SECURITY AND HUMAN HEALTH

16 – 17 NOVEMBER, 2022

DISTINGUISHED PROF
UMEZURUIKE LINUS OPARA
Convener & Conference Chair

PROF BENJAMIN UBI
CO-Chair & Coordinator
of Scientific Programme

APPENDIX XVI: Certificate of Conference Participation

