EVALUATION OF THE ANTHELMINTIC EFFICACY OF EXTRACTS OF *CITRUS AURANTIFOLIA* (CHRISTM) SWINGLE FRUIT PEELS IN MICE EXPERIMENTALLY INFECTED WITH *HELIGMOSOMOIDES BAKERI*

BY

ONYILOFE SUNDAY ENEJOH

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY,

FACULTY OF VETERINARY MEDICINE

AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA

SEPTEMBER, 2014

EVALUATION OF THE ANTHELMINTIC EFFICACY OF EXTRACTS OF *CITRUS AURANTIFOLIA* (CHRISTM) SWINGLE FRUIT PEELS IN MICE EXPERIMENTALLY INFECTED WITH *HELIGMOSOMOIDES BAKERI*

BY

Onyilofe Sunday ENEJOH, DVM (A.B.U) 2010
MSc/VET-MED/2129/2011-2012

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER DEGREE IN VETERINARY PHARMACOLOGY

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY,
FACULTY OF VETERINARY MEDICINE
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA

SEPTEMBER, 2014

DECLARATION

I declare that the work in this thesis entitled **"Evaluation of the anthelmintic efficacy of extracts of *Citrus aurantifolia* (Christm) Swingle fruit peels in mice experimentally infected with *Heligmosomoides bakeri*"** has been carried out by me in the Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine. The information derived from the literature has been duly acknowledged 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 other institution.

Onyilofe Sunday ENEJOH _____ _____

Signature **Date**

CERTIFICATION

This thesis entitled **"EVALUATION OF THE ANTHELMINTIC EFFICACY OF EXTRACTS OF *CITRUS AURANTIFOLIA* (CHRISTM) SWINGLE FRUIT PEELS IN MICE EXPERIMENTALLY INFECTED WITH *HELIGMOSOMOIDES BAKERI*"** by Onyilofe Sunday ENEJOH meets the regulations governing the award of the degree of Master of Veterinary Pharmacology of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Dr. M.M. Suleiman Chairman, Supervisory Committee	Signature	Date
Prof. S.F. Ambali Member, Supervisory Committee	Signature	Date
Prof. J.O. Ajanusi Member, Supervisory Committee	Signature	Date
Dr. M.M. Suleiman Head, Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria	Signature	Date
Prof. A. A. Joshua Dean, School of Postgraduate Studies, Ahmadu Bello University, Zaria	Signature	Date

ACKNOWLEDGEMENTS

I thank God Almighty for sparing my life and for granting me the privilege to study at this time. I appreciate Him for making ways for me in the midst of difficult situations, to Him be all the glory, honour, power and praises, now and forever (Amen).

My profound gratitude goes to my supervisors, Dr. M.M. Suleiman, Prof. S.F. Ambali, and Prof. J.O. Ajanusi for their guidance, counselling and corrections during the course of this research. I have benefited greately from their wealth of knowledge and experiences. I also want to appreciate them for the time devoted to the reading and corrections of this thesis. May God reward all their efforts (Amen).

I want to appreciate the effort of Dr. Ahmed Abubarkar from the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences for guiding me through the phytochemical analysis of the plant. My sincere gratitude to Dr. L.A. Ngongeh from the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka for providing the helminth parasite used for this study. I am also grateful to Dr. David Oshadu for guiding me through the laboratory procedures for culturing the parasite.

I am grateful to all technical staffs of the Department of Veterinary Physiology, Pharmacology and Toxicology laboratory especially, Mr. M. E. Nwosu, Mr. Dennis Otie, Mr. A. Sani, Mr. D.D. Dagai, Mr. Idris and Mr. S. Wakala for their technical assistance. In the same vein, I want to thank Mr. Amadi. N, Mr. Yusuf and Mr. Sha'aya'u from Veterinary helminthology laboratory section, Department of Parasitology and Entomology, as well as Mr. Kabir from Pharmacognosy and Drug development laboratory, Faculty of Pharmaceutical Sciences for their technical assistance. I am grateful to Mallam U.S. Gallah of the Department of Biological Sciences for assisting me in the identification of the plant, Dr. Sambo of the Department of Veterinary Pathology for examining, interpreting and taking photomicrographs of the histopathologic slides.

I want to appreciate all my colleagues who have contributed immensely to the success of this research. They include: Dr. Tauheed Mohammed, Dr. Buhari Habibu, Dr. Bobwealth Omontese, Dr. Delia Ussa, Dr. Enokela Idoga, Dr. Uchendu Chidebere, Dr. Olusola Oladipo, Dr. Smart Madu Bala, Dr. I. Ifeanyi, Dr. Friday Zakari, Dr. Khadija Shaibu and Dr. Danladi Baba. I want to appreciate Mr. Jacob Eneojo, Mr. Jonathan Otokpa, Mr. Jolly Kachi, Dr. Sotonye Isaiah Oruene, Dr. Ogechukwu Peace Ani, Dr. Fabian Oku Umoh, Mr. Sunday Apeh and Miss. Ekaete Constant Udoh for their care and support, and also, for their financial assistance during the course of my research. I'm particularly grateful to Miss. Mary Ogberefor, my beloved friend and sister who has been supportive all the time. Special thanks to all the DLCF P.G ABU, Zaria members, particularly, Victor Babatunde,

Micheal Yakubu, Bello Ibrahim, Ibrahim Isah, Francis Peter, Esther Benjamin, Ronke Oyeleke, Solomon Balogun, and Racheal Akpa for their prayers, encouragement and moral support.

I want to thank my beloved uncle; Mr. Shaibu Enejoh for his fatherly support. I want to appreciate him for sponsoring my post-graduate programme, and also, for financing this research. I lack words to express my profound gratitude. God will continue to increase him more and more. I want to thank my family members especially my mum; Veronica. P. Enejoh and my siblings; Victory, Pheobe, Favour, Onyemowo, Echeofun, Olofu and Idoko. They are wonderful to me and I appreciate all their supports.

DEDICATION

This thesis is dedicated to God Almighty and to my late father, Mr. Peter Enejoh.

ABSTRACT

Citrus aurantifolia, commonly known as Lime orange belonging to the family Rutaceae is one of the widely used plants in African and Asian traditional medicine. The plant is commonly available in Nigeria. This present study aimed at evaluating the *in vitro* and *in vivo* anthelmintic activity of the fruit peels of *Citrus aurantifolia* using *Heligmosomoides bakeri* as a model. The fruits of *Citrus aurantifolia* were washed, peeled, dried, pulverized and 821 g of it extracted with methanol which yielded 110 g of crude methanol extract (CME) which upon partitioning of 70 g yielded 2.0 g (2.9 %), 4.7 g (6.7 %), 17.5 g (25 %) and 30.7 g (43.9 %) of hexane extract (HE), ethylacetate extract (EE), butanol extract (BE), and aqueous methanol extract (AME), respectively. Preliminary phytochemical screening of the extracts revealed the presence of carbohydrates, glycosides, steroids, flavonoids, phenols, alkaloids, saponins, triterpenes and condensed tannins. Acute toxicity study of the crude methanol extract in mice showed that the extracts were non-toxic even at a dose of 5000 mg/kg. The *in vitro* anthelmintic studies involve the evaluation of ovicidal (Egg hatch inhibition test) and larvicidal activities of the extracts on the eggs and first stage larvae of *H. bakeri*. Different concentrations (0.625, 1.25, 2.5, 5, 10, and 20 mg/ml) of the extracts were prepared by dissolving the extracts in distilled water. Two hundred microlitres of each concentration was incubated with the larvae and eggs of *H. bakeri* contained in 0.2 ml solution in a 96-well microtitre plates and incubated at room temperature for 24 and 48 hours, respectively. Distilled water and albendazole were used as non-treated and treated controls, respectively. In the *in vivo* anthelmintic study, seventy mice infected with *H. bakeri* were randomly allocated into 14 groups of 5 mice each. Groups 1-3, 4-6, 7-9, 10-12 were treated with crude methanol extract (CME), aqueous

methanol extract (AME), ethylacetate extract (EE) and butanol extract (BE), respectively at doses of 250, 500 and 1000 mg/kg. Groups 13 and 14 were treated with albendazole (10 mg/kg) and distilled water (5 ml/kg), respectively, and serve as treated and non-treated controls. All treatments were administered orally on the 16^{th}, 17^{th} and 18^{th} day post infection. Anthelmintic activity was assessed by comparing the faecal egg count reduction and the worms recovered from the treated groups with the non-treated control group.

At 20 mg/ml, the CME, EE, HE, AME and BE inhibited the hatching of *H. bakeri* eggs by 100, 98.2, 97.8, 97.7, and 28.2 %, respectively. Similarly, CME, BE, AME, EE and HE caused death of the larvae of *H. bakeri* by 100, 94.4, 90.8, 85.9 and 83.3 %, respectively. All the extracts showed significant ($p<0.001$) anthelmintic effect when compared with albendazole and the non-treated (DW) control. In the *in vivo* anthelmintic study, at the dose of 1000 mg/kg, CME, BE, EE and AME produced a faecal egg count reduction rate of 91, 80, 71 and 58 %, respectively. Similarly, BE, CME, EE and AME caused a deparasitization rate of 79, 75, 75 and 51 %, respectively. CME, BE and EE produced a significant ($p<0.05$) deparasitization rate. This study demostrates that *Citrus aurantifolia* fruit peels possess anthelmintic activity that might be caused by one or more of the secondary metabolites contained in the plant.

TABLE OF CONTENTS

Page

Title Page……...……ii

Declaration……..……..iii

Certification……..…iv

Acknowledgements……v

Dedication……vii

Abstract ……..…… …….viii

Table of Contents……..x

List of Figures……xvi

List of Tables…….xvii

List of Plates……xix

List of Appendices ……..xx

List of Abbreviations……...xxi

List of symbols…….……..xxii

1.0 INTRODUCTION……..1

1.1 Statement of Research Problems……...3

1.2 Justification…….....4

1.3 Aim and Objectives…….6

1.4 Null Hypothesis ……..6

2.0 LITERATURE REVIEW……..7

2.1 The Plant *Citrus aurantifolia* (Christm) Swingle…….…7

2.1.1 Plant Description……7

2.1.2 Brief History and Geographical Distribution of *Citrus aurantifolia*…….8

2.2 Domestic Uses of *Citrus aurantifolia* ……11

2.3 Ethnomedical Uses of *Citrus aurantifolia*……..……...12

2.3.1 Leaves…..……12

2.3.2 Juice…….....13

2.3.3 Other parts of the plant…….13

2.4 Pharmacological Activities of *Citrus* aurantifolia……...14

2.4.1 Antibacterial activity……14

2.4.2 Antifungal and antiaflatoxigenic activity…….14

2.4.3 Anticancer/ cytotoxic activity…….15

2.4.4 Antioxidant activity ……15

2.4.5 Immuno-modulatory activity ……..16

2.4.6 Antiobesity activity ……16

2.4.7 Antifertility activity……17

2.4.8 Anticholinesterase and antiplatelet activity……17

2.4.9 Cardiovascular activity…….18

2.4.10 Effects on the bone ……..18

2.4.11 Other effects ……18

2.4.12 Anthelmintic activity……18

2.4.13 Toxicity studies……20

2.5 Phytochemical Constituent of *Citrus aurantifolia*……...20

2.5.1 Nutritional analysis of *Citrus aurantifolia* peels and juice…….21

2.6 Health Benefits of Citrus Phytochemicals……24

2.6.1 Limonoids……24

2.6.2 Flavonoids……24

2.6.3 Carotenoids ……..…..25

2.7 The Helminth; *Heligmosomoides bakeri*……..26

2.7.1 Life cycle of *H. bakeri*……28

2.8 Anthelmintic Screening of Drugs or Plants…….31

2.8.1 *In vitro* anthelmintic activity……31

2.8.2 *In vivo* anthelmintic activity…….33

2.9 Control of Helminth Infection……..33

2.9.1 The use of anthelmintics……..33

2.9.2 Targeted drenching……...35

2.9.3 Grazing management……35

2.9.4 Biological control…….35

2.9.5 Vaccines……...36

2.9.6 Resistant and resilient breeds……...36

2.9.7 Strategic nutritional supplementation and feeding with antiparasitic plants……36

2.9.8 Hygiene……37

2.9.9 Anthelmintic plants……..37

3.0 MATERIALS AND METHODS…… …….41

3.1 Location of the Experiment…….....41

3.2 List of Materials Used……41

3.2.1 Equipment and apparatus……41

3.2.2 Reagents, drugs and chemicals……...42

3.3 Plants Collection, Identification and Preparation……...42

3.3.1 Plant methanol extraction process……42

3.3.2 Solvent partitioning of the methanol extracts……..43

3.4 Phytochemical Screening of the Extracts……45

3.4.1 Test for carbohydrates……..45

3.4.2 Test for free anthraquinones (Borntrager's test)……..45

3.4.3 Test for cardiac glycosides……..46

3.4.4 Test for saponins (frothing test)……..46

3.4.5 Test for steroids triterpenes (Lieberman-Burchards test)……47

3.4.6 Test for flavonoids……...47

3.4.7 Test for tannins (ferric chloride test)……47

3.4.8 Test for alkaloids……..48

3.5 Thin Layer Chromatography (TLC) ……...48

3.5.1 TLC Retention factor (R_f) calculation……....50

3.6 Experimental Animals……...50

3.7 Acute Toxicity Studies of Crude Methanol Extract of *Citrus aurantifolia* Fruit Peels…….. …… ……..51

3.7.1 Post-mortem and histopathological studies…….51

3.8 Faecal Culture and Larval Recovery……...52

3.8.1 Source of *H. bakeri* and *H. bakeri* infected mice…….....52

3.8.2 Recovery of *H. bakeri* eggs…….....52

3.8.3 Culture of *H. bakeri* eggs……53

3.8.4 Harvesting and preservation of infective *H. bakeri* larvae…….. ……..53

3.8.5 Determination of larval count for artificial infection and larvicidal test……54

3.9 *In vitro* Anthelmintic Screening of *C. aurantifolia* Fruit Peel Extracts……56

3.9.1 Evaluation of ovicidal activity of the extracts (egg hatch inhibition test)……...56

3.9.2 Evaluation of larvicidal activity of the extracts……...57

3.10 *In vivo* Anthelmintic Screening of the Extracts……...57

3.10.1 Fecal egg count reduction test (FECRT)……58

3.10.2 Post mortem worm counts……59

3.11 Data Analysis……..60

4.0 RESULTS……61

4.1 Extraction Yield of Plant Material…….....61

4.2 Phytochemical Screening of *Citrus aurantifolia* Fruit Peels Extracts……61

4.3 Thin Layer Chromatography (TLC) Results……..61

4.3.1 Retention factor (R_f) of different compounds of *C. aurantifolia* fruit peels extracts.....65

4.4 Acute Toxicity Study of Extracts of *Citrus aurantifolia* Fruit Peels……..65

4.4.1 Histopathological examination……65

4.5 *In vitro* Anthelmintic Activities of *Citrus aurantifolia* Fruit Peels……72

4.5.1 Egg hatch inhibition……72

4.5.2 Larvicidal activity of *Citrus aurantifolia* fruit peel extracts……72

4.5.3 Ic_{50} Determination for egg hatch inhibition……75

4.5.4 Lc_{50} Determination for larvicidal test……..75

4.6 *In vivo* Anthelmintic Activity of *Citrus aurantifolia* Fruit Peels in Mice…….78

4.6.1 4.6.1 Fecal egg count reduction……..78

4.6.2 Post-mortem worm count……. ……78

5.0 DISCUSSION…….....81

6.0 CONCLUSION AND RECOMMENDATIONS……88

REFERENCES…….....90

APPENDICES……..109

LIST OF FIGURES

Page

Figure 2.1: Life cycle of *H. bakeri* (formerly callded *H. polygyrus*)……...30

Figure 3.1: Flow chart of the extraction and solvent partitioning of *C. aurantifolia* peels....……44

Figure 3.2: Operations involved in the experimental infection of mice with *H. bakeri* ……...55

LIST OF TABLES

Page

Table 2.1: Anthelmintic Activities of *Citrus species*……..19

Table 2.2: Nutritional Values of Citrus Fruit Peels……..…….22

Table 2.3: Nutritional Value of *C. aurantifolia* Juice (raw)…….……23

Table 2.4: The Parameters often measured during *in vitro* Anthelmintic Studies… ……..32

Table 2.5: Plants Evaluated for Anthelmintic Activity……38

Table 3.1: TLC Staining Reagents and the Staining Methods Used……49

Table 4.1: Yield and colour of Semi-solid portions of *C. aurantifolia* fruit peels extract after solvent partitioning..……62

Table 4.2: Phytochemical Screening of Hexane, Ethylacetate, Butanol, Aqueous methanol and Crude methanol extracts of *C. aurantifolia* Fruit Peels.....63

Table 4.3: Observation on Thin Layer Chromatography of extracts of *C. aurantifolia* fruit peel sprayed with staining reagents……..……64

Table 4.4: Retention Factor (R_f) of Different Compounds of the Extracts…..…….....66

Table 4.5: Mean (±SD) Percentage Egg Hatch Inhibition of *H. bakeri* by *C. aurantifolia* Fruit Peels Extracts at Different Concentrations……..……..73

Table 4.6: Mean (±SD) Percentage Larvicidal Effect of *C. aurantifolia* Fruit Peels Extracts at Different Concentration on L_1 stage of *H. bakeri*…….....74

Table 4.7: The Log Concentration-inhibition Effect of the Extracts of *C. aurantifolia* Fruit Peels against *H. bakeri* eggs……..…….....76

Table 4.8: The Log Concentration-larvicidal Effect of the Extracts of *C. aurantifolia* Fruit Peels Against *H. bakeri*……...……..77

Table 4.9: EPG/ Percentage Fecal egg Count Reduction Rates in Mice Treated with *C. aurantifolia* Fruit Peels Extracts at Different Doses…….....79

Table 4.10: Mean Worm Counts (±SD) in Mice Treated With Varying Doses of *C. aurantifolia* Fruit Peel Extracts 16-days post-infection with 200 L_3 of *H. bakeri*……..80

LIST OF PLATES

Page

Plate I: *Citrus aurantifolia* fruits……...……... 9

Plate II: *Citrus aurantifolia* tree……..……...10

Plate III: Adult stages of *H. bakeri* (×10 magnification)……..……..27

Plate IV: TLC Plate of *C. aurantifolia* fruit peel extracts sprayed with P-Anisaldehyde+sulphuric acid (A and B), Ferric chloride (C) and Dragendoff (D)..67

Plate V: Histologic section of the liver of mice 3 days after treatment with distilled water (5 ml/kg). H&E ×320. No observable pathology. ……..….....68

Plate VI: Histologic section of the liver of mice 3 days after treatment with crude methanol extract of *C. aurantifolia* fruit peels at 5000 mg/kg. H&E ×500. No observable pathology……...……...69

Plate VII: Histologic section of the kidney of mice 3 days after treatment with distilled water (5 ml/kg). H&E ×400. No observable pathology.……..……..70

Plate VIII: Histologic section of the kidney of mice 3 days after treatment with crude methanol extract of *C. aurantifolia* fruit peels at 5000 mg/kg. H&E ×400. No observable pathology…….. ……..71

LIST OF APPENDICES

Page

Appendix I**:** Inhibitory effects of different concentrations (0.625 – 20 mg/ml) of crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE), ethylacetate extract (EE), hexane extract (HE) of *C. aurantifolia* peels and albendazole (ABZ) (0.0625 – 2 mg) on the egg hatching of *H. bakeri* after 48 hours of incubation. Values are mean (± S.D)……109

Appendix II**:** Effects of different concentrations (0.625 – 20 mg/ml) of the crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE), ethylacetate extract (EE), hexane extract (HE) of *C. aurantifolia* peels and albendazole (ABZ) (0.0625 – 2.0 mg/ml) on the L_1 Larvae of *H. bakeri* after 24 hours of incubation at 25°C. Values are mean (± SD)…….....110

Appendix III**:** Mean faecal egg count reduction rate in mice experimentally infected with *H.bakeri* and treated with crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE) and ethylacetate extract (EE) of *C. aurantifolia* fruit peels and albendazole (ABZ)…….....111

Appendix IV**:** Composition of Reagents used for Phytochemical Screening and TLC analysis……...112

LIST OF ABBREVIATIONS

ABU	Ahmadu Bello University
ABZ	Albendazole
AME	Aqueous methanol extract of *Citrus aurantifolia* fruit peels.
ANOVA	Analysis of variance
BE	Butanol extract of *Citrus aurantifolia* fruit peels.
CME	Crude methanol extract of *Citrus aurantifolia* fruit peels.
DW	Distilled water.
EE	Ethylacetate extract of *Citrus aurantifolia* fruit peels.
EPG	Egg per gram
FAO	Food and Agricultural Organisation
FEC	Faecal Egg Counts
H&E	Haematoxylin and Eosin
HE	Hexane extract of *Citrus aurantifolia* fruit peels
IC_{50}	50 % inhibitory concentration
LC_{50}	50 % larvicidal concentration
LD_{50}	Median lethal dose
R_f	Retention factor
rpm	revolutions per minute
SD	Standard Deviation
TLC	Thin Layer Chromatography
USDA	United State Department of Agriculture

LIST OF SYMBOLS

□	Greater than
%	Percent
˚C	Degree centigrade
<	Less than
µg	Microgram
µl	Microlitres
cm	Centimetres
g	Gram
IU	International unit
kg	Kilogram
L	Litres
L_1	First stage larvae
L_2	Second stage larvae
L_3	Third stage larvae
mg	Milligram
ml	Millilitres
P	Probability

CHAPTER ONE

INTRODUCTION

Parasitic diseases have been of great medical concern over the years as they are considered to cause severe economic losses to livestock production (Coles, 1999). Among the parasitic diseases is helminthosis which poses a major constraint to livestock production globally (Githiori *et al*., 2004) especially in the tropics and subtropics (Perry *et al*., 2002). In the developed world, the major impact of helminthosis results probably from the cost of controlling the disease while in the developing countries like Nigeria, the greatest impact of the disease is seen in the direct and potential production losses (Perry and Randolph, 1999). The productivity losses result from the severe morbidity and mortality of the livestock through; destruction of the gastrointestinal tract resulting in decrease in feed intake, decrease in nutrient absorption, altered feed utilization (Parkins and Holmes, 1989); impairment of behavior, reduced growth rates, low fertility, low birth weight, reduced animal products such as milk, wool, meat, hide and skin (Waller, 1997). Clinical signs manifested in helminthosis include: inappetance, lethargy dullness, loss of general body condition, rough or starry hair coat, pallor of mucous membrane, depression and anaemia (Okewole and Oduye, 2001). Helminthosis is severe in the tropics especially in Nigeria during the wet season when the infection rate is high up to 100 % in cattle but the infection persist throughout the year in small ruminants (Fakae, 1990). These high infection rates prevent the maximum performance of the animals which are under the traditional husbandry system resulting in financial loss to the farmer (Akhtar *et al.*, 2000).

Strategies adopted generally to tackle helminthosis include biological control, vaccination, breeding of livestock that are genetically resistant to parasites (Manuch,

1997, Fakae *et al*., 2003), the use of plants with anthelmintic properties especially feeding animals with forages rich in condensed tannins (Niezen *et al.*, 1998), the use of traditional herbal remedies (Waller, 1999), nutrient supplementation, 'Farmacha' system (Van Wyk *et al*., 1997), chemotherapy including strategic and tactical use of anthelmintics, and pasture management such as careful management of grazing lands; including control of stocking rates and proper rotation strategies (Akhtar *et al*., 2000). Despite the different control measures available, the major control strategy adopted against helminths parasites infection in Nigeria is the use of synthetic anthelmintics (Ibrahim *et al*., 1983).

Citrus aurantifolia (Christm) Swingle (*C. aurantifolia*) belonging to the family Rutaceae is one of the most widely used plants especially in African and Asian traditional medicine. The plant is used to treat several diseases such as gastrointestinal disorders, fever, scabies and malaria (Das *et al*., 2008; Abo *et al*., 2008). The plant has been shown to possess antioxidant, laxative, anti-inflammatory, anticancer, antiviral and antibacterial properties (Ebana *et al*., 1991; Souza *et al*., 2002; Ibukun *et al*., 2007; Jaiprakash *et al*., 2009; Souza *et al*., 2011).

Some members of the Citrus (Rutaceae) family have been shown to possess anthelmintic properties (Rajarajan *et al*., 2009; Bairagi *et al*, 2011). The traditional use of *C. aurantifolia* fruits juice, fruit peels and leaves to treat parasitic helminths in human and animals necessitate the need to evaluate the anthelmintic potential of *C. aurantifolia* fruit peels so as to verify the traditional claims.

Heligmosomoides bakeri (*H. bakeri*) is a trichostrongyliod that occurs naturally in wild mouse populations and has clearly evolved a high level of adaptation to the murine immune system. This worm has been used extensively as laboratory model in screening

for anthelmintic drugs because of its unique characteristics and its resistantance to a number of anthelmintics (Wabo *et al*., 2013). It has a direct life cycle. The life cycle of *H. bakeri* has many similarities to those of the economically important trichostrongyloid nematodes of sheep and cattle (Behnke, *et al.*, 2009).

1.1 Statement of Research Problems

Livestock farming is an important sector that contributes to the income of farmers. It is an important tool for alleviating extreme hunger and poverty as it creates source of livelihood to many families involved in farming. It also plays a role in promoting human health by supplying animal protein in form of meat, milk and eggs. Substantial amount of income is also generated through foreign exchange by exporting leather and products made from bones, horns, teeth and blood. These great prospects in livestock farming are constrained by parasitic diseases particularly, helminthosis, as they cause clinical and subclinical infections that reduce animal survival and depress growth rate, wool and milk production, reproductive performance and finally leads to the death of the animals (Waller, 1997).

Chemotherapy still remains the most effective way employed in the treatment of helminthosis in the world. However, the inappropriate use of these drugs and the high cost coupled with wide spread use of low quality anthelmintics have led to the development of resistance of most helminth parasites to the available anthelmintics and thus, limiting the usefulness of these drugs (Prichard, 1994; Monteiro *et al*, 1997, Jackson and Coop, 2000; Saeed *et al*., 2007). Other limiting factors to the currently used anthelmintics include: unavailability to rural farmers and problems of drug residues in animals intended for human consumption, toxicity and slow development of

new drugs due to high cost of developing and licensing a new drug. (Gasbarre *et al*., 2001; Jabbar *et al*, 2007).

In Africa, particularly, Nigeria, there is a decline in funding of Veterinary services resulting in a rise in the cost of these services, thus, making it difficult for resource-poor farmers to have access to quality Veterinary drugs and services (Abdu and Faya, 2000). This has led to increasing use of medicinal herbs by African small holder livestock producers. More so, traditional Veterinary practice continues to play important role in livestock health and management in Nigeria and throughout Africa (Kudi and Myint, 1999).

1.2 Justification

There is an increasing interest in ethnomedical and ethnoveterinary practices across the world especially as it relates to the use of medicinal plants in treating various diseases (Gasbarre *et al*., 2001). This move is in response to the need to produce animals that are free from drug residues in their meat, milk and eggs. Also, it is a step toward discovering new therapeutic substances of natural origin that are effective and with low or no toxic effect to man and animals (Guarrera, 1999). Therefore, the search for alternatives for treating helminthosis from plant origin may provide lasting solution to the problems of adverse drug effects, toxicity, residual effects and resistance to the currently used anthelmintic drugs. It is also a step toward minimizing the economic loss incured by farmers through morbidity and mortality of livestock and the expenses made on the purchase of synthetic anthelmintics. Eighty percent of the world`s population now rely entirely on drugs obtained from plant sources since plant-based drugs are

cheap and relatively safe (Sofowora, 1993). Many of these medicinal plants have been used to treat parasitic infections in man and animals (Akhtar *et al*., 2000).

In addition, *C. aurantifolia* fruits are cheap and readily available across all the states in Nigeria and many other countries in the world. The fruit peels are often removed and discarded as waste products especially in food processing industries. These fruit peels are also rarely consumed by ruminants making the plant a less competative source of cheap and readily available drug. There is always an increased attention in bringing useful products from waste materials and citrus wastes are no exceptions. Thus, the results of this research can provide alternative anthelmintics from industrial waste products.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study is to determine the anthelmintic effects of the extracts of *Citrus aurantifolia* fruit peels against *Heligmosomoides bakeri*.

1.3.2 Objectives

The objectives of the study are to carry out investigations on the:

i. phytochemical constituents present in the extracts of *C. aurantifolia* fruit peels.
ii. safety of the extracts of *C. aurantifolia* fruit peels in mice.
iii. *in vitro* anthelmintic effects of extracts of *C. aurantifolia* fruit peels on eggs and larvae of *Heligmosomoides bakeri*.
iv. *in vivo* anthelmintic effects of extracts of *C. aurantifolia* fruit peels on *Heligmosomoides bakeri* in experimentally infected mice.

1.4 Null Hypothesis

Extracts of *C. aurantifolia* fruit peels do not have anthelmintic effects against *Heligmosomoides bakeri*.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Plant *Citrus aurantifolia* (Christm.) Swingle (Lime)

Citrus aurantifolia (Christm) Swingle (*C. aurantifolia*) is a polyembryonic plant cultivated in many countries all over the world and grows in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and the West Indies (Patil, 2009). The plant belongs to the kingdom: Plantae; Phylum: Magnoliophyta; Class: Magnoliopsida; Order: Sapindales; Family: Rutaceae; Genus: Citrus and Species: *Citrus aurantifolia* (Sethpakdee, 1992).

C. aurantifolia is commonly called Lime (Nigeria), Key lime, Mexican lime, Sour lime, Dayap, bilolo, Indian lime, Egyptian lime (USDA, 2013).

Important species of citrus grown in Nigeria include: *Citrus sinensis* (sweet orange), *Citrus paradisi* (Grape fruit), *Citrus limon* (Lemon), *Citrus reticulate* or *tangerine* (Tangerine), *Citrus aurantium* (Bitter orange) and *Citrus aurantifolia* (Lime) (Raiyemo, 2013).

2.1.1 Plant Description

C. aurantifolia is a small shrubby tree, about 5 m tall. It is an evergreen and ever bearing tree that is densely and irregularly branched and possesses short and stiff spines (thorns). The leaves are alternate; elliptical to oblong-oviate (4 - 8 cm × 2 - 5 cm) shaped and has a crenulate margin (Plate 2.2). The flowers are 1 inche in diameter and are yellowish white with a light purple tinge on the margin. The fruits are globose to ovoid berry of about 3 - 6 cm in diameter and sometimes have apical papilla (Plate 2.1). It is yellow when ripe but usually picked green commercially. The fruits and flowers appear throughout the year but are most abundant from May to September in the

Northern hemisphere. The fruit peels are very thin with densely glandular segments with yellow-green pulp vesicles. The fruit juice is acidic and fragrant, sour as lemon juice but more aromatic. It is usually valued for its unique flavor compared to other limes. The seeds are small, plump, ovoid, pale, and smooth with white embryo (Sethpakdee, 1992; Golob, 1999; Okwu, 2008).

2.1.2 Brief history and geographical distribution of *C. aurantifolia*

C. aurantifolia is believed to have originated from the south East Asia around 4000 BC and it is native to the Indo-Malayan region. It was assumed to have been carried to North Africa and the near east by the Arabs; and was taken by crusaders from Palestine to Mediterranean Europe. It was introduced to the Caribbean Island and Mexico by the Spaniards and readily became naturalized in the West Indies and Mexico (Morton, 1987). The current major citrus producing countries in the world are Spain, USA, Israel, Morocco, South Africa, Japan, India, Brazil, Turkey and Cuba (Patil, 2009).

In Nigeria, about 930,000 tons of citrus fruits are produced annually from an estimated 3 million hectares of land (FAO, 2006). The major citrus producing States in Nigeria are: Benue, Nassarawa, Kogi, Ogun, Oyo, Ebonyi, Kaduna, Taraba, Ekiti, Imo, Kwara, Edo and Delta (Taiwo, 2005).



Plate I: ***Citrus aurantifolia*** **fruits**



Plate II: ***Citrus aurantifolia*** **tree**

2.2 Domestic Uses of *Citrus aurantifolia*

Citrus fruits are consumed worldwide in form of fresh fruit or are processed into citrus products and citrus-by-products. Approximately, one third of total citrus production is utilized for processing (Okwu, 2008). *C. aurantifolia* juice is used as flavorings in beverages (Yano *et al*., 1999); and it is also used by juice producing companies to produce fruit juice which are presented inform of freshly squeezed orange juice or frozen concentrated orange juice. The juice is squeezed from fresh fruit and packaged in paper cartons, glass or plastic containers, without being pasteurized (Gorinstein *et al*., 2005). In addition, *C. aurantifolia* fruits can be processed to obtain other food products such as dehydrated citrus products or marmalades, jams, sorbet, pickles, jellies, candies and sugar boiled (Okwu, 2008).

Citrus essential oils are another by-product of citrus fruits. Essential oils are volatile oils obtained from the citrus fruit peels' sacks. They are used by the food industry to give flavor to drinks and foods. They are also a component for the pharmaceutical industry for the preparation of drugs, soaps, perfumes, hair cream, body oil and other cosmetics as well as for home cleaning products (Ferguson, 1990). *C. aurantifolia* juice and sometimes fruit peels are combined with vinegar to produce disinfectant; it is combined with salt to clean copper pot. It is used to polish brass, aluminum and copper. It is combined with peroxide or used alone to bleach cloth, remove ink, rusty and mineral stains from cloths; whitening tannin shoe and soften fabrics (Ferguson, 1990). *C. aurantifolia* fruit juice has been used to preserve the quality of "zobo" drink in Nigeria. It was reported to decrease the total coliform and other bacterial counts in "zobo" drink. Thus, the plant can be used to prolong the shelf life of "zobo" drink (Nwachukwu *et al*., 2007).

C. aurantifolia fruit peels have been found very useful domestically. In the kitchen, it is used for cooking, to add flavour to food, cakes and roasted chicken. It is used to garnish salad and to add flavor to drinking water. It is used for bathing and washing of hair, and also, used as a deodorizer to freshen up smelly garbage and composite pile. It also serves as natural room freshener (Lyle, 2006). It has been found useful as a repellant; used to repel mosquitoes from the body (Effiom *et al*., 2012), moth from cloth and cats from gardens (Lyle, 2006). Citrus fruit peels have been transformed into volatile gases through a high-powered microwave. The gasses are then distilled into liquid that is used for making plastics (Nelson, 2011). The pulp of citrus fruit peels is used as cereal substitute in ruminant feeds due to its high energy content and good digestibility in ruminant species (Heuze *et al*., 2012).

2.3 Ethnomedical Uses of *Citrus aurantifolia*

Lime in its natural state is widely used in West Africa, particularly in Nigeria where it is employed in herbal medicine to treat several illnesses. It forms an essential ingredient in the preparation of most herbal concoctions (Aibinu *et al*., 2007).

Different parts of lime tree have been used traditionally to cure some illnesses:

2.3.1 Leaves

The decoction of pounded leaves is drunk for stomach ache, used as eye wash and to bath feverish patient. Poultice of leaves are applied to ulcer wounds, used for skin disease and also applied to abdomen after child birth. Crushed leaves are applied to forehead to treat headache and it is squeezed near the nostril for irritant inhalation to treat nausea and resuscitate fainting individuals (Khan and Abourashed, 2010). Infusion

of *C. aurantifolia* leaves have been given to treat fever with jaundice, sore throat and oral thrush (Kunow, 2003). A decoction of the flower is believed to help induce sleep for those with insomnia (Khan and Abourashed, 2010). In southwest Nigeria, the roots, bark, stem twigs, leaves and fruits are used in the treatment of malaria (Khare, 2007).

2.3.2 Juice

In Nigeria, *C. aurantifolia* fruit juice is added to sugar and palm oil or honey to relief cough. In Malayan medicine, the juice is considered as tonic for libido and as antidote for poison. It is also used to increase stamina, treat dysfunctional uterine bleeding, used as facial wash to rejuvenate the skin and remove stain, drunk to control epistaxis and given in pure form as remedy for athralgia, diabetes and atherosclerosis. The diluted form of the *C. aurantifolia* fruit juice is used for mouth wash to treat sore mouth and sore throat (Khare, 2007; Aibinu *et al*., 2007). The juice has been found useful to treat irritation, diarrhoea and swelling due to mosquito bites. It is sometimes mixed with oil and used as vermifuge and also incorporated into weight management diet (Akhtar, 2013).

2.3.3 Other parts of the plant

Decoction of roots is used to treat dysentery, diarrhea, colic, gonorrhea and fever (Kunow, 2003). The rind is burnt in some homes to act as insecticide against mosquitoes. The mesocarp is used for facial scrubbing to prevent pimples (Aibinu *et al.*, 2007). The *C. aurantifolia* oil extracted by steam distillation of the fruit rinds is used for cold, sore throat, bronchitis, asthma, arthritis and obesity. It is used as an astringent and toning action to clear oily skin acne and treat cuts. Decoction of the bark can help relieve flatulence (Kunow, 2003).

2.4 Pharmacological Activities of *C. aurantifolia*

Pharmacological activities of the extract of different parts of *C. aurantifolia* have been studied and it has been shown that the plant possesses the following pharmacological activities:

2.4.1 Antibacterial activity

Studies revealed that the extracts of root of *C. aurantifolia* have been found effective in inhibiting the growth of *Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Beta-haemolytic streptococci, Escherichia coli* and *Neisseria gonorrhoeae (Aibinu et al., 2007).* The fruit on the other hand was found to inhibit anaerobic facultative bacteria as well *(Ebana et al., 1991; Rahman et al., 2011). C. aurantifolia* have been proved to have significant antimycobacterial activity especially against the isoniazid-resistant strain of *Mycobacteria* (Camacho-corona *et al*., 2008). The antimycobacterial activity have been attributed to the presence of some phytochemicals in *C. aurantifolia* which include: 5,8-dimethoxypsoralen, 5-geranyloxypsoralen, palmitic acid, linoleic acid, oleic acid, 4-hexan-3-one and citral (Nallely *et al*., 2012).

2.4.2 Antifungal and antiaflatoxigenic activity

Studies on the essential oil of *C. aurantifolia* shows that the plant has inhibitory action against *Phaeoramularia angolensis* (Dongmo *et al.,* 2009), *Aspergillus niger, Aspergillus parasiticus* and its aflatoxins, and *Candida albicans* (Aibinu *et al.,* 2007). The antifungal activity of the plant have been attributed to the presence of monoterpenes and the plant is currently used as a fungicide for citrus fruit crop, and it has also been suggested that the plant may be a potential candidate used for the

protection of food and feeds from toxigenic fungal growth as well as their aflatoxin contamination (Dongmo *et al.*, 2009).

2.4.3 Anticancer/ cytotoxic activity

C. aurantifolia have been shown to inhibit colon cancer (Jayaprakasha *et al*., 2008), breast cancer, neuroblastoma (Poulose *et al*., 2005), pancreas cancer (Patil *et al.*, 2009) and prostate cancer cells (Gao *et al*., 2006). D-limonene, D-dihydrocarvone, limoniod and flavonoid are among the major phytoconstituent in *C. aurantifolia* responsible for the anticancer activity (Rooprai *et al.*, 2001; Jayaprakasha *et al*., 2007). The essential oil of *C. aurantifolia* has 78 % inhibition of human colon cancer cells, DNA fragmentation and apoptosis induction as revealed from a study and it is suggestive of the potential use of the plant for prevention of cancer especially colon cancer (Poulose *et al*., 2005).

2.4.4 Antioxidant activity

Studies of the juice and fruit peels (Boshtam *et al*., 2011) as well as leaves (Loizzo *et al*., 2012) of *C. aurantifolia* revealed that the plant has a concentration-dependent effect on low density lipoprotein (LDL) oxidation. The antioxidant activity of *C. aurantifolia* was ascribed to their hydrogen donating ability which may be due to the presence of flavonoids, carotenoids and Vitamin C (Halliwel, 2001; Boshtam *et al*., 2011). Flavonoids present in *C. aurantifolia* fruit juice and peels demonstrate their antioxidant activity by inhibiting the enzymes responsible for superoxide anion production such as xanthine oxidase and protein kinase C (Hanasaki *et al*., 1994). Flavonoids also inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase (St), mitochondrial succinoxidase and NADH oxidase; all involved in generating reactive oxygen species (Korkina *et al*., 1996). Vitamin C is predominant in *C.*

aurantifolia and it acts as antioxidant both *in vitro* and *in vivo* (Bendich *et al*., 1986). Vitamin C protects the plasma lipids and LDL against peroxidative damage induced by various types of oxidation (Retsky *et al*., 1993).

2.4.5 Immuno-modulatory activity

C. aurantifolia juice has been shown to possess immune modulatory activity when it was tested in mitogen activated culture mononuclear cells. The result revealed that the juice could significantly inhibit the proliferation of phytohaemaglutinin activated mononuclear cells at 250 and 500 microgram per litre of the juice extract (Gharagozloo and Ghaderi, 2001). Studies by Pourhossein *et al.*, 2014 on the effects of different levels of sweet orange (*Citrus sinensis*) peel extract (SOPE) on humoral immune system responses in broiler chickens showed that dietary supplementation of Citrus peels extract caused the improvement of immune response and disease resistance of the broiler. In addition, Vitamin C, one of the components of Citrus juice and peels have been shown to play an important role in enhancing immune system, fighting against the formation of free radicals that cause muscle damage. Several cells of the immune system, especially phagocytes and t-cells accumulate vitamin C and need the vitamin to perform their task. Thus, Vitamin C deficiency results in a reduced resistance against certain pathogens whilst a higher supply enhances several immune system parameters (Strohle and Hahn, 2009).

2.4.6 Antiobesity activity

Studies by Asnaashari *et al*. (2010) on the effect of essential oil of *C. aurantifolia* on weight gain in mice showed that mice treated with the extract displayed a reduction in both the amount of food intake and body weight compared with the control group.

Another interesting finding was that co-administration of *C. aurantifolia* essential oil and ketotifen caused a significant suppression of weight gain and also decreased weight gain in mice. The weight loss was ascribed to the fact that *C. aurantifolia* may possibly promote anorexia which may play significant role on weight loss.

2.4.7 Antifertility activity

C. aurantifolia juice have been shown to reduce the number of ova shed in Sprague-Dawley rats and also caused irregularities of the estrous cycle, partially blocked ovulation and also caused irregularities in the histology of the ovaries and uterus and thus may possibly compromise fertility. More so, *C. aurantifolia* juice showed abortificient effect, although it does not possess teratogenic effect on foetus (Bakare *et al*., 2012a). In Nigeria, a survey conducted by Imade *et al*. (2005) in Jos and Mairiga *et al*. (2010) in Borno revealed that it is a common practice for women to use *C. aurantifolia* juice solutions as a post coital douche with the believe that it prevents pregnancy or sexually transmitted diseases such as HIV, and that it enhances sexual pleasure. Though recent research by Bakare *et al*. (2012b) has proved the contraceptive effect of *C. aurantifolia* juice but there are no reports on its ability to prevent sexual transmission of HIV infections.

2.4.8 Anticholinesterase and antiplatelet activity

The extracts of the fruit peels, leaves and the essential oil of the leaves of *C. aurantifolia* showed anticholinesterase and butyrylcholinesterase activity (Loizzo *et al*., 2012; Chaiyana and Okongi, 2012). Similarly, the hydroalcohol extract of *C. aurantifolia* in the form of tincture has been found to have anti-platelet aggregation activity. It was found to have significantly inhibited ADP and epinephrine-induced platelet aggregation in a concentration-dependent manner (Piccinelli *et al*., 2008).

2.4.9 Cardiovascular activity

Akhtar (2013) evaluated the cardiovascular effects of *C. aurantifolia* fruit on three hypertensive models and found that the methanol extract of the fruit produces hypotensive and antihypertensive effects. Mechanisms responsible for these had been proposed by Souza *et al*. (2011) to be related to cardiodepression and vaso-relaxation.

2.4.10 Effects on the bone

The efficacy of *C. aurantifolia* and *C. sinensis* against osteoporosis was evaluated in an ovariectomized rat model, and the result revealed that the administration of citrus extracts increased the trabecular bone mineral content, and bone mineral density of tibia, improved the levels of phosphorus and calcium. The result demonstrates that *C. aurantifolia* possesses the ability to reduce bone loss (Shalaby *et al*., 2011).

2.4.11 Other effects

Citrus fruits are highly recommended for persons suffering from kidney stones, gout and arthritis. *C. aurantifolia* juice contains potassium citrate which prevents the formation of kidney stones and eases their dissolution (Roger, 2002). Due to the high content of vitamin C, citrus fruits are used in the treatment of scurvy. The anti-scurvy effect of citrus fruits is very strong because of the balanced composition of organic acids and minerals (Okwu, 2008).

2.4.12 Anthelmintic activity:

In vitro and *in vivo* anthelmintic evaluation of ethanol extract of three species of citrus fruit peels against *Ascaridia galli* by Abdelqader *et al*. (2012) showed that the extracts caused a significant reduction in worm burden and fecal egg count in chicken compared with the control treated with fenbendazole. Other anthelmintic studies using citrus species are given in Table 2.1.

Table 2.1: Anthelmintic Activities of *Citrus species*

Citrus species	**Parts used/ Extract type**	**Parasites**	**Target**	**Reference**
Citrus medica	Aqueous and alcohol extract of leaves	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Manoj and Rajesh, 2011
Citrus limon	Methanol, petroleum ether, chloroform and alcohol extracts of juice sacks	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Sonali *et al*., 2011
Citrus sinensis	Petroleum ether extract of fruit peels	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Rajarajan *et al*., 2009
Citrus medica	Petroleum ether extract of leaves	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Bairagi *et al*., 2011
Citrus limon	Ethanol extract of fruit peels	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Bairagi *et al*., 2011
Cirus aurantium	Fruit juice	Adult Indian worm: *Pheritima posthuma* (Annelida)	*In vitro*	Bidkar, 2011
Citrus sinensis Citrus limon and citrus reticulate	Ethanol extracts of fruit peels	*Ascaridia galli*	*In vitro and in vivo* (Chicken).	Abdelqader *et al*., 2012

2.4.13 Toxicity studies

Acute and subchronic toxicity studies of water extract from root of *C. aurantifolia* in rats showed no signs of toxicity and no significant histopathological changes in the internal organs; but the expressed *C. aurantifolia* oil contained certain coumarins which are known to cause phototoxicity in humans and has been found to promote tumor formation on the skin and fore stomach epithelium of mice (Khan and Abourashed, 2010). Recent acute toxicity of the fruit by Akhtar (2013) showed that doses above 3.5 g were toxic to rats.

2.5 Phytochemical Constituent of C*itrus aurantifolia*

Until recently, health-promoting properties of citrus had always been associated with their content of Vitamin C. However, studies within the last decade have focused on identifying the bioactive compounds (Patil *et al.*, 2009). Some of the major classes of compounds in citrus include: flavonoids, limonoids, coumarins and phytosterols (Wang *et al*., 2007). Preliminary screening of *C. aurantifolia* fruit and other parts showed the presence of alkaloids, flavanoids, tannins, saponins, steroids, cardiac glycosides, carbohydrates, phenols and reducing sugars (Dongmo *et al.*, 2009; Bairagi *et al*., 2011; Pathan *et al*., 2012; Reddy *et al*., 2012; Akhtar, 2013).

Detailed analysis of *C. aurantifolia* using High-performance Lipid Chromatography (HPLC) and Gas chromatography Mass spectrometry have led to the identification of phytochemicals from the plant, which include 1,4-cineole, linalool; 1,8-cineole; 2,4,6-trichloroanisole; 5-geranoxy-7-methoxy-coumarin; 6,7-dimethoxycoumarin; 8-geranoxypsoralen; 9,10-dimethyl-1,2-benxanthracene; a-bergamotene; a-phellandrene; a-pinene; a-terpineol; b-bisabolene; b-caryophyllene; b-pinene, d-limonene; camphene; g-terpenene; p-cymene; apigenin; apigenin-6-C-[alpha-l-arabinopyranosyl-(1-->6)]-O-

beta-D-glucopyranoside; apigenin-8-C-[alpha-L-arabinopyranosyl-(1-->6)]-O-beta-D-glucopyranoside; benzo-[a]-pyrene; berfapten; bergamottin; ciral; citronellol; diosmetin-6,8-di-C-beta-d-glucopyranoside; fenchol; germacrene B; imperatorin; isoimperatorin; isopimpinellin; isovitexin; kaempferol; kaempferol 3-O-beta-D-glucopyranoside-6"-(3-hydroxy-3-methyl glutarate); kaempferol 3-O-beta-D-glucopyranoside-6-(3-hydroxy-3-methyl glutarate)-7-O-beta-D-glucopyranoside; l-camphor; *C. aurantifolia*ttin; limonene; nobiletin; o-cymene; oxypeucedanin hydrate; phellopterin; quercetin; rutin; sabinene; terpinolene; vicenin II; vitexin (Berhow *et al*., 1994; Khan and Abourashed, 2010; Chaiyana and Okonogi, 2012; Loizzo *et al*., 2012).

Other bioactive compounds present in *citrus* include: pectin, furocoucarins, coumarins, lycopene (in grape fruit), pyrano-coumarins, sitosterol, monoterpenes and sesquiterpenes (Patil, 2009; Loizzo *et al*., 2012).

2.5.1 Nutritional analysis of *C. aurantifolia* fruit peels and juice

The detail nutritional content of raw *C. aurantifolia* fruit peels and juice as reported by the United State Department of Agriculture (USDA), 2013 are given in the Tables 2.2 and 2.3, respectively.

Table 2.2: Nutritional values of Citrus fruit peels

NUTRITIONAL VALUES OF CITRUS FRUIT PEELS (RAW)				
Nutrient	**Unit**	**Value per 100 g**	**Value per table spoon (6g)**	**Value per table spoon (2g)**
Water	G	72.50	4.35	1.45
Energy	Kcal	97	6	2
Protein	G	1.50	0.09	0.03
Total lipid (fat)	G	0.20	0.01	0.00
Carbohydrate	G	25.00	1.50	0.50
Fiber, total dietary	G	10.6	0.6	0.2
Calcium	Mg	161	10	3
Iron	Mg	0.80	0.05	0.02
Magnesium	Mg	22	1	0
Phosphorus	Mg	21	1	0
Potassium	Mg	212	13	4
Sodium	Mg	3	0	0
Zinc	Mg	0.25	0.02	0.00
Vitamin C	Mg	136.0	8.2	2.7
Thiamin	Mg	0.120	0.007	0.002
Riboflavin	Mg	0.090	0.005	0.002
Niacin	Mg	0.900	0.054	0.018
Vitamin B-6	Mg	0.176	0.011	0.004
Folate, DFE	µg	30	2	1
Vitamin B-12	µg	0.00	0.00	0.00
Vitamin A	IU	420	25	8
Vitamin E (alpha-tocopherol)	Mg	0.25	0.02	0.00
Fatty acids, total saturated	G	0.024	0.001	0.000
Fatty acids, total monounsaturated	G	0.036	0.002	0.001
Fatty acids, total polyunsaturated	G	0.040	0.002	0.001
Cholesterol	Mg	0	0	0

Source: United State Department of Agriculture (USDA), 2013.

Table 2.3: Nutritional Value of *C. aurantifolia* Juice (raw)

NUTRIENTS	**Value per 242 g (1 cup)**
Water	220 g
Total calories	60.5 KJ
Protein and amino acid	1.0 g
Total carbohydrate	20.4 g
Dietary fiber	1.0 g
Sugar	4.1 g
Total fat	0.2 g
VITAMINS	
Vitamin A	121 IU
Vitamin C	72.6 mg
Vitamin E	0.5 mg
Vitamin K	1.5 mcg
Thiamin	0.1 mg
Riboflavin	0.0 mg
Niacin	0.3 mg
Vitamin B6	0.1 mg
Folate	24.2 mcg
Vitamin B12	0.0 mg
Pantothenic acid	0.3 mg
Choline	12.3 mg
Betaine	0.5 mg
MINERALS	
Calcium	33.9 mg
Iron	0.2 mg
Magnesium	19.4 mg
Phosphorus	33.9 mg
Sodium	4.8 mg
Zinc	0.2 mg
Copper	0.1 mg
Selenium	0.2 mg
Ash	0.8 mg

Source: United State Department of Agriculture (USDA), 2013.

2.6 Health Benefits of Phytochemicals Obtained From Citrus

2.6.1 Limonoids

There are about 37 limonoid aglycones and 19 glycosides in *C. aurantifolia* and their hybrids (Patil *et al*., 2009). Limonoids are principally found in citrus fruit peels where they produce the bitter taste and the zest aroma. They are also found in large amount in the citrus fruit juice, tissue and seeds. Some of the commonly found limonoids in citrus include: limonin, nomilin LG, LNA, ILNA, deacetyl nomilinic acid glucoside, limonexic acid and isolimonexic acid (Jayaprakasha *et al*., 2008). Limonoids possess the ability to inhibit tumor formation by stimulating the enzyme glutathione S-transferase (GST) which is a detoxifying enzyme that catalyzes the reaction of glutathione to form less toxic and more importantly water soluble compounds that can be easily excreted from the body (Craig, 2002). Orange and lemon oil contain substantial amounts of GST that also possess anti-cancer activity (Craig, 2002).

2.6.2 Flavonoids

Citrus is rich in flavonoids and the most abundant flavonoids in *C. aurantifolia* extracts include: apigenin, rutin, quercetin, kaempferol, nobiletin, hesperidin, hesperitin, and neohesperidin (Jayaprakasha *et al*., 2008; Loizzo *et al*., 2012). The flavonoids have strong inherent ability to modify the body's reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity (Okwu, 2005).

These flavonoids are responsible for the bitter taste of some grape fruits and lemons. It has been demostrated that quercetin, one of the most active flavonoids possess significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation. For example, it inhibits the production of histamine and

other allergic/inflammatory mediators (Roger, 2002). Quercetin also showed remarkable anti-tumor properties and may have positive effects in combating or helping to prevent cancer, prostatitis, heart diseases, cataracts, allergies/inflammations and respiratory diseases such as bronchitis and asthma (Yano *et al*., 1999).

Hesperidin is a flavonoid glycoside and it is believed to play a role in plant defense, acts as antioxidant, reduces cholesterol and has anti-inflammatory effects (Del-Rio *et al*., 1997). Hesperitin also showed an ability to penetrate the blood-brain barrier in an *in vitro* model (Yano *et al*., 1999).

Another useful citrus flavonoid glycoside is rutin, also known as rutoside or quercetin-3-rutinoside. In humans, rutin attaches to the Fe^{2+} ion and prevents it from binding to hydrogen peroxide that may create highly reactive free radicals that may damage body cells. It is also an antioxidant and therefore, plays an important role in inhibiting some cancers. Furthermore, rutin strengthens the capillaries and therefore, can reduce the symptoms of hemophilia. It also helps to prevent edema of the legs. Rutin can reduce the cytotoxicity of oxidized low-density lipoprotein (LDL) cholesterol and lower the risk of heart diseases (Del-Rio *et al*., 1997).

2.6.3 Carotenoids

They are also found in citrus and are believed to reduce the incidence of age-related macular degeneration, the leading cause of blindness in human after the age of sixty five (Seddon *et al*., 1994). They play essential roles as sources of Vitamin A. The most active role is protection against serious disorders such as cancer, heart diseases and degenerative eye diseases. It is an antioxidant and acts as regulators of the immune system. Carotenoids commonly found in citrus are β-carotene, lutein, zeaxanthin and cryptoxanthin (Mangels *et al*., 1993).

Citrus is one of the main sources of Vitamin C (Okwu and Emenike, 2006). Ascorbic acid in the body aids in iron absorption from the intestines. It is required for connective tissue metabolism especially the scar tissue, bones and teeth. It is necessary as an anti-stress and protector against cold, chills and damp. It prevents muscle fatigue and scurvy. It is needed for normal wound healing (Okwu and Emenike, 2007). It acts also as antioxidants in the skin by scavenging and destroying free radicals generated by ultra violet radiation. The production of collagens is also dependent on Vitamin C. It helps in the promotion and restoration of skin (Roger, 2002).

2.7 The Helminth; *Heligmosomoides bakeri*

Heligmosomoides bakeri (*H. bakeri*) is a trichostrongylid that occurs naturally in wild mouse populations and has clearly evolved a high level of adaptation to the murine immune system. The adult worms are 5-20 mm in length and bright-red due to the pigmentation of their tissues. They are usually heavily coiled (Plate III), with the female having 12-15 coils and the male 8-12. The male can be distinguished from the female by a prominent copulatory bursa and two long, thin spicules at the posterior end. The worm was formerly called *Nematospiroides dubius* (Spurlock, 1943) and later *Heligmosomoides polygyrus* (Forrester and Neilson, 1973) but now called *Heligmosomoides bakeri* (Named after N.F Baker, one of the earliest workers on this species; Baker, 1954) to distinguish it from a closely related murine helminth designated as *Heligmosoides polygyrus polygyrus* (Behnke and Harris, 2009).



Plate III: Adult stages of *H. bakeri* (×10 magnification)

This worm has been used extensively as laboratory model in screening for anthelmintic drugs because of its unique characteristics. *H. bakeri* differs markedly from other murine intestinal nematodes because the primary infections by this parasite are tolerated by mice for very long periods of time. Whereas nematodes such as *Nippostrongylus brasilensis* and *Trichinella spiralis* lasted only 2–3 weeks in the guts of their hosts when administered at typical relatively high dose rates, *H. bakeri* could survive for 10 months in the host (Day *et al*. 1979; Robinson *et al*. 1989). *H. bakeri* is also unique compared to other models in that it is easy to maintain in mice since it does not have to be passaged as frequently as the other species of helminth. It is usually enough just to infect a batch of mice and keep them for most of the year, with backup stocks of infective larvae in the fridge. The larvae could be stored at 4°C for months and still retain 50 % of infectivity at this storage temperature (Kerboeuf, 1978; Behnke *et al*., 2009).

2.7.1 Life cycle of *H. bakeri*

H. bakeri has a direct life cycle as described by Bryant, 1973 (Figure 2.1). This begins when the larvae enter the mice by the oral route and invade the duodenal mucosa, penetrating the entire muscular layer to reside beneath the serosal membrane. About 8 days later, the parasites return as adult worms into lumen, where they mate to produce eggs that are passed out through the faeces into the environment. After two days the eggs hatch as larvae which are about 300 μm in length. The larvae moult 3 days later but retain the shed cuticle for protection. It is at this point they become infective. The larvae shed the outer protective sheath after they are ingested by mice. The larvae then penetrate the submucosa of the duodenum where they undergo two further moults to become adult as said earlier. The complete life cycle from egg to egg takes a minimum

of 15 days, and the female worms will live inside their host for 10 months. Adult worms coil around villi and remain closely apposed to the proximal intestinal epithelium (Telford *et al.,* 1998) where they are thought to feed on the epithelial cell layer rather than penetrating it to access blood like the hookworms (Bansemir and Sukhdeo, 1994; Behnke *et al*., 2009).

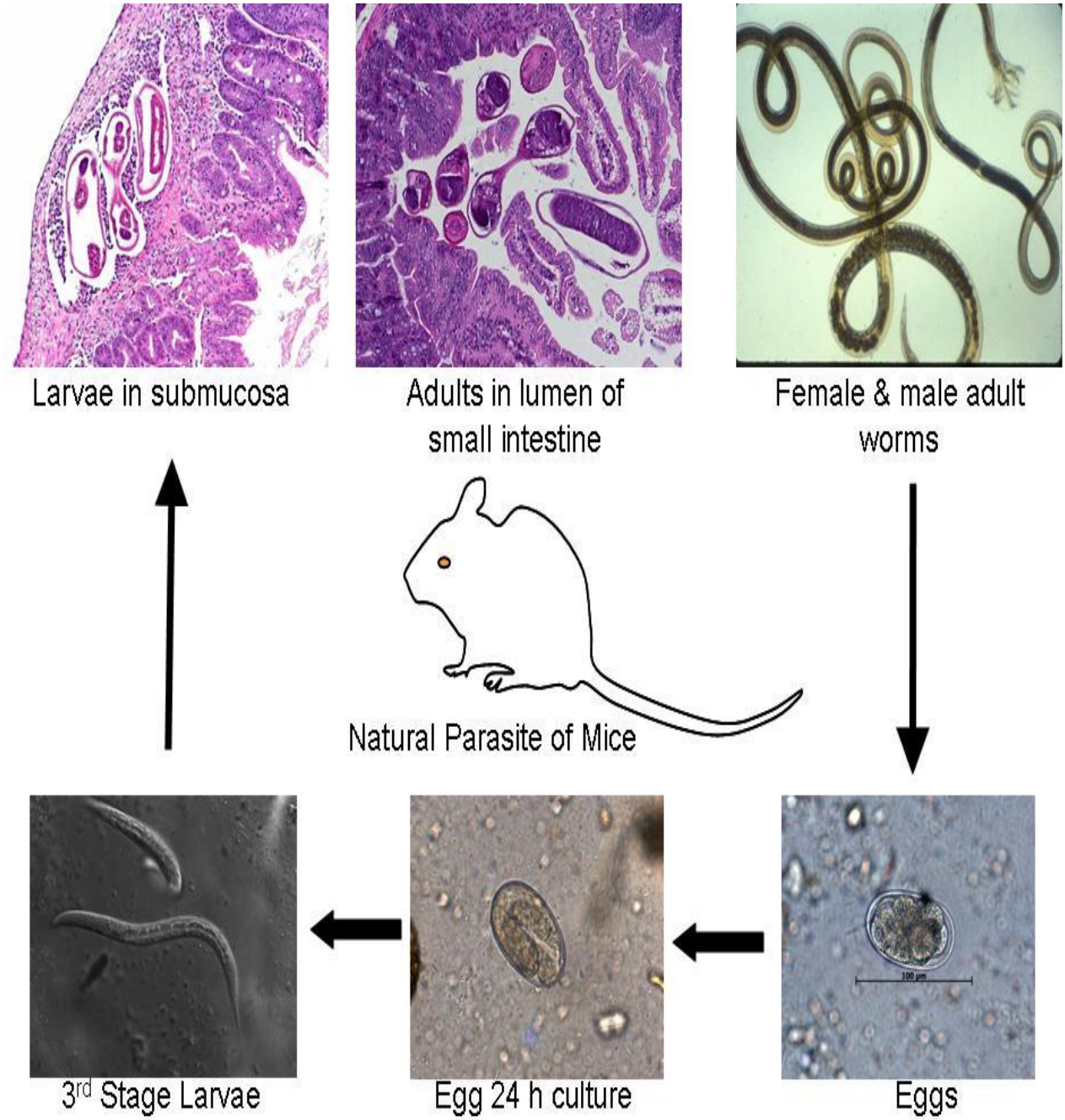


Figure 2.1: Life cycle of *H. bakeri* (formerly callded *H. polygyrus*) (Bryant, 1973)

2.8 Anthelmintic Screening of Drugs or Plants

2.8.1 *In vitro* anthelmintic activity

Most anthelmintic screening of medicinal plants reported in literatures are *in vitro* studies using some worm sample like Indian adult earthworm; *Pheretima posthuma* (Manoj and Rajesh, 2011), *Ascaridia galli* (Abdelquader *et al*., 2012), tapeworm; *Ascaris lumbricoides* (Mali and Mehta, 2007), hookworm; *Haemonchus contortus* (Alawa *et al*., 2003; Ademola and Eloff, 2011), trichostrongylid; *Heligmosomoides bakeri* (Wabo *et al*., 2011; Payne *et al*., 2013), *Trichostrongylus colubriformis* (Bizimenyenra *et al*., 2006), *Oesophagostomum* and *Cooperia* (Chiezey *et al*., 2000). A recent model developed for *in vitro* anthelmintic studies is the use of house fly worm (from *Stomoxys calcitrans*) reported by Murugamani *et al*. (2012). This new model is quite easy, prominent, eco-friendly, and the method is reproducible in all aspects such as equal age, size, and weight of worms used for the experiment (Murugamani *et al*., 2012). The parameters often measured during *in vitro* anthelmintic studies are shown in Table 2.4.

In vitro studies are important because they give the basis for further *in vivo* studies (Mali and Mehta, 2007).

Table 2.4: The Parameters often measured during *in vitro* Anthelmintic Studies.

S/N	BIOASSAY	TARGET STAGE(S)	PROCESS DISRUPTED
1	Egg hatch test	Eggs	Hatching of eggs to L_1
2	Larval migration inhibition test	L_3	Locomotion of L_3
3	Larval feeding inhibition test	L_1	Disruption of feeding of L_1
4	Larval exsheathment assay	L_3	Exsheathment of L_3
5	Larval development assay	Eggs, L_1, L_2, L_3	Disruption of development to L_3
6	Adult motility test	L_5/Adult worm	Motility (Viability) of adult worms

Source: Jackson and Gordon, 2013

2.8.2 *In vivo a*nthelmintic activity

Some *in vivo* studies of anthelmintic activity of plant extracts have been conducted using parasites such as: *Nippostrongylus brasiliensis* in rats (Suleiman *et al*., 2005; Jegede *et al*., 2009), *Ancylostoma caninum* in dogs (Adedapo *et al*., 2005), *Haemonchus contortus* in sheep and goat (Alawa *et al*., 2003; Hussain *et al*., 2008, 2011), *Heligmosomoides bakeri* in mice (Ngongeh and Fakae, 2011), *Ascaris suum* in pigs (Satrija *et al*., 1994), *Ascaridia galli* in chicken (Abdelquader *et al*., 2012). The parameters for such activity included expulsion of worms from their hosts or reduction in the number of eggs per gram of faeces (EPG) passed by the infected hosts compared to commercial anthelmintic treated animals (Jegede *et al*., 2009). Others also measured the weight and the Packed Cell Volume (PCV) of the animals (Githiori, 2004; Ngongeh and Fakae, 2011).

2.9 Control of Helminth Infection

Several strategies are now employed for the control of helminth infection. Some of these strategies include:

2.9.1 The use of anthelmintics

Livestock producers have generally derived substantial benefits from the use of anthelmintics in controlling livestock parasitosis (Abdu and Faya, 2000). By definition, anthelmintics are drugs that reduce parasite burdens in the animals to a tolerable level; they kill the parasites (vermicide), inhibit their growth or paralyse them (vermifuge). They also reduce the build-up of infective worm larva on the pasture, or eggs in the environment (Aliu, 2006). The major classes of synthetic anthelmintics used for control of helminth parasites of ruminant livestock are:

2.9.1.1 The benzimidazole/probenzimidazoles group

These drugs act by interference with polymerization of microtubules (Harder, 2002). They bind to the protein tubulin of the parasite, therefore causing death by starvation (Martin, 1997). Examples include: albendazole, cabendazole, fenbendazole, flubendazole, mebendazole, oxfendazole, oxibendazole, parbendazole and triclabendazole (Aliu, 2006).

2.9.1.2 The imidazothiazoles group: These drugs affect acetylcholine neuro-transmission by interfering with nicotinic acetylcholine receptors leading to spastic paralysis of susceptible nematode by selectively gating acetylcholine receptor ion channels on nerve and muscle. Examples are levamisole and butamisole (Aceves and Martinez-Maranon, 1970; Robertson and Martin 1993; Roos, 1997; Harder, 2002).

2.9.1.3 Tetrahydropyrimidines group

Members of this group of anthelmintics are: pyrantel, morantel and oxantel. Their mode of action is similar to that of levamisole above (Martin, 1997).

2.9.1.4 The macrocyclic lactones (MLs) or avermectins/milbemycins group

The MLs are thought to interact with chloride channels on helminth gammaaminobutyric acid (GABA) receptor complexes, and also inhibit pharyngeal pumping (and hence feeding), motility and fecundity in susceptible nematodes, resulting in paralysis and ultimately elimination from the host (Cavier, 1973; Harder, 2002).

Examples of other anthelmintics commonly used include naphthalophos, salicylanilides and substituted phenols (closantel, oxyclozanide and nitroxynil), piperizine, and organophosphate compounds (Riviere and Papich, 2009).

The use of anthelmintics is however limited due to their high cost, uncertain availability of the drugs, especially to rural farmers, poor quality especially with increasing rates of fake and substandard drugs in the country, increasing resistance of helminth parasites to the currently available drugs, toxicity and drug residues (Prichard, 1994; Hammond *et al*., 1997; Monteiro *et al.*, 1997; Bizimenyenra *et al*., 2006).

2.9.2 Targeted drenching

Targeted drenching is based on selective treatment of those individual animals that are diagnosed as heavily infected, and with clinical symptoms of the disease. The 'FAMACHA' system was founded on this concept, and developed in South Africa for the treatment of small ruminants infected with *H. contortus* (Malan and van Wyk, 1992). The system utilizes clinical identification of anaemia in animals infected with a particular parasite by use of a chart indicating the degree of anaemia. Thus, instead of treating the whole flock, only those animals with infections inducing anaemia are treated with an effective anthelmintic (vanWyk and Bath, 2002; Vatta *et al.*, 2002).

2.9.3 Grazing management

Pasture management is designed to prevent infection of ruminants with internal parasites, and requires long-term planning. Grazing management approach includes the rapid rotational grazing systems, tethering and zero grazing, which separate the host from infective larvae on pasture (Semenye *et al.*, 1992).

2.9.4 Biological control

Biological control of parasitic nematodes in livestock aims at establishing a situation where grazing animals are exposed to a low level of infective larvae, but at a level that

will secure the development of naturally acquired immunity in the same animals (Thamsborg *et al*., 1999).

2.9.5 Vaccines

Vaccination against *Dictyocaulus viviparus* (bovine lungworm) has been quite successful using irradiated L_3 larval form of the parasite. Similarly, irradiated L_3 form of *H. contortus* had offered good protection against haemonchosis in sheep older than six months when administered (Gray, 1997; Dalton *et al*., 2003; Geary and Thompson, 2003; Smith *et al.*, 2003).

2.9.6 Resistant and resilient breeds

Resistance has been described as the ability of the host to prevent or limit the establishment or development of infection. Resilience is the ability of an animal to maintain reasonable levels of production when subjected to parasitic challenge. Considerable research over decades has been undertaken in many sheep rearing countries to identify breeds that have natural resistance to GI nematode parasites infections (van Houtert and Sykes, 1996).

2.9.7 Strategic nutritional supplementation and feeding with antiparasitic plants

Studies had shown that nutritional supplementation, especially improved dietary protein supply, reduces production losses and mortality from infection by GI nematode parasites (Sykes and Coop, 2001). More so, Plants that contain condensed tannins, a class of phenolic secondary metabolites, have antiparasitic properties and have been used to feed animals for controlling helminth infections. Condensed tannins are powerful antimetabolites, and in amounts exceeding 5 %, which is common in tropical

forages, can impair microbial functioning of ruminants (Singh and Bhat, 2003). Investigation into the effects of condensed tannins on GI nematode parasites, either in experimental or in grazing conditions, have been conducted using sheep (Athanasiadou *et al.*, 2007) and goats (Paolini *et al.*, 2003).

2.9.8 Hygiene

Good hygienic practice is important in livestock production. Regular cleaning of the animal house and prevention of fecal contamination of feed and water can reduce the incidence of gastrointestinal helminth infections of healthy ones (Smyth, 1994).

2.9.9 Anthelmintic plants

Medicinal plants have been found useful in the treatment of parasitic diseases including helminthosis. Anthelmintic screening of medicinal plants have been conducted over the years and hundreds of plants were reported to possess anthelmintic activity. Plants known to possess anthelmintic activity have been surveyed and reported in India (Mali and Mehta, 2007), Indian subcontinent, Indo-Pakistan subcontinent (Akhtar *et al*., 2000), Eastern and Southern Africa (Watt and Breyer-Brandwijk, 1962), East Africa (Kokwaro, 1993), West Africa (Ibrahim, 1984), Nigeria (Nwude and Ibrahim, 1980; Fajimi and Taiwo, 2004), Kenya (Githiori, 2004), Africa (Bizimana, 1994), and the world (Tagboto and Townson, 2001). Some of these anthelmintic plants are shown in Table 2.5.

Table 2.5: Plants Evaluated for Anthelmintic Activity

Plant species	**Parts used/ Extract type**	**Parasite targeted**	**Method**	**Reference**
Anarcadium occidentale	Acetone extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Ademola and Eloff, 2011
Annona sanegalensis	Water extract of stem bark	*Haemonchus contortus* eggs and larvae	*in vitro*	Alawa *et al*., 2003
Occimum gratissimum	Leaves essential oil	*Haemonchus contortus* eggs and larvae	*in vitro*	Pessoa *et al*., 2002
Artemisia brevifolia	Crude methanol extract of whole plant	*Haemonchus contortus* eggs, larvae and adult	*in vitro* and *in vivo*	Iqbal *et al*., 2004
Mucuna pruriens	Leaves	*Haemonchus contortus* adult	*in vivo* in lamb	Huisdena *et al*., 2010
Azadiracha indica	Ethanol extract of powdered seed	*Haemonchus contortus* eggs and larvae	*in vitro*	Hordegen *et al*., 2006
Musa paradisiaca	Crude methanol extract of leaves	*Haemonchus contortus* eggs, larvae and adult	*in vitro* and *in vivo*	Hussain *et al*., 2011
Peltrophorum africanum	Acetone extract of leaves, root and stem bark	*Trichostrongylus colubriformis*	*in vitro*	Bizimenyenra *et al*., 2006
Euphorbia hirta	Aqueous crude extract of leaves	*Ancylostoma caninum, Toxocara canis, Dipylidium caninum*	*in vivo* in dogs	Adedapo *et al*., 2005
Zingiber officinale	Methanol extracts of Rhizomes	*Haemonchus contortus* adult	*in vitro*	Iqbal *et al*., 2001

Allium sativum	Methanol extract of bulb	*Haemonchus contortus* eggs and larvae	*in vitro*	Iqbal *et al*., 2001
Moringa olifera	Ethanol extract of leaves and stem bark	*Ascaris suum* adult	*in vitro*	Wasswa and Olila, 2006
Carica papaya	Ethanol extract of leaves	*Ascaris suum* adult	*in vitro*	Wasswa and Olila, 2006
Daniellia oliveri	Stem bark	*Haemonchus contortus* eggs and larvae	*in vitro*	Adama *et al*., 2009
Picrolemma sprucei	Water and ethanol extract of stem bark and root	*Haemonchus contortus* eggs and larvae	*in vitro*	Nunomura *et al*., 2006
Rumex abyssinicus	Alcohol extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Eguale *et al*., 2011
Aframomum danielli	Water and ethanol extract of seed	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Kollins *et al*., 2012
Embelia rowlandii	Cold and hot water and ethanol extract of seed	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Payne *et al*., 2013
Dichrocephala integrifolia	Aqueous and ethanol extract of leave	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Wabo *et al*., 2013
Schinopsis spp (Quebracho tannin)	Extract of the bark dissolved in water	*Heligmosomoides bakeri* eggs and larvae	*in vivo* in mice	Ngongeh and Fakae., 2011
Chenopodium ambrosoides	Hexane and ethanol extract of leaves	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Wabo *et al*., 2011

Spigelia anthelmia	Crude ethanol, chloroform and ethylacetate extract of plant	*Nippostrongylus braziliensis*	*in vivo* in rats	Jegede *et al*., 2009
Saraka indica	Ethanol extract of leaves	*Pheritima posthuma (*indian earthworm)	*in vitro*	Sarojini *et al*., 2011
Corallocarpus epigaeus	Alcohol and water extract of root	*Pheritima posthuma*	*in vitro*	Kirubha *et al*., 2011
Xylopia aethiopica	Crude methanol extract of seeds	*Nippostrongylus braziliensis*	*in vivo* in rats	Suleiman *et al*., 2005
Persicaria hydropiper	Methanol extract of the aerial part	*Pheritima posthuma*	*in vitro*	Raihan *et al*., 2012
Cassia auriculata	Acetone and ethanol extract of the plant	*Eicinia fetida* (Earthworm)	*in vitro*	Gaikwad *et al*., 2011
Afzelia africana	Crude methanol, chloroform and n-butanol extract of stem bark	*Nippostrongylus braziliensis*	*in vivo* in rats	Simon *et al*., 2012
Cymbopogon ciratus	Methanol and aqueous extract of leaves	*Pheritima posthuma*	*in vitro*	Tare *et al*., 2011
Combretum molle	Acetone extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Ademola and Ellof, 2010

CHAPTER THREE

MATERIALS AND METHODS

3.1 Location of the Experiment

The extraction of the plant was done in the Department of Veterinary Pharmacology and Toxicology, while the phytochemical screening of the plant was done in the Department of Pharmacognosy and Drug development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The *in vitro* and *in vivo* anthelmintic screenings were done in the Helminthology Laboratory of the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, A.B.U, Zaria.

3.2 List of Materials Used

3.2.1 Equipment and apparatus

Ninety six–well microtitre plates, light microscope, glass slides, cover slips, Pasteur pipettes, Petri dishes, filter papers, sample bottles, masking tape, permanent makers, digital weighing balance, beakers, measuring cylinders, stirring rod, funnels, test tubes (centrifuge tubes and flotation tubes), egg counter, McMaster slides, syringes of different sizes, conical flask, separatory funnels, cotton wool, gauze, water bath, retort stand, glass bottles and TLC Plate.

3.2.2 Reagents, drugs and chemicals

Methanol (Anala® BDH chemicals Ltd Poole England), hexane and Ethylacetate (JHD Guang dong Guanghua Sci-Tech Co Ltd, China), butanol (Merck CE, Merk Dormstd), distilled water, normal saline (0.9 % Nacl), albendazole, chloroform, tween 80, flotation media (saturated salt solution), formalin, thin layer chromatography spraying reagents (P-anisaldehyde + Sulfuric acid, Ferric chloride, Dragendoff, Aluminium chloride).

3.3 Plant Collection, Identification and Preparation

Samples of *C. aurantifolia* fruits (Plate I) were obtained from some backyard *C. aurantifolia* trees in Adum-ochi, Ankpa Local Government Area of Kogi State in the first quarter of the year. The plant material was identified at the Herbarium, Department of the Biological Sciences, Ahmadu Bello University, Zaria by a taxonomist, Mr. U.S. Gallah. A sample of the plant was deposited in the same Herbarium and given a voucher specimen number of 990. The fruits were washed, peeled manually and dried under shade to prevent heat destruction. The dried fruit peels were pounded to powder using wooden mortar and pestle.

3.3.1 Plant methanol extraction process

The dried pulverized *C. aurantifolia* fruit peels were weighed and extracted by cold extraction (maceration) technique according to method described by Handa *et al.* (2008). About 821 g of the pulverized peel was soaked in methanol in the ratio of 1:3 w/v in a seperatory funnel, whose neck was stucked with cotton wool to allow for filteration of the extract. The set up was allowed to stand for 48 hours and the extract was drained into clean bottles. The procedure was repeated twice using the plant

materials and solvent in the ratio of 1:2 w/v. The liquid methanol extract obtained were pooled together and concentrated to dryness over water baths at 50-60 ˚C. The brown, semi-solid, oily extract obtained was weighed and stored in a labeled bottle at room temperature (25˚C).

3.3.2 Solvent partitioning of the methanol extracts

The solvent partitioning of the methanol extracts was done according to methods described by Handa *et al.* (2008). Seventy grams of the crude methanol extract was used for solvent partitioning by dissolving it in 700 ml of distilled water (ratio of 1:10 w/v) to form an aqueous methanol extract (AME) and serially partitioned with hexane, ethylacetate and butanol as solvents. Each partition process was repeated twice using equal volume (700 ml) of solvent, and the individual fractions were pooled together and concentrated *in-vacuo*.

The flow chart in Figure 3.1 shows the summary of the extraction and solvent partitioning of *C. aurantifolia* fruit peels.

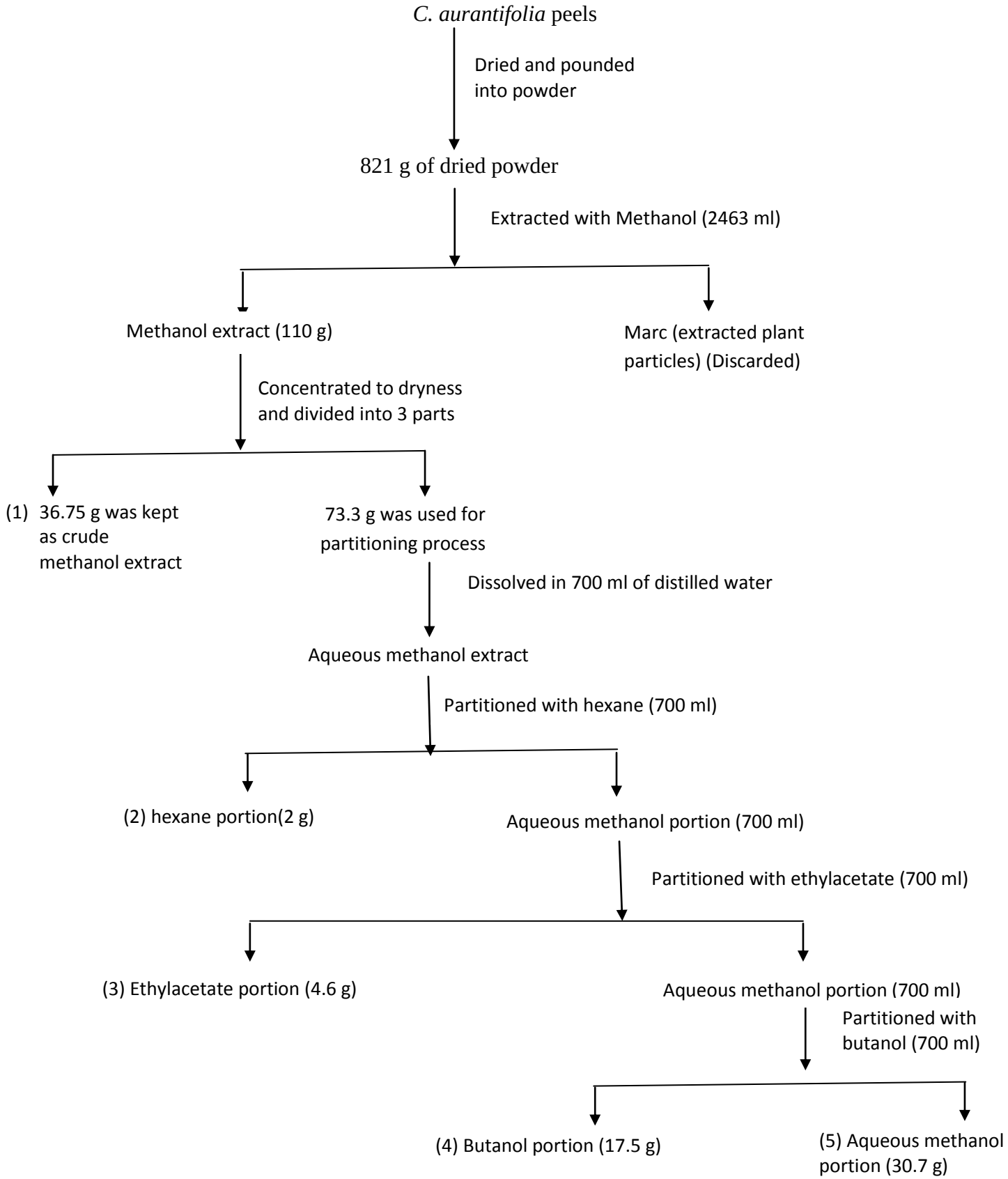


Figure 3.1: Flow chart of the extraction and solvent partitioning of *C. aurantifolia*

3.4 Phytochemical Screening of the Extracts

The crude methanol extract (CME), aqueous methanol extract (AME), hexane extract (HE), ethylacetate extract (EE), and butanol extract (BE) were subjected to phytochemical screening using methods described by Trease and Evans (2007) and Tiwari *et al*. (2011). One gram of each portion of the extract was dissolved in 100 ml of distilled water and the following phytochemical tests were conducted:

3.4.1 Test for carbohydrates

3.4.1.1 Molisch's test

Few drops of Molisch's reagent was added to a 3 ml of the dissolved extract in a test tube followed by the addition of 2 ml of 99.9 % sulphuric acid which was carefully poured down the side of the test tube. A purple to violet colour at the interface was indicative of the presence of carbohydrate.

3.4.1.2 Fehling's test

Two milliliters of each portion of the extract was poured into a test tube and 5 mls of the mixture of Fehlings solution A and B (Appendix IV) in the ratio of 1:1 were added. The mixture was boiled for 2 minutes. The appearance of a brick-red precipitation was indicative of the presence of glycone due to hydrolysis of glycosides.

3.4.2 Test for free anthraquinones (Borntrager's test)

About 1g of the extract was mixed with 10 ml of 99.9 % benzene and filtered and 5 ml of 10 % ammonia solution was added to the filtrate and stirred. The production of pink-red colouration was indicative of free anthraquinones.

3.4.3 Test for cardiac glycosides

3.4.3.1 Kella-Killiani Test

One gram of the extract was dissolved in 2 ml glacial acetic acid in a test tube. Thereafter the test tube was held at an angle of 45 degrees while carefully adding 1ml of 99.9 % sulphuric acid down the side using a pipette. The appearance of a purple ring colour at the interface indicated the presence of cardiac glycosides.

3.4.3.2 Salkowski test

About 0.5 g of the extract was dissolved in 2 ml of chloroform and 2 drops of 99.9 % sulphuric acid were added to form a lower layer. The presence of a reddish-brown ring colour at the interface indicated the presence of a steroid.

3.4.3.3 Kadde test

One mililitre of 2 % 3, 5-dinitrobenzoic acid in 95 % alcohol was added to 1 ml of the extract followed by the addition of 5% sodium hydroxide to make the solution alkaline. The appearance of purple-blue colour ring indicates the presence of cardenoides.

3.4.4 Test for saponins (frothing test)

About 0.5 g of the extract was dissolved in 10 ml of distilled water. The mixture was vigorously shaken for 30 seconds and allowed to stand for 30 minutes. The formation of a honey-comb froth that persisted for more than 30 minutes was indicative of the presence of saponins.

3.4.5 Test for steroids triterpenes (Lieberman-Burchards test)

One mililitre of 99.9 % acetic anhydride was added to 1 ml of the extract. Furthermore another 1 ml of 99.9 % sulphuric acid was added down the side of the test tube. Red, pink or purple colour observed immediately after the addition of the acid indicated the presence of triterpenes while blue or blue-green coloration observed much later indicated the presence of steroids.

3.4.6 Test for flavonoids

3.4.6.1 Sodium hydroxide test

About 0.5 g of the extract was dissolved in 5 ml of water in a test tube. Three drops of 5% sodium hydroxide were then added to the solution. The appearance of yellow colouration indicated the presence of flavonoids.

3.4.6.2 Shinoda Test

About 0.5 g of each portion of the extract was dissolved in 2 ml of 50 % methanol and heated. This was followed by the addition of metallic magnesium chips and four drops of 99.9 % hydrochloric acid using the pipette. A red or orange colour was indicative of the presence of flavanoids aglycones.

3.4.7 Test for tannins (ferric chloride test)

One gram of each portion of the extract was dissolved in 10 ml of distilled water and the solution was filtered. Two drops of ferric chloride solution were added to the filtrate. The formation of a blue-black precipitate indicated the presence of hydrolysable tannins, while a green precipitate indicated the presence of condensed tannins.

3.4.8 Test for alkaloids

3.4.7.1 Meyer's test

Few drops of Meyer's reagent (Appendix IV) were added to 5 ml of the extract already dissolved in distilled water. The appearance of a creamy precipitate indicated the presence of alkaloids.

3.4.7.2 Dragendoff's test

The extract (0.5 g) was dissolved in 10 ml of distilled water and 3 drops of Dragendoff's reagent were added to the solution. Red-rose precipitate indicated the presence of alkaloids.

3.4.7.3 Wagner's test

One gram of each portion of the extract was dissolved in 10 ml of distilled water in a test tube and 2 drops of Wagner's reagent were added to the solution. The presence of a white precipitate indicated the presence of alkaloids (Brain and Turner, 1975).

3.5 Thin Layer Chromatography (TLC)

Two grams of each extract was dissolved in 2 ml of 99.9 % methanol. Each extract was spotted on four different TLC plates and eluted with 99.9% butanol, 99.9 % Acetic acid and Water in the ratio of 16:1:1, respectively. Individual plates were sprayed with P-anisaldehyde and sulphuric acid, ferric chloride in alcohol, dragendoff reagent and aluminium chloride as detecting agents (Trease and Evans, 2007).

Table 3.1: TLC Staining Reagents and the Staining Methods Used

TLC STAINING REAGENTS	TLC STAINS RECIPE	STAINING METHOD	INFERENCE
P-Anisaldehyde+ Sulfuric acid	0.5 ml of P-Anisaldehyde in 50 ml of glacial acetic acid and 1ml of 97 % sulfuric acid (H_2SO_4)	The Plate was sprayed with the reagent and heated in an oven to 105 ^{0}C and then monitored until the spots were clearly visible	Phenol ------- Violet Terpenes ---- Red Sugar----- Gray/green Allylic alcohol…green.
Alcohol ferric Chloride	1 g of ferric (III) chloride was dissolved in 50 ml of aqueous methanol and 50 ml of deionized water. The mixture was stirred into a homogenous solution.	The TLC plate was sprayed with alcohol ferric chloride and then observed for colour change on the spots.	Blue coloration indicates the presence of Phenolic compound.
Dragendoff	5 ml of Stock solution A and B (mixed at ration 1:1), 10 g of tartaric acid and 50 ml of distilled water.	The plate was first sprayed with Dragendoff reagent and then later sprayed with 99.9 % HCl to intensify the spots.	Orange spots developed which were intensified by the spray of 99.9 % HCl indicate the presence of alkaloids or quaternary nitrogen compounds.
Aluminum chloride	1% ethanol solution of aluminum chloride	The plate was sprayed with 1% ethanol aluminium chloride and then observed under a long wave length ultraviolet light (360 nm).	A yellow fluorescence under the UV light indicates the presence of flavonoids.

3.5.1 TLC retention factor (R_f) calculation

The R_f gives the relative distance moved by the different compounds present in the extract in a particular solvent system. The movement and separation of compounds in an extract depends on their polarities. R_f values is calculated by measuring the solvent front (distance travelled by the solvent) and distance travelled by the compound as given by the formula described by Ettre (1993).

$$R_f = \frac{\text{Distance travelled by compound (middle of the spot)}}{\text{Distance travelled by the solvent (solvent front)}}$$

3.6 Experimental Animals

Eighty five apparently healthy albino mice (*Mus musculus*) of both sexes within the ages of 8 to 10 weeks were used for the experiment. The mice were purchased from the Animal House, National Veterinary Research Institute, Vom, Plateau State, Nigeria. The mice were maintained on commercial chick growers mash (Vital feed®) (Jegede *et al.,* 2009). Water and feed were provided *ad libitum*. Wood shavings were used as bedding and were changed every two days. The mice were grouped and kept in cages. Each group consisted of five mice which were identified by marks on their tails using permanent markers of different colours.

The mice were preconditioned to laboratory environment for two weeks. During this period, those intended for anthelmintic studies were screened for helminth parasites and those positive to the screening were dewormed using albendazole (7.5 mg/kg) orally.

3.7 Acute Toxicity Studies on the Crude Methanol Extract of *C. aurantifolia* Fruit Peels

The method of Lorke (1983) was used to determine the median lethal dose (LD_{50}) of the crude methanol extract in mice. Male mice were used for this study. In the first phase, the mice were randomly divided into three groups consisting of 3 mice each. Groups 1, 2 and 3 were dosed per os with the extract at 10, 100, 1000 mg/kg, respectively. The mice were observed over a period of 48 hours for signs of toxicity and mortality. In the second phase of the trial, new set of mice were divided into three groups of one mouse each. Groups 1, 2 and 3 were treated with 1600, 2900 and 5000 mg/kg of the extract, respectively. Group 4 was given distilled water at 5 ml/kg (maximum volume administrable to rats/mice at a time) (Ibrahim *et al.*, 1983). The animals were observed for 24 hours for signs of toxicity and mortality.

3.7.1 Post-mortem and histopathological studies

At the end of the trials, all mice administered with the extract and those treated with distilled water were euthanized and the internal organs were grossly examined for any pathological changes. The livers, kidneys, stomach and intestine were removed, fixed in 10 % buffered neutral formalin and processed for histopathological examination. The slides were stained with haematoxylin and eosin stains and examined under a light microscope at ×10 magnification for any histhological changes (Luna, 1960).

3.8 Faecal Culture and Larval Recovery

3.8.1 Source of *H. bakeri* and *H. bakeri* infected mice

Infective third stage larvae (L_3) of *H. bakeri* and mice infected with the parasite were obtained from the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. Helminth-free mice were infected orally with 200 L_3 contained in 0.2 ml of water using a blunted tip 18 G needle mounted on a 1 ml syringe. Two weeks post infection, droppings from the infected mice were obtained by placing the mice in clean plastic cages (Figure 3.2) for 20 minutes and faeces produced by the mice were collected into a labeled container and examined for the presence of *H. bakeri* eggs using the simple flotation method (Soulsby, 1982).

3.8.2 Recovery of *H. bakeri* eggs

The method of Micheal *et al*. (2001) with modification was used to obtain fresh eggs of *H. bakeri*. Briefly, 3 grams of faeces was collected in a centrifuge tube and homogenized using a pestle in 12 ml of saturated sodium chloride solution. The solution was filtered through a tea sieve and the filtrate centrifuged at 250 rpm for 5 minutes. The supernatant was poured into a beaker and 100 ml of distilled water was added to it. The supernatant added to the distilled water was again centrifuged at 250 rpm for 5 minutes. Using a 10 ml syringe, the supernatant was aspirated and discarded and the sediment re-centrifuged for 5 minutes at 250 revolutions per minutes after adding more water. The sediment obtained after aspirating the supernatant with a syringe was examined under the light microscope at ×10 objective for the presence of *H. bakeri* eggs. The recovered eggs were used for the *in vitro* egg hatch test or cultured to obtain the third stage larvae of *H. bakeri* used to infect other mice for *in vivo* test

3.8.3 Culture of *H. bakeri* eggs

Two methods were used to obtain the larval forms of *H. bakeri*. The first method was done according to the description of Kollins *et al*. (2012). Briefly, 3 ml of egg suspension recovered earlier was poured into Petri dishes having their bottom covered with Whatsman filter paper. The Petri dishes were covered to prevent excessive evaporation and contamination by flies, and maintained a high relative humidity of about 65 – 67 %. The Petri dishes were allowed to stand at room temperature (25˚C) for 7 days to allow hatching of the eggs and development to the L_3 stage.

The second method was done as described by Chiejina and Fakae (1984). Four grams of faecal sample was collected from infected mice and homogenized in 50 ml centrifuge tube using distilled water. The fecal slurry was filtered using a tea sieve or doubly folded gauze placed on a funnel. The filtrate was centrifuged at 250 rpm for 5 minutes and the supernatant aspirated using 10 ml syringe. The sediment was then poured into petri dishes having their bottom covered with Whatman's filter paper. The Petri dishes were covered, left at room temperature (25˚C) and moistened daily with distilled water for 7 days to obtain the L_3 stage.

3.8.4 Harvesting and preservation of infective *H. bakeri* larvae

The filter paper on Day 7 of culture was lifted with a forcep and the top and bottom of it sprayed with distilled water to ensure the larvae were washed into the Petri dish. Thereafter, the filter paper was removed and discarded. Using a Pasteur pipette, 2 or 3 drops of the water in the Petri dish containing the larvae were placed on a glass slide and examined under the light microscope for the presence of moving/mobile L_3. The harvested larvae were preserved in a clean, labelled sample bottle (Figure 3.2) and kept in a refrigerator at the temperature of 4˚C.

3.8.5 Determination of larval count for artificial infection and larvicidal test

The method described by Suleiman (2002) was adopted. The larval suspension was placed in a beaker and stirred. Using a 1 ml syringe, 0.1 ml of the solution was aspirated and stripped on a glass slide followed by the addition of a drop of iodine to immobilize the larvae to make counting easier and the slide examined at ×10 magnification of light microscope. The process was repeated five times and the average number of the larva counted was recorded. A concentration and dilution technique was used and the volume adjusted such that about 200 infective larvae were contained in 0.2 ml of the solution.

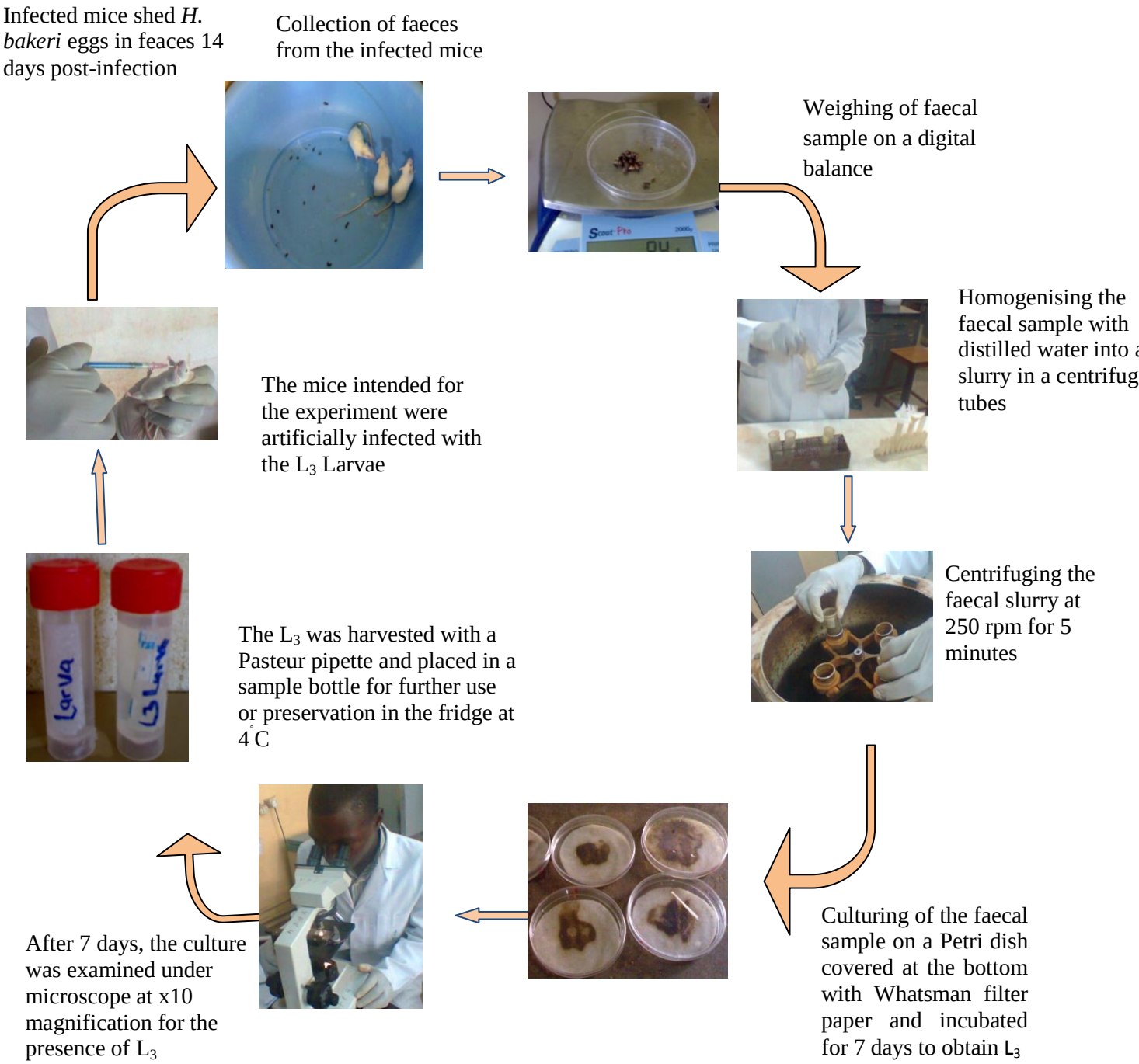


Figure 3.2: Operations involved in the experimental infection of mice with *H. bakeri*

3.9 *In Vitro* Anthelmintic Screening of *C. aurantifolia* Fruit Peel Extracts

3.9.1 Evaluation of ovicidal activity of the extracts (Egg hatch inhibition test)

The *in vitro* ovicidal effect of the extracts was done according to methods described by Coles *et al.* (1992) and Wabo *et al*. (2011). Two hundred microliters of egg suspension containing about 100 fresh eggs was aspirated using a pipette and put into each well of a labeled 96-well microtitre plate. In addition, 200 µL of different concentrations (20, 10, 5, 2.5, 1.25 and 0.625 mg/ml) of each extract were added. Each concentration was tested in triplicate. Similarly, albendazole (0.2 ml in each well) of different concentrations (2, 1, 0.5, 0.25, 0.125, 0.0625 mg/ml) and distilled water (0.2 ml in each well) were used as treated and untreated controls, respectively. Each experimental set up was done in triplicate. The plates were covered with foil papers to prevent evaporation and then left to stand at room temperature for 48 hours. Thereafter, using a pipette, the content of each well of the microtitre plate was placed on a glass slide and examined microscopically at ×10 magnification. All the unhatched eggs as well as the L_1 in each well were counted and recorded.

The percentage inhibition of egg hatching was calculated using the formula described by Wabo *et al*. (2010):

$$\text{Hatching rate (\%)} = \frac{\text{Number of } L_1 \text{ larvae}}{\text{Number of eggs cultured}} \times 100$$

3.9.2 Evaluation of larvicidal activity of the extracts

The evaluation of the larvicidal activities of the different portions of the extract was done according to methods described by Njoku and Asuzu (1998) and Wabo *et al*. (2011). Albendazole at different concentration and distilled water were used as treated and untreated controls, respectively. About 100 larvae of *H. bakeri* contained in 0.2 ml of water was added into each of a labeled 96-well flat-bottom microtitre plate. Thereafter, 0.2 ml of the different concentrations (20, 10, 5, 2.5, 1.25 and 0.625 mg/ml) of the extracts were added. Each test was done in triplicate. The plates were covered with foil paper and left at room temperature (25˚C) for 24 hours. Thereafter, the content of each well was stirred and pipetted onto a clean glass slide and then examined under the microscope at ×4 magnifications to count the number of larvae that were dead or alive. A larva was considered alive if it moved any part of its body or migrated from one point to another; but if the larva showed no observable motion after 5-10 seconds interval, it was considered dead.

3.10 *In vivo* Anthelmintic Screening of the Extracts

The *in vivo* anthelmintic trials of the crude methanol, ethylacetate, butanol and aqueous methanol extracts of *C. aurantifolia* fruit peels was conducted based on methods described by Ngongeh and Fakae (2011). Seventy mice infected with *H. bakeri* were randomly allocated into 14 groups of 5 mice each. Groups 1-3, 4-6, 7-9, 10-12 were treated with crude methanol extract (CME), aqueous methanol extract (AME), ethylacetate extract (EE) and butanol extract (BE), respectively. Each extract was tested at doses of 250, 500, 1000 mg/kg. Group 13 and 14 were treated with albendazole (10 mg/kg) and distilled water (5 ml/kg), respectively and served as treated and untreated

controls, respectively. All treatments were administered orally on the 16th, 17th and 18th day post-infection.

3.10.1 Fecal egg count reduction test (FECRT)

One gram of faecal sample was collected daily from each group of infected and treated as well as infected and untreated (control) mice for four consecutive days starting from the 16th day post infection. The modified Mcmaster technique was employed for the faecal egg count. One gram of faeces from each group was homogenized in 12 ml of saturated sodium chloride/sucrose solution of specific gravity of 1.28 and then centrifuged at 11 *g* for 5 minutes. The supernatant was poured into a labeled sample bottle and the sediment discarded. Aliquot of the supernatant from each of sample was put into both chambers of McMaster slides and allowed to stand for 5 minutes. The slides were examined microscopically at ×10 magnification. Egg per gram (epg) of faeces was calculated by multiplying the faecal egg count by 40 (Soulsby, 1982; MAFF, 1977).

The anthelmintic effects of each extract of *C. aurantifolia* fruit peel was accessed by comparing the EPG of the treated groups with that of those treated with distilled water (untreated control). The percentage reduction in *H. bakeri* eggs in the treatment groups was calculated using the formula described by Abdelqader *et al*. (2012):

$$\frac{N - n}{N} \times 100$$

Where N= Mean faecal egg counts in untreated control group (distilled water)

n= Mean faecal egg counts in the treated group.

3.10.2 Post mortem worm counts

At the end of the treatment (18 days post-infection), all mice were deprived of food but not water for 24 hours so as to empty the gastrointestinal tract and ease the worm counting process. The mice were euthanized in chloroform chamber and the gastrointestinal tract removed immediately. The method described by Ngongeh (2004) with little modifications was employed for the worm count. The entire length of the small intestine of each mouse was removed and splited longitudinally with a pair of fine surgical scissors. The adult worms were recovered by suspending the small intestine in normal saline. The solution was incubated at room temperature (25˚C) for 20 hours to enable the migration of the worms into the solution. The solution was centrifuged and the supernatant discarded. The sediment containing the worms was poured into a Petri dish and then observed under a stereomicroscope. The adult worms seen were counted and recorded. Further more, the intestines removed from the normal saline earlier were fixed in 10 % formalin and then observed under the stereomicroscope for counting of adult worms that did not migrate into the normal saline.

The percentage deparasitization was calculated using the formula described by Suleiman *et al*. (2005):

$$\frac{N - n}{N} \times 100$$

Where "N" = mean number of worms found in untreated control mice.

"n" = mean number of worms found in treated mice.

Percentage deparasitization of 70 and above was considered significant in this experiment.

3.11 Data Analysis

Results obtained were expressed as mean ± standard deviation (±SD). Analysis of variance (ANOVA) using GraphPad Prism Version 5.0 (San Diego, 2010) was used to compare the anthelmintic effects of the different extracts of *C. aurantifolia* fruit peels to albendazole and the non-treated (distilled water) group. The mean in different group was compared using Tukey Post hoc test. Value of $P< 0.05$ was considered significant.

CHAPTER FOUR

RESULTS

4.1 Extraction Yield of Plant Material

After extracting 821 g of pulverized *C. aurantifolia* fruit peels three times with methanol and concentrating the extract to dryness over water baths, a brown, semi-solid crude methanol extract weighing 110 g was obtained.

The yields of the different portions after serial partitioning of the crude methanol extract with different solvents are given on Table 4.1.

4.2 Phytochemical Screening of *C. aurantifolia* Fruit Peels Extracts

Different secondary metabolites that were detected in the extracts are presented in Table 4.2.

4.3 Thin Layer Chromatography (TLC) Results

The TLC phytochemical results are presented in Table 4.3. The results showed the presence of triterpenes, sugars and alkaloids in all the extracts. Phenols were present in all extracts except in hexane and aqueous methanol extracts. In addition, crude methanol, butanol and aqueous methanol extracts contained flavonoids.

Table 4.1: Yield and colour of Semi-solid portions of *C. aurantifolia* fruit peels extract after solvent partitioning

Extracts	**Yield (grams)**	**Percentage yield**	**Colour**
Hexane	2.0	2.9	Dark brown
Ethylacetate	4.7	6.7	Brown
Butanol	17.5	25	Brown
Aqueous methanol	30.7	43.9	Light brown

Table 4.2: Phytochemical screening of hexane, ethylacetate, butanol, aqueous methanol and crude methanol extracts of *C. aurantifolia* fruit peels

Components tested for	Phytochemical Test employed	Etracts of *C. aurantifolia* fruit peels				
		CME	HE	EE	BE	AME
Carbohydrate	Molisch test	+	+	+	+	+
	Fehling test	+	+	+	+	+
Free Anthraquinone	Borntrager`s test	_	_	_	_	_
Glycosides	Kella-killiani test	+	+	+	+	+
	Kadde`s test	-	-	-	-	-
	Salwoski`s test	+	+	+	+	+
Saponins	Frothing test	+	-	+	+	+
Steroids	Lieberman-Burchards test	+	+	+	+	+
Triterpenes	Lieberman-Burchards	+	-	+	+	+
Flavonoids	Shinoda test	+	+	+	+	+
	Sodium hydroxide test	+	+	+	+	+
Tannins	Lead sub-acetate test	+	+	+	+	+
Condensed tannins	Ferric chloride test	+	+	+	+	+
Alkaloids	Mayer`s test	+	-	+	+	-
	Dragendorff`s test	+	-	+	+	-
	Wagner`s test	+	-	+	+	-

Key: + = positive or present
- = negative or absent
CME = crude methanol extract
HE = hexane extract
EE = ethylacetate extract
BE = butanol extract
AME = aqueous methanol extract (Residue)

Table 4.3: Observation on Thin Layer Chromatography of extracts of *C. aurantifolia* fruit peel sprayed with staining reagents

Secondary metabolites	**Extracts of *C. aurantifolia* fruit peels**				
	CME	**HE**	**EE**	**BE**	**AME**
Triterpenes	+	+	+	+	-
Sugars	+	+	-	+	+
Phenols	+	-	+	+	-
Alkaloids	+	+	+	+	+
Flavonoids	+	-	-	+	+

Key: + = positive or present
- = negative or absent
CME = crude methanol extract
HE = hexane extract
EE = ethylacetate extract
BE = butanol extract
AME = aqueous methanol extract

4.3.1 Retention factor (R_f) of different compounds of *C. aurantifolia* fruit peels extracts

When the eluted plates were sprayed with the different reagents (Plate IV), the separated compounds had R_f values ranging from 0.27 – 0.97 (Table 4.4). Since the eluting solvent (16 parts butanol: 1 part acetic acid: 1 part water) is polar in nature, it therefore implies that compounds with R_f of 0.5 or greater are relatively polar in nature.

4.4 Acute Toxicity Study of Extract of *C. aurantifolia* Fruit Peels

There were no deaths following the oral route administration of the crude methanol extract of *C. aurantifolia* fruit peels even at the dose of 5000 mg/kg. The clinical signs observed include dullness and anorexia. At post mortem, the euthanized animals showed petchial haemorrhage of the stomach and intestine.

4.4.1 Histopathological examination

There were no observable histopathological lesions in the livers and kidneys of mice in the group dosed with crude methanol extract (Plate VI and VII).

Table 4.4: Retention Factor (R_f) of Different Compounds of the Extracts

TLC STAINING REAGENTS	CME	HE	EE	BE	AME
P-Anisaldehyde + Sulfuric acid	0.68	0.75	0.68	0.64	0.27
Alcohol ferric chloride	0.49	-	0.76	0.64	-
Dragendoff	0.91	0.92	0.95	0.93	0.97
Aluminum chloride	0.30	-	-	0.34	0.30

Key: - = absent
CME = crude methanol extract
HE = hexane extract
EE = ethylacetate extract
BE = butanol extract
AME = aqueous methanol extract

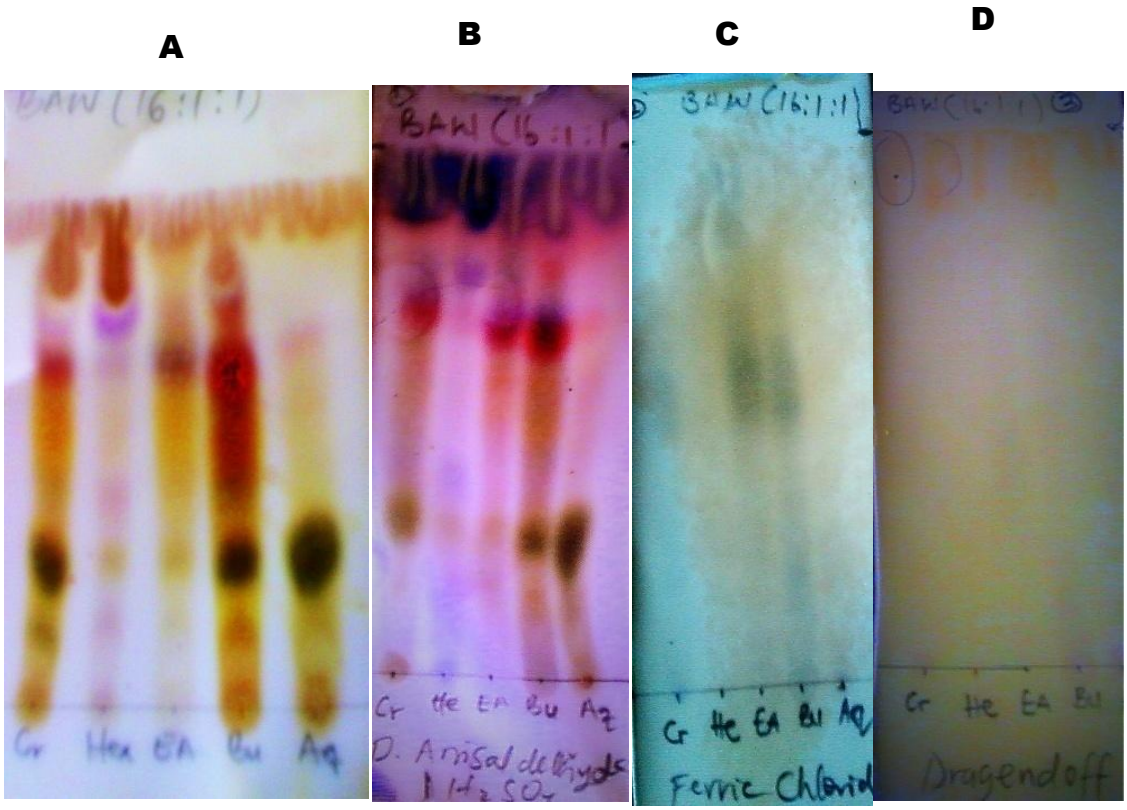


Plate IV: TLC Plate of *C. aurantifolia* fruit peel extracts sprayed with P-Anisaldehyde+sulphuric acid (A and B), Ferric chloride (C) and Dragendoff (D).

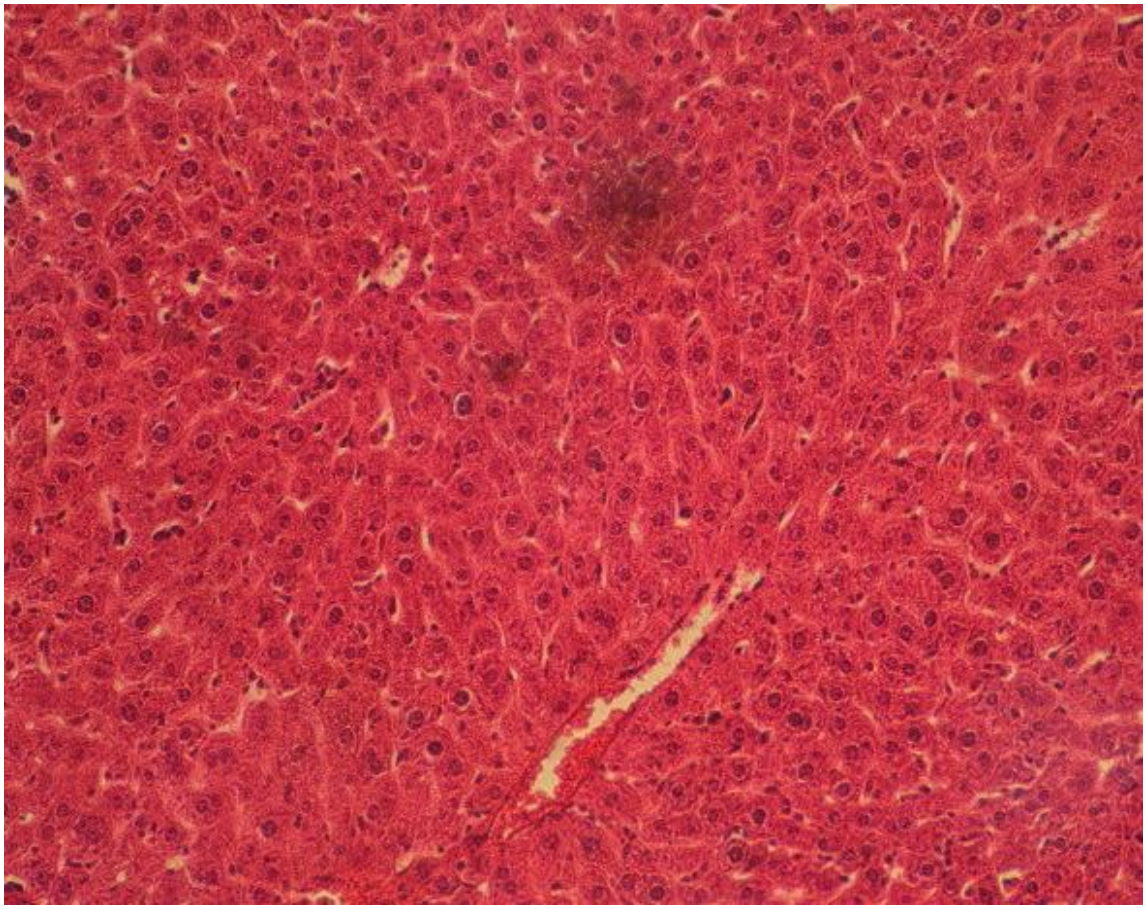


Plate V: Histologic section of the liver of mice 3 days after treatment with distilled water (5 ml/kg). H&E ×320. No observable pathology.

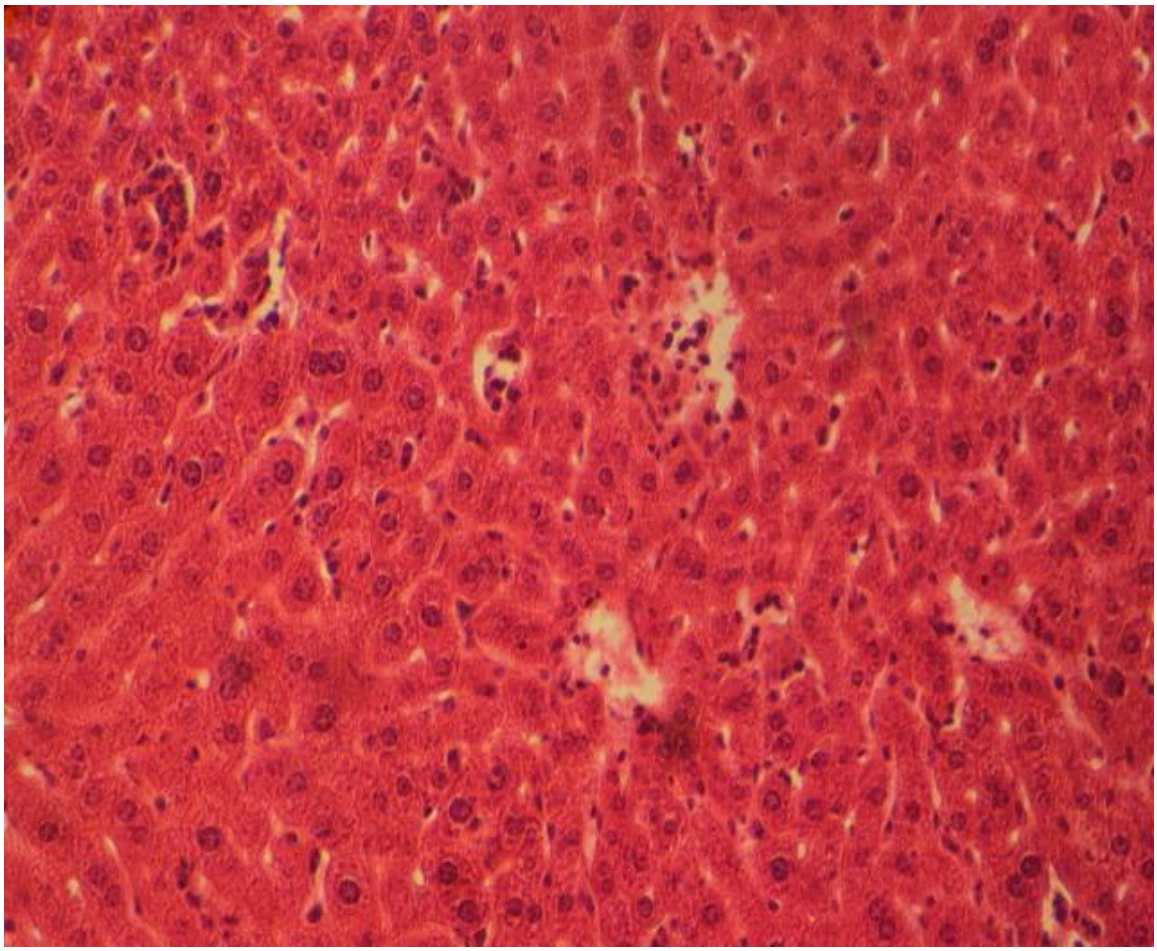


Plate VI: Histologic section of the liver of mice 3 days after treatment with crude methanol extract of *C. aurantifolia* fruit peels at 5000 mg/kg. H&E ×500. No observable pathology.

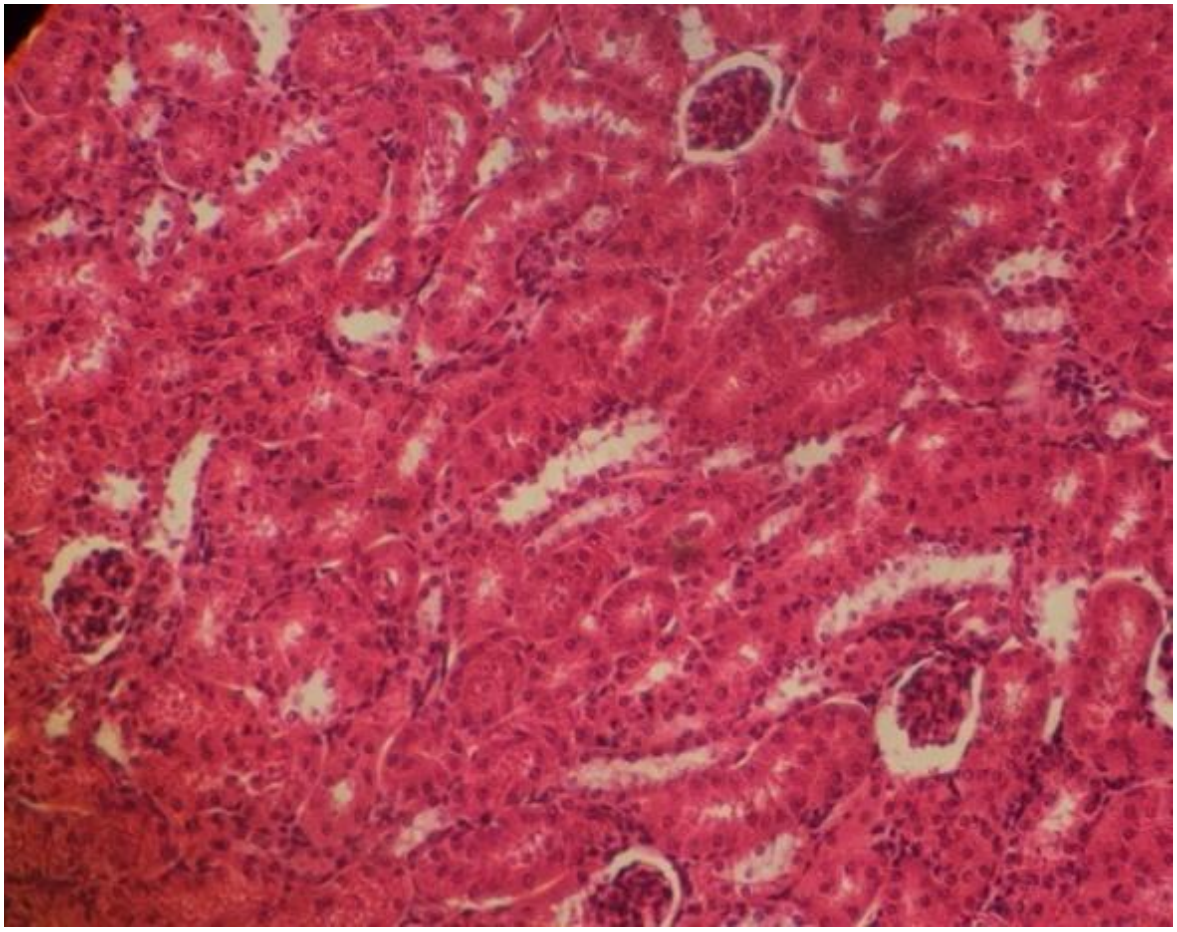


Plate VII: Histologic section of the kidney of mice 3 days after treatment with distilled water (5 ml/kg). H&E ×400. No observable pathology.

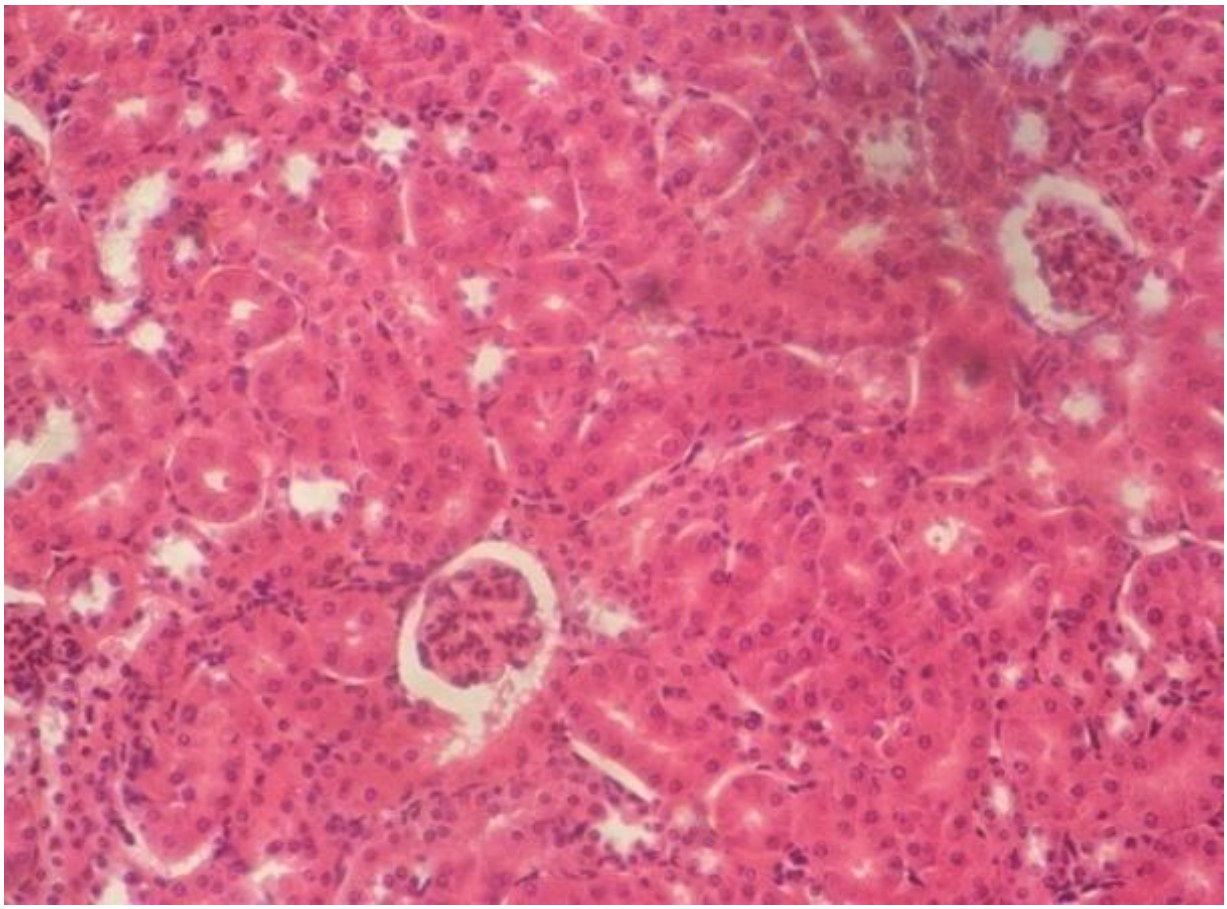


Plate VIII: Histologic section of the kidney of mice 3 days after treatment with crude methanol extract of *C. aurantifolia* fruit peels at 5000 mg/kg. H&E ×400. No observable pathology.

4.5 *In vitro* Anthelmintic Activities of *C. aurantifolia* Fruit Peels

4.5.1 Egg hatch inhibition

The extracts of *C. aurantifolia* fruit peels significantly ($p < 0.05$) inhibited the hatching of eggs of *H. bakeri* in a concentration-dependent manner (Table 4.5). There was a positive correlation between the concentrations of CME, AME, HE and ABZ and the rates of egg hatch inhibition i.e., as the drug concentration increased, the egg hatch inhibition rate increased. However, the opposite was the case for BE.

4.5.2 Larvicidal activity of *C. aurantifolia* fruit peel extracts

All the extracts of *C. aurantifolia* fruit peels had significant ($p< 0.01$) larvicidal activity against L_1 of *H. bakeri*. The effect is concentration-dependent. The mean (±SD) percentage larvicidal effect of the extracts is shown in Table 4.6.

Table 4.5: Mean (±SD) percentage Egg Hatch Inhibition of *H. bakeri* by *C. aurantifolia* fruit peels extracts at different concentrations.

Extracts	Mean (±SD) percentage egg hatch inhibition at different concentrations of extracts					
	0.625 mg/ml	**1.25 mg/ml**	**2.5 mg/ml**	**5.0 mg/ml**	**10.0 mg/ml**	**20.0 mg/ml**
CME	23.6 ±15.29[c]	37.6 ±27.31[c]	90.2 ± 2.10[a]	97.1 ± 0.55[a]	100.0 ±0.00[a]	100.0 ±0.00[a]
AME	23.8 ± 0.80[c]	17.8 ± 6.27[c]	35.9 ±18.88[c]	87.4±11.15[a]	90.7 ±11.92[a]	97.7 ± 2.86[a]
BE	82.4 ±3.76[a]	81.9 ±4.76[a]	65.1 ±17.75[b]	31.0 ±4.3[c]	20.8± 12.77[c]	28.2 ±11.60[c]
EE	28.2 ±24.47[c]	42.0 ± 9.61[c]	37.3 ± 5.48[c]	35.3 ± 3.11[c]	63.0 ± 9.62[b]	98.4 ± 2.77[a]
HE	6.1 ± 6.99[c]	21.6 ±18.16[c]	66.5 ±15.36[b]	77.9 ± 7.85[b]	97.9 ± 4.79[a]	97.8 ± 0.68[a]
ABZ*	87.0 ± 0.75[a]	91.9 ± 2.00[a]	97.0 ± 5.25[a]	100.0 ±0.00	100.0 ±0.00[a]	100.0 ± 0.0[a]
DW	0.00 ±0.00					

Means with different superscript letter (a, b, c) differ significantly ([a] $p < 0.0001$, [b] $p < 0.001$ and [c] $p < 0.05$) from the distilled water-treated control group.

CME = crude methanol extract of *C. aurantifolia* fruit peels.
AME = aqueous methanol extract of *C. aurantifolia* fruit peels.
BE = butanol extract of *C. aurantifolia* fruit peels.
EE = ethylacetate extract of *C. aurantifolia* fruit peels.
HE = hexane extract of *C. aurantifolia* fruit peels
ABZ* = albendazole (at concentration of 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml).
DW = distilled water.

Table 4.6: Mean (±SD) percentage larvicidal effect of *C. aurantifolia* fruit peels extracts at different concentration on L_1 stage of *H. bakeri*.

Extracts	Mean (±SD) percentage larvicidal effect of *C. aurantifolia* peels at different concentrations					
	0.625 mg/ml	**1.25 mg/ml**	**2.5 mg/ml**	**5.0 mg/ml**	**10.0 mg/ml**	**20.0 mg/ml**
CME	58.2±9.83[b]	67.9 ±7.47[b]	74.8±1.69[b]	78.6±3.52[a]	94.6±9.35[a]	100.0±0.0[a]
AQE	68.8±9.35[b]	75.1 ±13.31[b]	80.0±3.80[a]	84.5±7.83[a]	87.1±7.18[a]	90.8±8.11[a]
BNE	55.1±5.27[b]	60.9±16.65[b]	80.8±9.01[a]	64.6±5.12[b]	75.9±7.90[b]	94.4±5.13[a]
EAE	35.7±17.99[c]	56.5±15.30[b]	74.0±15.36[b]	67.5±12.35[b]	72.2±11.65[b]	85.9±6.59[a]
HXE	17.5±11.03[c]	45.0±15.88[c]	62.4±8.86[b]	63.8±11.55[b]	82.8±11.55[a]	83.3±6.88[a]
ABZ*	73.5± 4.95 [b]	100.0±0.0 [a]	100.0±0.0 [a]	100.0±0.0 [a]	100.0±0.0 [a]	100.0±0.0 [a]
DW	16. 7±0.00					

Means with different superscript letter (a, b, c) differ significantly ([a] $p < 0.0001$, [b] $p < 0.001$ and [c] $p < 0.05$) from the distilled water-treated control group.

CME = crude methanol extract of *C. aurantifolia* fruit peels.
AME = aqueous methanol extract of *C. aurantifolia* fruit peels.
BE = butanol extract of *C. aurantifolia* fruit peels.
EE = ethylacetate extract of *C. aurantifolia* fruit peels.
HE = hexane extract of *C. aurantifolia* fruit peels
ABZ* = albendazole (at concentration of 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml).
DW = distilled water.

4.5.3 IC_{50} determination for egg hatch inhibition

The concentrations of the extracts that inhibited 50 % of *H. bakeri* eggs from hatching (IC_{50}) were determined from a log concentration-response curve plotted on Microsoft excel. The correlation/regression equation, coefficient and the IC_{50} values are shown in Table 4.7.

The IC_{50} was calculated by substituting Y with 50 in the equation given on Table 4.7, and using the equation to find the value of X. The antilog of X gives the concentration of the extracts that inhibited 50 % of the eggs from hatching during the test period.

4.5.4 LC_{50} determination for larvicidal test

The concentration of the extracts that killed 50 % of the larvae (LC_{50}) was determined from the equation of the log concentration-response curve and the values are presented in Table 4.8.

The LC_{50} was calculated by substituting Y with 50 in the equation given on the Table and using the equation to find the value of X. The antilog of X gives the concentration of the extracts that kill 50 % of the larvae (LC_{50}) during the test period.

Table 4.7: The log concentration-inhibition effect of the extracts of *C. aurantifolia* fruit peels against *H. bakeri* eggs

Treatments	**Correlation equation**	**Coefficient of determination (R^2) & (%)**	**IC_{50} (mg/ml)**
ABZ	Y = 51.69X + 50.99	0.05 (5%)	0.96
CME	Y = 73.50X + 17.81	0.92 (92%)	2.74
EE	$Y = 39.35X^2 + 29.13$	0.71 (71%)	3.39
BE	Y = -46.44X + 77.01	0.86 (86%)	3.80
AME	Y = 61.31X + 12.31	0.91 (91%)	4.12
HE	Y = 71.02X + 5.974	0.95 (95%)	4.17

CME = crude methanol extract of *C. aurantifolia* fruit peels.

AME = aqueous methanol extract of *C. aurantifolia* fruit peels.
BE = butanol extract of *C. aurantifolia* fruit peels.
EE = ethylacetate extract of *C. aurantifolia* fruit peels.
HE = hexane extract of *C. aurantifolia* fruit peels
ABZ* = albendazole (at concentration of 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml).
Y = 50 %
X = concentration of extracts (mg/ml) that inhibited 50 % of eggs of *H. bakeri* from hathing

Table 4.8: The log concentration-larvicidal effect of the extracts of *C. aurantifolia* fruit peels against *H. bakeri*.

Treatments	**Correlation Equation**	**Coefficient of Determination (R^2) & (%)**	**LC_{50} (mg/ml)**
ABZ	Y = 12.58X + 88.68	0.43 (43 %)	0.00084
AME	Y = 14.32X + 73.20	0.98 (98 %)	0.024
CME	Y = 27.92X + 63.72	0.98 (98 %)	0.32
BE	Y = 21.52X + 60.15	0.69 (69 %)	0.34
EE	Y = 27.77X + 50.08	0.81 (81 %)	0.99
HE	Y = 42.27X + 35.97	0.90 (90 %)	2.15

CME = crude methanol extract of *C. aurantifolia* fruit peels.
AME = aqueous methanol extract of *C. aurantifolia* fruit peels.
BE = butanol extract of *C. aurantifolia* fruit peels.
EE = ethylacetate extract of *C. aurantifolia* fruit peels.
HE = hexane extract of *C. aurantifolia* fruit peels
ABZ* = albendazole (at concentration of 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml).
Y = 50 %
X = concentration of extracts (mg/ml) that killed 50 % of *H. bakeri*

4.6 *In vivo* Anthelmintic Activity of *C. Aurantifolia* Fruit Peels in Mice

4.6.1 Fecal egg count reduction

There was a significant ($p<0.05$) reduction in fecal egg counts (FEC) in all the extract-treated groups when compared with the distilled water treated group. The FEC reduction rates after the 3 days of treatment are presented in Table 4.9. There was however, no significant ($p>0.05$) difference in the FEC reduction rate within mice treated with the extracts.

4.6.2 Post-mortem worm counts

The mean worm counts and the deparasitization rates are shown in Table 4.10. Statiscal analysis has showed that there was no significant difference ($p \square 0.05$) in the worm counts of mice treated with the individual extracts at 250, 500 and 1000 mg/kg. However, CME at 500 and 1000 mg/kg, BE at 1000 mg/kg and EE at 250, 500, 1000 mg/kg produced percentage deparasitization $\geq$ 70 and which was considered significant. Also, the deparasitization rates caused by CME, BE and EE were all significantly ($p<0.05$) lower than that caused by ABZ (10 mg/kg).

Table 4.9: EPG/ Percentage fecal egg count reduction rates in mice treated with *C. aurantifolia* fruit peels extracts at different doses.

Treatment	**Doses (mg/kg)**	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Crude	250	810	1116	1668	1122 (81)
methanol	500	640	492	748	614 (90)
extract	1000	320	332	248	508 (91)
Aqueous	250	1008	1008	1478	2456 (58)
methanol	500	1268	782	1382	1690 (71)
extract	1000	840	840	520	688 (88)
Butanol extract	250	804	1266	996	1178 (80)
	500	326	446	762	750 (87%)
	1000	278	310	408	672 (89%)
Ethylacetate	250	518	164	704	1684 (71)
extract	500	444	154	682	1332 (77)
	1000	178	130	364	132 (98)
Control					
Albendazole	10	304	32	16	0 (100)
Distilled water	5 ml/kg	2464	2880	3088	5872

Table 4.10: Mean Worm Counts (±SD) in mice treated with varying doses of *C. aurantifolia* fruit peel extracts 16-days post-infection with 200 L_3 of *H. bakeri*

Treatments	Doses (mg/kg)	Mean worm counts (±SD: n=5)	Percentage (%) deparasitization
Crude methanol extract (CME)	250	15.2 ± 7.36^{d}	62
	500	11.6 ± 3.78^{b}	71
	1000	10.0 ± 2.45^{b}	75
Aqueous methanol extract (AME)	250	26.2 ± 4.15^{c}	35
	500	22.6 ± 6.27^{c}	44
	1000	19.8 ± 3.83^{c}	51
Butanol extract (BE)	250	14.8 ± 4.72^{d}	63
	500	12.4 ± 2.30^{b}	69
	1000	8.6 ± 3.85^{b}	79
Ethylacetate extract (EE)	250	11.6 ± 3.21^{b}	71
	500	11.0 ± 4.85^{b}	73
	1000	10.2 ± 3.96^{b}	75
Controls			
Albendazole (+ve)	10	1.5 ± 1.29^{a}	96
Dist. water (-ve)	5ml/kg	40.0 ± 4.35^{e}	0

Means with different superscript letter (a, b, c, d, e) differ significantly ($p<0.05$) from one another.

CHAPTER FIVE

DISCUSSION

In this study, the methanol extraction of *C. aurantifolia* fruit peels yielded 13.4 % of crude methanol extract. Methanol is commonly used for the extraction of plant materials because of its ability to extract almost all the chemical components of the plant due to its high polarity (Cock, 2011). Li *et al*. (2006) reported a high yield (74 %) of *Citrus* fruit peels following extraction with ethanol. The crude methanol extract of *C. aurantifolia* fruit peels was partitioned with different solvents to separate the phytochemical components of the crude methanol extract based on their polarity and solubility. Partitioning of plant extract has been established as one of the methods used in separating plant components based on their relative solubilities in the solvents used (Harbone, 1973). From the results of the partitioning process, butanol and aqueous methanol (residue) gave more yields when compared to the hexane and ethylacetate portions. Nwauzoma *et al*. (2013) reported a higher yield of aqueous methanol extract of *C. aurantifolia* fruit peels. Also, the yield from hexane extraction of 1400 gram of *C. aurantifolia* fruit peels by Nallely *et al.* (2012) gave 16 g (1.14 %) of extract. This showed that extraction of *Citrus* fruit peels with non-polar solvents (hexane) is usually small. The variations in the extract yields from *C. aurantifolia* fruit peels using different solvents might be explained by the polarity of extracted components (Shabbir *et al*., 2011). It could also be due to the availability of the different extractable components of the plant as a result of varied chemical compositions (Hsu and Coupar, 2006), or due to the efficiency of the extracting solvents to dissolve endogenous compounds present in the plant (Siddhuraju and Becker, 2003; Sultana *et al*., 2007) and may also be due to the geographical location of the plant (Cock, 2011).

The preliminary phytochemical screening and TLC analysis of *C. aurantifolia* fruit peel extracts showed that the plant contained carbohydrate, glycosides, steroids, flavonoids, phenol, alkaloids and condensed tannins. However, alkaloids were absent in the hexane and aqueous methanol extracts. Saponins and triterpenes were present in all the portions except in the hexane extract portion. The finding of this study agrees with what was reported earlier by other workers (Berhow *et al*., 1994; Wang *et al*., 2007; Dongmo *et al.*, 2009; Khan and Abourashed, 2010; Bairagi *et al*., 2011; Pathan *et al*., 2012; Chaiyana and Okonogi, 2012; Loizzo *et al*., 2012; Reddy *et al*., 2012; Akhtar, 2013). These phyto-constituents in *C. aurantifolia* fruit peels have been suggested to be responsible for the different pharmacologic activities of the plant (Abdelqader *et al*., 2012).

The results of the acute toxicity studies showed that extracts of *C. aurantifolia* fruit peels were non-toxic to mice even at a dose up to 5000 mg/kg per os. Grossly, the pathological findings in mice treated with the extract showed slight haemorrhage of the stomach and intestine. Histopathological examination revealed no observable lesions on different organs of the treated mice. This result which showed that the extracts were non-toxic agrees with findings by Chunlaratthanaphorn *et al*. (2007) that reported the non-toxic effect of water extract of the plant in both acute and subchronic toxicity studies, although in this work subchronic toxicity study was not conducted. Similarly, subchronic toxicity study on a related species; *Citrus aurantium* indicated a very low toxic effect of the plant in mice (Arbo *et al*., 2009). However, acute toxicity studies on the fruit juice of *C. aurantifolia* showed that the fruit juice is toxic to rats when given at doses above 3.5 g/kg (Akhtar, 2013).

The results of the *in vitro* and *in vivo* anthelmintic study revealed that CME, AME, BE, EE and HE significantly inhibited the hatching of *H. bakeri* eggs and killed the larvae

of the helminth as well as reduced the worm burdens in mice in a concentration-dependent manner. All the extracts of *C. aurantifolia* showed significant inhibitory effect on hatching of *H. bakeri* eggs. Even though there were differences in activity between the extracts of the plant, that was shown to be statiscally non-significant ($p>0.05$). The effect of CME, AME, BE on egg hatching of *H. bakeri* eggs was not significantly ($p>0.05$) different from the effect produced by albendazole (standard drug). Studies by other workers had shown that plant extract usually produce concentration – dependent effects when tested on helminth eggs (Alawa *et al*., 2003; Wasswa and Olila, 2006; Ademola and Eloff, 2011; Kollins *et al*., 2012). The egg hatch inhibitory activities of these extracts were more effective with the crude methanol extract. The higher effect produced by crude methanol extract may be due to the synergistic effects of the different chemical constituents of the extract that interacted in complex ways to produce an effect higher than the individual components (Cock, 2011). The ovicidal effect produced by these extracts might be caused by the penetration of the active phytochemicals into the helminth's egg shell which stopped the segmentation of the blastomere, thus, inhibiting the hatching of the eggs (Kollins *et al.*, 2012).

This study also showed an increase in the mean larval mortality rates with increase in concentration of all the extracts tested. In general, the extract with higher concentrations showed more activity when compared to extract with lower concentration. An increase in concentration represents a supplementary input of different active compound (Wabo *et al.*, 2011). The extracts showed a highly significant ($p<0.001$) larva mortality rates at the concentration range of 2.5-20 mg/ml. The larvicidal effect of CME, AME, BE on *H. bakeri* larvae was not significantly ($p>0.05$) different from effect produced by albendazole. Significant difference ($p<0.05$)

however existed when the larvicidal effect of EE and HE was compared to albendazole. The larvicidal effect produced by these extracts was due to the penetration of the active chemical constituents of the extract across the cuticle of the larvae into their circulatory system when the larvae were brought in contact with the extracts (Payne *et al*., 2013).

The *in vivo* evaluation of the extracts of *C. aurantifolia* fruit peels showed that the plant has a remarkable anthelmintic effect. All the extract portions caused a significant ($p<0.05$) reduction in fecal egg count (FEC) when compared with the negative control. The reduction in FEC might be due to the reduction of the worm burden in mice as a result of treatment with the extracts (Paolini *et al*., 2003; Ngongeh and Fakae, 2011). Abdelqader *et al*. (2012) reported a dose - dependent reduction in FEC in chicken infected with *Ascaridia galli* and treated with ethanol extracts of *Citrus* fruit peels. Similar findings were reported using different medicinal plants (Asuzu and Onu, 1994; Javed *et al*., 1994; Athanasiadou *et al*., 2007; Tariq *et al*., 2009; Squires *et al*., 2010; Ngongeh and Fakae, 2011).

Post-mortem worm counts indicated that *C. aurantifolia* fruit peels possess anthelmintic activity. The AME did not show a significant ($p>0.05$) deparasitization rate compared to CME, BE and EE. This is similar to the findings of Abdeqader *et al*. (2012) where the aqueous extract of *Citrus* peel had the least anthelmintic activity.

In this study, the anthelmintic effect of *C. aurantifolia* fruit peels could be attributed to one or more of the phytochemicals present in the plants. Limonene was believed to be the active principle in *citrus* fruit peels that caused its anthelmintic activity in ruminants (Rosskopf *et al*., 2008; Squires *et al*., 2010). The mode of action of limonene is unknown; however investigations suggested that it has inhibitory effects on nematode

growth and also interfere with parasite enzymes (Kaur *et al*., 2009; Squires *et al*., 2010).

Previous anthelmintic trials of medicinal plants have shown that the presence of secondary metabolites like alkaloids, saponin, polyphenols, carotenoids, tannins, coumarine, cardenolides, triterpenes, saponoside, embeline, sesquiterpenlactones may be responsible for the plants anthelmintic effect (Suleiman *et al*., 2005; Athanasiadou *et al*., 2007; Vagionas *et al*., 2007; Ajayi *et al*., 2008; Adama *et al*., 2009; Kabore *et al*., 2009; Marie-Magdeleine *et al*., 2009; Katiki *et al*., 2011). Similarly, Eguale *et al*. (2011) and Wabo *et al*. (2011) have shown that, carotenoids, triterpenes, saponin, steroids, coumarines, tannins and other chemical compounds of plants like glycosides, enzymes, anthraquinones, essential oils, lipid, protein, fibers if present in a plant implies that plant might have anthelmintic activity. The tannins contained in plants have been reported to posses antiviral (Cheng *et al*., 2002), antibacterial (Perumal *et al*., 1998) and anthelmintic (Paolini *et al*., 2003; Ademola *et al*., 2005) activities. The anthelmintic effects of tannins may be attributed to its ability to bind free protein available for larval nutrition and thus reduced nutrient availability which could result in larval starvation or decrease in gastrointestinal metabolism directly through inhibition of oxidative phosphorylation causing larval death. Tannins can also bind to free proteins in the gastrointestinal tract of host animal (Athanasiadou *et al*., 2007) or glycoprotein on the cuticle of the parasite (Thompson and Geary, 1995) and cause death but this effect of tannins depends on the type and content of tannins in the plant (Athanasiadou *et al*., 2007). The anthelmintic effect of tannins is similar to some synthetic phenolic anthelmintics like niclosamide, oxyclozanide, bithionol, nitroxynil, etc; which are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (Bate-Smith, 1962; Martin, 1997). More so,

tannins or their metabolites have been shown to have a direct effect on the viability of the preparasitic stages of helminths (Duncan, 1996). Saponin contained in plants has been shown to be responsible for the anthelmintic activities of some medicinal plants (McAllister *et al*., 2001). Monodesmoside saponins act by destabilizing the membranes and increasing the cell permeability in helminth parasites (Price *et al*., 1987; Gee and Johnson, 1988).

Though the active principles responsible for the anthelmintic activity in *C. aurantifolia* fruit peels is not known; condensed tannin and other phytochemicals present in the extracts might be responsible for the anthelmintic effect of the plant. Also, the fact that condensed tannins were present in all the extracts of the plant, perhaps, that is why all the extracts showed anthelmintic activities.

From this study, there was a variation between the *in vitro* and *in vivo* anthelmintic activity of *C. aurantifolia* fruit peels extracts. In the *in vitro* anthelmintic test, some of the extracts showed activity as high as 100 % at the concentration of 10 – 20 mg/ml, whereas, the *in vivo* anthelmintic test produced an average efficacy of 70 % at the dose of 500 – 1000 mg/kg. The differences between *in vitro* and *in vivo* efficacies could be attributed to the gastro intestinal tract environment as well as the physiology of the animals which may alter the helminth's response to treatment. In addition, oral drug administration is limited by many factors such as: first pass effect, drug forming complexes with food, drug being destroyed by gastric acid or enzymes and the effect of gastric pH on the drug. These might have limited the efficacy of the extracts *in vivo.* However, in the *in vitro* study, the extracts had direct contact with the parasites resulting in the high efficacy. Other workers reported similar differences between the *in*

vitro and *in vivo* anthelmintic activity of medicinal plants (Alawa *et al*., 2003; Iqbal *et al*., 2004; Adedapo *et al*., 2007; Ademola and Eloff, 2011).

The main advantages of using *in vitro* assays to test for the anti-parasitic properties of plant extracts is that the study is done at a low cost and there are rapid turnover which allows large scale screening of plants. Most anthelmintic activities of plants reported so far were based on *in vitro* studies (Mali and Mehta, 2007).

In this study, there was variation in the level of larvicidal and ovicidal effects shown by the extracts. The extract showed more larvicidal effect. This difference could be attributed to the egg shell resistance. The egg shell comprises three layers; an external lipoprotein layer, a middle chitinous protein layer and an inner lipid layer. The middle and inner layers are resistant to salts and chemicals and also protects the eggs from desiccation, strong acid and bases, oxidants, reductive agents, detergents and proteolytic compounds (Jimenez-Cisneros *et al*., 2007). This egg shell resistance limited the penetration of the extracts into the egg and thus, lowered the effects of the extracts had on the egg when compared to that of the larvae.

Albendazole showed a higher anthelmintic activity than *C. aurantifolia* fruit peels extracts in this study. This superior anthelmintic activity of albendazole could perhaps be attributed to its purity when compared with the extracts which may have other compounds contained in it. Albendazole is a broad-spectrum anthelmintics; and it has been shown to possess both ovicidal and larvicidal activities (Rivere and Papich, 2009).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The findings from this study suggest that the extracts of *C. aurantifolia* fruit peels possess anthelmintic activity. While the *in vitro* anthelmintic activity of the extract is comparable to that of albendazole, the *in vivo* activity was relatively lower. The results obtained therefore indicate that the extract of *C. aurantifolia* fruit peels might serve as a potentially useful and effective drug for the control of gastrointestinal nematodes in livestock and humans, since *H. bakeri*, the helminth used for this screening is a trichostrongyle like *Haemonchus*, the important abomasal parasite of sheep.

The preliminary phytochemical screening of *C. aurantifolia* fruit peels extracts showed that the plant contained carbohydrate, glycosides, steroids, flavonoids, phenol, alkaloids, saponins, triterpenes and condensed tannins, some of which might be responsible for the anthelmintic activity of the plant.

Acute toxicity studies of crude methanol extract of *C. aurantifolia* fruit peels showed that the plant has no apparent toxic effect in mice even at the dose of 5000 mg/kg.

6.2 RECOMENDATIONS

1. There is need to isolate and characterize the active principles from extracts of *C. aurantifolia* fruit peels. This could provide a safe and effective anthelmintic drug.
2. Further *in vivo* studies involving the target helminth parasites for specific animals should be conducted to evaluate the efficacy of this plant on these animals.
3. Detail toxicological studies including, sub-chronic and chronic toxicity effect of *C. aurantifolia* fruit peels should be conducted.

REFERENCES

Abdelqader, A., Bassam, Q., Diya, A. and Gürbüz, D. (2012). Anthelmintic effects of citrus fruit peels ethanolic extracts against *Ascaridia galli. Veterinary Parasitology*, 1-7. doi:10.1016.

Abdu, P.A. and Faya, J.N. (2000). Efficacy of some Nigerian plants on helminthes found in local chickens. In: J.O. Gefu. P.A. Abdu. and C.B.I. Alawa (Eds.), *Ethnoveterinary practices, research and development. Proceedings of the International Workshop on Ethnoveterinary Practices* (pp. 14-18). Kaduna, Nigeria: NAPRI/ABU, Zaria.

Abo, K.A., Fred-Jaiyessimi, A.A. and Jaiyesimi, A.E.A. (2008). Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. *Journal of Ethnopharmacology*, 115: 67-71.

Aceves, J., Erlij, D. and Martinez-Maranon, R. (1970). The mechanism of the paralyzing action of tetramisole on *Ascaris* somatic muscle. *British Journal of Pharmacology*, 38: 602–607.

Adama, K., Belem, A.M.G., Tamboura, H.H., Traore, A. and Sawadogo, L. (2009). *In vitro* antelmintic effect of two medicinal plants (*Anogeissus leiocarpus* and *Daniellia oliveri*) on *Haemonchus contortus*, an abomasal nematode of sheep in Burkina Faso. *African Journal of Biotechnology*, 8(18): 4690-4695.

Adedapo, A. A., Shabi, O.O. and Adedokun, O.A. (2005). Anthelmintic efficacy of the aqueous crude extract of *Euphorbia hirta* Linn in Nigerian dogs. *Veterinary arhiv*, 75: 39-47.

Adedapo, A.A., Otesile, A.T. and Soetan, K.O. (2007). Assessment of the anthelmintic efficacy of an aqueous crude extract of *Vernonia amygdalina*. *Pharmaceutical Biology*, 45: 564–568.

Ademola, I.O., Akanbi, A.I. and Idowu, S.O. (2005). Anthelmintic activity of *Leucaena leucocephala* chromatographic seed fractions on gastrointestinal sheep nematodes. *Pharmaceutical Biology*, 45(7): 599-604.

Ademola, I.O. and Eloff, J.N. (2010). *In vitro* anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae. *Veterinary Parasitology*, 169 (2010): 198–203.

Ademola, O. and Eloff. J.N. (2011). Anthelmintic efficacy of cashew (*Anarcadium occidentale* L.) on *in vitro* susceptibility of the ova and larvae of *Haemonchus contortus*. *African Journal of Biotechnology*, 10(47): 9700-9705.

Aibinu, I., Adenipekun, T., Adelowotan, T., Ogunsanya, T. and Odugbemi, T. (2007). Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. *African Journal of Traditional Complement and Alternative Medicine*, 4(2): 185-90.

Ajayi, I.A., Ademola, I.O. and Okotie, S.V. (2008). *In vitro* larvicidal effects of *Aframomum danielli* seed extracts against gastrointestinal nematodes of sheep. *African Journal of Traditional Complement and Alternative Medicine*, 5(3): 244-246.

Akhtar, M. S., Iqbal, M. N. and Khan, M. L. (2000). Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo Pakistan subcontinent. *Small Ruminant Research*, 38: 99-107.

Akhtar, S. S. (2013). Evaluation of Cardiovascular Effects of *Citrus aurantifolia* (Linn.) Fruit. *Social Science Research Network*: Retrieved from http://ssrn.com/abstract=2279447. Retrieved June 13, 2013.

Alawa, C.B.I., Adamu, A.M., Gefu, J.O., Ajanusi, O.J., Abdu, P.A., Chiezey, N.P., Alawa, J.N. and Bowmana, D.D. (2003). *In vitro* screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. *Veterinary Parasitology*, 113 (2003): 73–81.

Aliu, Y.O. (2006). *Veterinary Pharmacology*. 1st ed. Zaria, Tamaza publishing company, pp. 467-482.

Arbo, M.D., Schmitt, G.C., Limberger, M.F., Charao, M.F., Moro, A.M., Ribeiro, G.L., Dallegrave, E., Garcia, S.C., Leal, M.B. and Limberger, R.P. (2009). Subchronic toxicity of *Citrus aurantium* L. (Rutaceae) extract and p-synephrine in mice. *Regulatory Toxicology and Pharmacology*, 54(2009): 114–117.

Asnaashari, S., Delazar, A., Habibi, B., Vasfi, R., Nahar, L., Hamedeyazdan, S. and Sarker, S.D. (2010). Essential oil from *Citrus aurantifolia* prevents ketotifen-induced weight-gain in mice. *Phytotherapeutic Resource*, 24(12): 1893-1897.

Asuzu, I.U. and Onu, O.U. (1994). Anthelmintic activity of the ethanolic extract of *Piliostigma thonningii* bark in *Ascaridia galli* infected chickens. *Fitoterapia*, 54(65): 291–297.

Athanasiadou, S., Githiori, J. and Kyriazakis, I. (2007). Medicinal plants for helminth parasite control: facts and fiction. *Animal*, 1: 1392–1400.

Bairagi, G.B., Kabra, A.O. and Mandade, R.J. (2011). Anthelmintic activity of *Citrus limon* L. (burm) fruit peels extract in Indian adult earthworm. *International Journal of Pharmacy and Technology*, 3(2): 664-667.

Bakare, A. A., Bassey, R. B., Okoko, I. E., Sanyaolu, A. O., Ashamu, A.E., and Ademola. A.O. (2012a). Effect of Lime Juice (*Citrus aurantifolia*) on histomorphological alterations of the ovaries and uterus of cyclic Sprague-Dawley rats. *European Journal of Scientific Research*, 67(4): 607-616.

Bakare, A.A., Basiono, R.B., Onyeka, C.A. and Duru, F.I. (2012b). Lime Juice (*Citrus aurantifolia*): Effect on fetal parameters of pregnant Sprague-Dawley rats. *International Journal of Medicine and Medical Sciences*, 2(5): 114-116.

Baker, N. F. (1954). Trichostrongylidosis – the mouse as an experimental animal. *Proceedings of the American Veterinary Medical Association,* 91: 185–191.

Bakunzi, F. R. and Serumaga-zake, P.A.E. (2000). The effect of strategic anthelmintic treatment on internal parasites in communally grazed sheep in a Semi-Arid area as reflected in the faecal nematodal egg count. *Tropical Animal Health Production,* 32: 295-302.

Bansemir, A.D. and Sukhdeo, M.V.K. (1994). The food resource of adult *Heligmosomoides polygyrus* in the small intestine. *Journal of Parasitology*, 80: 24-28.

Bate-Smith, E. C. (1962). The phenolic constituents of plants and their taxonomic significance. *Journal of Linn Society of Botany*, 58: 95-173.

Behnke, J. and Harris, P. (2009). *Heligmosomoides bakeri* or *Heligmosomoides polygyrus? Ameriacan Society of Tropical Medicine,* 80(4): 684-685.

Behnke, J.M., Menge, D.M. and Noyes, H. (2009). *Heligmosomoides bakeri*: a model for exploring the biology and genetics of resistance to chronic gastrointestinal nematode infections. *Parasitology*, doi:10.1017 /S0031182009006003.

Behnke, J. and Harris, P.D. (2010). *Heligmosomoides bakeri:* a new name for an old worm. *Trends Parasitology*, 26(11): 524-529.

Bendich, A., Machlina, L.J., Scandurraa, O., Burtonb, O.G. and Wayner, D.D. (1986). The antioxidant role of vitamin C. *Advances in Free Radical Biology and Medicine,* 2(2): 419–44.

Berhow, M.A., Bennett, R.D., Poling, S.M., Vannier, S., Hidaka, T. and Omura, M. (1994). Acylated flavonoids in callus cultures of *Citrus aurantifolia*. *Phytochemistry*, 36(5): 1225-7.

Bidkar, J.S., Bhujbal, M.D., Ghanwat, D.D. and Dama, G.Y. (2011). Anthelmentic Activity of *Citrus aurantium* Linn. *International Journal of Pharmaceutical Research and Development,* 3(3): 10.

Bizimana, N. (1994). *Traditional Veterinary practices in Africa*. The pharmaceutical Press Londons, British Veterinary Codex, pp. 231- 241.

Bizimenyenra, E.S., Githiori, J.B., Eloff, J.N. and Swan, G.E. (2006). *In vitro* activity of *Peltophorum africanum* Sond. (Fabaceae) extract on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*. *Veterinary Parasitology*, 142: 336–343.

Boshtam, M., Moshtaghian, J., Naderi, G., Asgary, S. and Nayeri, H. (2011). Antioxidant effects of *Citrus aurantifolia* (Christm) juice and peel extract on LDL oxidation. *Journal of Resource and Medical Science*. 16(7): 951-955.

Brain, K. R. and Turner, T.D. (1975). *The practical evaluation of Phytopharmaceuticals*. Wright-Scientechnica, Bristol, pp.10-30.

Bryant. V. (1973). Life cycle of *Heligmosomoides polygyrus*. *Journal of Helminthology*, 3: 263-268.

Camacho-Corona, M. R., Ramírez-Cabrera, M.A., Santiago, O.G., Garza-González, E., Palacios, I. P., and Luna-Herrera, J. (2008). Activity against drug resistant-tuberculosis strains of plants used in Mexican traditional medicine to treat tuberculosis and other respiratory diseases. *Phytotherapeutic Resource*, 22(1): 82-5.

Cavier, R. (1973). *Chemotherapy of intestinal nematodes*. Pergamon, Press Oxford. pp. 215-436.

Chaiyana, W. and Okonogi, S. (2012). Inhibition of cholinesterase by essential oil from food plant. *Phytomedicine*, 19(8-9): 836-9.

Cheng, H.Y., Lin, C.C. and Lin, T.C. (2002). Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. *Antiviral Research*, 55: 447–455.

Chiejina, S.N. and Fakae, B.B. (1984). Development and survival of infective larvae of gastrointestinal nematode parasites of cattle on pasture in eastern Nigeria. *Research of Veterinary Science*, 37: 148-153.

Chiezey, N.P., Gefu, J.O., Jagun, A.G., Abdu, P.A., Alawa, C.B.I., Magaji, S.O., Adeyinka, I.A., and Eduvie, L. O. (2000). Evaluation of some Nigerian plants for anthelmintic activity in young cattle. *Proceedings of an international workshop on Ethnoveterinary Practices held between 14 – 18 August, 2000,* Kaduna. Published by the National Animal Production Research Institute, Ahmadu Bello University, Zaria, Nigeria. pp. 38-48.

Chunlaratthanaphorn, S., Lertprasertsuke, N., Srisawat, U., Thuppia, A., Ngamjariyawat, A., Suwanlikhid, N., and Jaijoy, K. (2007). Acute and subchronic toxicity study of the water extract, from root of *Citrus aurantifolia* (Christm.) Swingle in rats, *Journal of Science and Technology*, 29(1): 125-139.

Cock, I.E. (2011). Problems of Reproducibility and Efficacy of bioassays using crude extracts, with reference to *Aloe vera. Pharmacognosy communications*, 1(1): 52-62.

Coles, G. C., Bauer, C., Borgsteede, F. H., Geerts, S., Klei, T. R., Taylor, M. A. and Waller, P. J. (1992). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of Veterinary importance. *Veterinary Parasitology,* 44: 35-44.

Coles, G. C. (1999). Anthelmintic resistance and the control of worms. *Journal of Medical Microbiology*, 48: 323–325.

Craig, E.J. (2002). Phytochemical Guardians of Our Health, *Journal of American Dietetic Association*, 97(2): 199-204.

Dalton, J.P., Brindley, P.J., Knox, D.P., Brady, C.P., Hotez, P.J., Donnelly, S., O'Neill, S.M., Mulcahy, G. and Loukas, A. (2003). Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. *International Journal for Parasitology*, 33: 621-640.

Das, F.A., Barua, I. and Das, D.D. (2008). Ethno-Medicinal practices: a case study among the Sonowal Kacharis of Dibrugarh Assam. *Ethno-Med*. 2(1): 33-37.

Das, K. Tiwari, R.K.S. and Shrivastava, D.K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*, 4(2): 104-111.

Day, K. P., Howard, J. R., Prowse, S. J., Chapman, C. B. and Mitchell, G. F. (1979). Studies on chronic versus transient intestinal nematode infections in mice. I. A comparison of responses to excretory/secretory (ES) products of *Nippostrongylus brasiliensis* and *Nematospiroides dubius* worms. *Parasite Immunology*, 1: 217–239.

Del –Rio, J.A., Obdulio, B.G., Castillo, J., Marin, F.R. and Ortuno, A. (1997). Uses and Properties of Citrus Flavonoids. *Journal of Agricultural and Food Chemistry*, 45(12): 4505-4514.

Dongmo, P.M. D., Tatsadjieu, L.N., Tchinda, E. S., Kuate, J., Amvam, P.H. Z. and Menut, C. (2009). Essential oils of *Citrus aurantifolia* from Cameroon and their antifungal activity against *Phaeoramularia angolensis. African Journal of Agricultural Research*, 4(4): 354-358.

Duncan, R. A. (1996). *An evaluation of suitable bioassays to measure the potential of condensed tannins to inhibit the viability of sheep nematode parasites*. Bachelor of Rural Science, Honours thesis, Animal Science, University of New England, Armidale, NSW, Australia, pp.34.

Ebana, R.U., Madunagu, B.E., Ekpe, E.D. and Otung, I.N. (1991). Microbiological exploitation of cardiac glycosides and alkaloids from *Garcinia kola, Borreria ocymoides, Kola nitida* and *Citrus aurantifolia*. *Journal of Applied Bacteriology*, 71(5): 398-401.

Effiom, O. E., Avoaja, D. A., and Ohaeri, C. C. (2012). Mosquito Repellent Activity of Phytochemical Extracts from Fruit peels of Citrus Fruit Species. *Global Journal of Science Frontier Research Interdiciplinary*, 12(1): 1.

Eguale, T., Tilahun, G., Debella, A., Feleke, A. and Makonnen, E. (2007). *In vitro* and *in vivo* anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus*. *Journal of Ethnopharmacology*, 110: 428–433.

Eguale, T., Dereje, T. and Mirutse, G. (2011). *In vitro* anthelmintic activity of crude extracts of five medicinal plants against egg-hatching and larval development of *Haemonchus contortus*. *Journal of Ethnopharmacology*, 137(1): 108-113.

Eloff, J.N. (1998).Which extractant should be used for screening and isolation of 396 antimicrobial components from plants? *Journal of Ethnopharmacology*, 60: 1–8.

Ettre, L. S. (1993). Nomenclature for chromatography (IUPAC Recommendations 1993) (retention factor, k in column chromatography). *Pure and Applied Chemistry*, 65 (4): 819-843, doi:10.1351/pac199365040819.

Fajimi, A. K. and Taiwo, A. A. (2004). Herbal remedies in animal parasitic diseases in Nigeria: a review. *African Journal of Biotechnology*, 4(4): 303-307.

Fakae, B.B. (1990). Epidemiology of helminthosis of small ruminants under the traditional husbandry system in eastern Nigeria. *Veterinary Research communications*, 14: 389-391.

Fakae, B. B., Chiejina, S. N., Musongong, G. A., Ngongeh, L. A., Behnke, J. M., and Wakelin, D. (2003). Prospects for genetic selection for resistance to gastrointestinal nematodes in West African Dwarf Goats. *Proceedings of the 28th Annual conference of the Nigerian Association for Animal Production*, vol. 28. pp. 65-70.

FAO. (2006). Citrus production. Retrieved from FAO Statistical data base.

Ferguson, U. (1990). *Citrus fruits processing*. Horticultural Science Florida, pp. 117-118.

Forrester, D. J. and Neilson, J. T. (1973). Comparative infectivity of *Heligmosomoides polygyrus* (=*Nematospiroides dubius*) in three species of Peromyscus. *Journal of Parasitology*, 59: 251–255.

Gaikwad, S.A., Kale, A.A., Jadhav, B.G., Deshpande, N.R. and Salvekar, J.P. (2011). Anthelmintic activity of *Cassia auriculata* L. extracts-*In vitro* study. *Journal of Natural Product and Plant Resource*, 1(2): 62-66.

Gao, K., Henning, S. M., Niu, Y., Youssefian, A. A., Seeram, N. P., Xu, A. and Heber, D. (2006). The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells. *Journal of Nutrition and Biochemistry*, 17(2): 89-95.

Gasbarre, C.L., Stout, L.W., and Leighton, A.E. (2001). Gastrointestinal nematodes of cattle in the northeastern US: results of a producer survey. *Veterinary Parasitology*, 101: 29–44.

Geary, T.G. and Thompson, D.P. (2003) Development of antiparasitic drugs in the 21st Century. *Veterinary Parasitology*, 115: 167-184.

Gee, J.M. and Johnson, I.T. (1988). Interaction between haemolytic saponins, bile salt and small intestinal mucosa in rats. *Journal of Nutrition*, 118: 1391-1397.

Gharagozloo, M. and Ghaderi, A. (2001). Immunomodulatory effect of concentrated lime juice extract on activated human mononuclear cells. *Journal of Ethnopharmacology*, 77(1): 85-90.

Gharagozloo, M., Doroudchi, M. and Ghaderi, A. (2002). Effects of *Citrus aurantifolia* concentrated extract on the spontaneous proliferation of MDA-MB-453 and RPMI-8866 tumor cell lines. *Phytomedicine*, 9: 475–477.

Githigia, S. M., Kimoro, C.O., Mwangi, G.M. and Gichanya, J. (1995): Prevalence and economic significance of oesophagostomum and other helminth parasites of ruminants surveyed in selected abattoirs around Nairobi, Kenya. Bull. *Animal Health and Production in Africa*, 43: 29-33.

Githiori, J.B. (2004). *Evaluation of anthelmintic properties of ethnoveterinary plants preparation used as livestock dewormers by pastoralist and small holder farmer in Kenya.* (Unpublished Doctoral dissertation), Department of Biomedical Sciences and Veterinary Public Health, SLU. Acta Universitatis Agricultural Suliae, pp. 76.

Githiori, J.B., Hoglund, J., Waller, P.J. and Baker, R.L. (2004). Evaluation of anthelmintic properties of some plants used as livestock dewormers against *Haemonchus contortus* infections in sheep. *Parasitology*, 129: 245–253.

Golob. P. (1999). Alphabetical List of Plant Families with Insecticidal and Fungicidal Properties. The use of spices and medicinals as bioactive protectants for grains. *Food and Agriculture Organization of the United Nations*. pp. 13. Retrieved June 19, 2013.

Gorinstein, S., Leontowicz, H., Leontowicz, M., Krzeminski, R., Gralak, M., Delgado-Licon, E. and Trakhtenberg, S. (2005). Changes in Plasma Lipid and Antioxidant Activity in Rats as a Result of Naringin and Red Grapefruit Supplementation. *Journal of Agriculture and Food Chemistry*, 53(8): 3223-3228.

Gray, G. D. (1997). The use of genetically resistant sheep to control nematode parasitism. *Veterinary Parasitology*. 72: 345-366.

Guarrera, M.P. (1999). Traditional antihelmintic, antiparasitic and repellant uses of plants in central Italy. *Journal of Ethnopharmacology*, 68: 183–192.

Halliwell, B. (2001). Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. *Drugs Ageing*, 18(9): 685-716.

Hammond, J. A., Fielding, D. and Bishop, S.C. (1997). Prospects for plant anthelmintics in tropical veterinary medicine. *Veterinary Research Communication*, 21: 213-228.

Hanasaki, Y., Ogawa, S. and Fukui, S. (1994). The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radical Biology and Medicine*, 16(6): 845-850.

Handa S.S., Khanuja, S.P.S., Longo, G. and Rakesh, D.D. (2008). *Extraction Technologies for Medicinal and Aromatic Plants*. International centre for science and high technology, Trieste, pp. 21-25.

Harborne. J.B. (1973). *Phytochemical methods- A guide to Modern techniques of plant analysis*. Chapman and Hall: London. pp.1.

Harder, A. (2002). Chemotherapeutic approaches to nematodes: current knowledge and outlook. *Parasitology Research*, 88: 272-277.

Hasegawa, S., Berhow, M. A. and Manners, G. D. (2000). *Citrus Limonoid Research: An Overview*. Washington DC, American Chemical Society, pp. 1-8.

Heuzé V., Tran G. and Hassoun P. (2012). Citrus pulp, dried. A programme by INRA, CIRAD, AFZ and FAO. Retrieved from http://www.feedipedia.org/node/680. on October 12, 2013.

Hordegen, P., Cabaret, J., Hertzberg, H., Langhans, W. and Maurer, V. (2006). *In vitro* screening of six anthelmintic plant products against larval *Haemonchus contortus* with a modified methyl-thiazolyl-tetrazolium reduction assay. *Journal of Ethnopharmacology*, 108(2006): 85–89.

Hsu, B. and Coupar, I.M. (2006). Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*. *Food Chemistry*, 98: 317-328.

Hubert, J. and Kerboeuf, D. (1992). A microlarval development assay for the detection of Anthelmintic resistance in sheep nematode. *Veterinary Record*, 130: 442-446.

Huisdena, C.M., Adesogana, A.T., Gaskinb, J.M., Courtney, C.H., Raji, A.M. and Kanga, T. (2010). Effect of feeding Mucuna pruriens on helminth parasite infestation in lambs. *Journal of Ethnopharmacology*, 127(2010): 669–673.

Hussain, A., Khan, M.N., Iqbal, Z. and Sajid, M.S. (2008). An account of the botanical anthelmintics used in traditional Veterinary practices in Sahiwal district of Punjab. Pak. *Journal of Ethnopharmacology*, 119: 185–190.

Hussain, A., Khan, M.N., Iqbal, Z.M.S. and Sajid, M. K. (2011). Anthelmintic activity of *Trianthema portulacastrum* L. and *Musa paradisiaca* L. against gastro intestinal nematodes of sheep. *Veterinary Parasitology*, (2011), doi:10.1016.

Ibrahim, M. A., Nwude, N., Ogunsussi, R.A. and Aliu, Y. O. (1983). Screenig of West African plants for anthelmintic activity. *International Livestock Centre for Africa*, 17: 19-23.

Ibrahim, M.A. (1984). *Evaluation of the activities of some West African traditional anthelmintic herbs against Nippostrongylus brasiliensis in rats.* M.Sc. Thesis, Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, pp. 116.

Ibukun, A., Tayo, A., Toyin, A., Tolu, O. and Tolu, O. (2007). Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. *Africa Journal of Traditional Medicine*, 4(2): 185-190.

Imade, G.E., Sagay, A.S., Onwuliri, V.A., Egah, D.Z., Potts, M. and Short, R.V. (2005). Use of lemon or lime juice douches in women in Jos, Nigeria. *Sex Health*, 2(4): 237-279.

Iqbal, Z., Nadeem, Q.K., Khan, M.N., Akhtar, M.S., and Waraich, F.N. (2001). *In Vitro* Anthelmintic Activity of *Allium sativum*, *Zingiber officinale*, *Curcurbita mexicana* and *Ficus religiosa*. *International Journal of Agriculture and Biology*, 3(4): 454 – 457.

Iqbal, Z., Lateef, M., Ashraf, M. and Jabbar, A. (2004). Anthelmintic activity of *Artemisia brevifolia* in sheep. *Journal of Ethnopharmacology*, 93(2004): 265–268.

Jabbar, A., Zaman, M.A., Iqbal, Z., Yaseen, M. and Shamim, A. (2007). Anthelmintic activity of *Chenopodium album* (L) and *Caesalpinia crista* (L) against trichostrongylid nematodes of sheep. *Journal of Ethnopharmacology*, 114: 86-91.

Jackson, F. and Coop, R.L. (2000). The development of Anthelmintic resistance in sheep nematodes. *Parasitology*, 120: 95-107.

Jackson, F and Gordon, Y. (2013). Screening plants for anthelmintic activity – a challenging situation. *Moredun Research Institute*, pp. 26.

Jaiprakash, R.P., Jayaprakasha, G.K., Mahadev, B.C. and Bhimanagouda, S.P. (2009). Bioactive compounds from maxican lime (*Citrus aurantifolia*) juice induce apoptosis in human pancreatic cells. *Journal of Agriculture and Food Chemistry*, 57(22): 10933-10942.

Javed, I., Akhtar, M.S., Rahman, Z.U., Khaliq, T. and Ahmad, M. (1994). Comparative anthelmintic efficacy and safety of *Caesalpinia crista* seed and piperazine adipate in chickens with artificially induced *Ascaridia galli* infection. *Acta of Veterinary*, 42: 103–109.

Jayaprakasha, G. K., Mandadi, K. K., Poulose, S. M., Jadegoud, Y., Nagana Gowda, G. A. and Patil, B. S. (2007). Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliata* (L.). *Bioorganic Medical Chemistry*, 15(14): 4923-4932.

Jayaprakasha, G. K., Mandadi, K. K., Poulose, S. M., Jadegoud, Y., Nagana Gowda, G.A. and Patil, B. S. (2008). Novel triterpenoid from *Citrus aurantium* L. possesses chemopreventive properties against human colon cancer cells. *Bioorganic Medical Chemistry*, 16(11): 5939-5951.

Jegede, O. C., Agbede, R.I.S., Ajanusi, J.O. and Adaudi, A.O. (2009). Anthelmintic screening of fractions of *Spigelia anthelmia* Linn extracts against experimental *Nippostrongylus braziliensis* in rats. *Veterinary arhiv*, 79: 189-197.

Jimenez-Cisneros, B.E. (2007). *Helminth ova control in waste water and sludge for Agricultural reuse*. In: W.O.K. Grabow, Ed. Encyclopedia of Life Support Systems (EOLSS), pp. 3-9.

Kabore, A., Belem, A., Gaston, M., Tamboura, H.H., Traore, A. and Sawadogo, L. (2009). *In vitro* anthelmintic effect of two medicinal plants (*Anogeissus leiocarpus* and *Daniellia oliveri*) on *Haemonchus contortus*, an abosomal nematode of sheep in Burkina Faso. *African Journal of Biotechnology*, 8(18): 4690-4695.

Kaemmerer, K. and Butenkotter, S. (1973). The problem of residues in meat of edible domestic animals after application or intake of organophosphate esters. *Residue Review*, 46, 1.

Katiki, L.M., Chagas, A.S., Bizzo, H.R., Ferreira, J.S., and Amarante, F.T. (2011). Antehlmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests. *Veterinary Parasitology*, 183:103-106.

Kaur, K., Jain, M., Kaur, T. and Jain, R. (2009). Antimalarials from nature. *Bioorganic of Medical Chemistry*, 17: 3229–3256.

Kerboeuf, D. (1978). The effects of time and temperature of storage on the infectivity of third-stage larvae of *Heligmosomoides polygyrus* (=*Nematospiroides dubius*): Effects on the development to the adult stage in mice. *Annales de Recherches Veterinaires*, 9: 153–159.

Khan, I.A. and Abourashed, E.A. (2010). *Leung's Encyclopedia of Common Natural Ingredients*. John Wiley and Sons Publication, New Jersey, pp. 422 – 423.

Khare, C.P. (2007). *Indian Medicinal Plants: An Illustrated Dictionary*. Springer, Berlin, pp. 154.

Kirubha, S.V., Senthamarai, K., Vasuki, A., Venkateswara, R. and Selvadurai, S. (2011). Anthelmintic activity of roots and rhizomes of *Corallocarpus epigaeus*. *Journal of Natural Product and Plant Resources*, 1(1): 81-84.

Kokwaro, J.O. (1993). *Medicinal plants of East Africa*, 2nd ed. Kenya Literature Bureau, Nairobi, pp.401.

Kollins, E.N., Wabo, P.J., Payne, V.K., Jeannette, Y. and Marie-Claire, K. (2012). *In vitro* Comparative Effect of Aqueous (Cold and Hot Water) and Ethanolic Extracts of the Seeds of *Aframomum Danielli* (Zingiberaceae) on Three Life Cycle Stages of the Parasitic Nematode *Heligmosomoides bakeri* (Nematoda; Heligmosomatidae), Parasite of the Laboratory Mice (*Mus Musculus*). *Medical and Aromatic Plants*, 1: 111. doi:10.4172/2167-0412.1000111.

Korkina, L. G., Afanas'ev, I. B. and Helmut, S. (1996). Antioxidant and Chelating Properties of Flavonoids. *Advances in Pharmacology, Academic Press*, 38: 151-163.

Kudi, A. C. and Myint, S.H. (1999). Antiviral activity of some Nigerian medicinal plant extracts. *Journal of Ethnophamarcology*, 68: 289-294.

Kumara, N.K. (2001). *Identification of strategies to improve research on medicinal plants used in Sri Lanka*. In: WHO Symposium. University of Ruhuna, Galle, Sri Lanka, pp. 6.

Kunow, M.A. (2003). *Maya Medicine: Traditional Healing in Yucatan*. UNM Press, New Mexico, pp. 117.

Li, .B.B., Smith, B. and Hossain, M.M. (2006). Extraction of phenolics from citrus fruit peels. *Solvent extraction method Separation and Purification Technology*, 48(2006): 182–188.

Loizzo, M.R., Tundis, R., Bonesi, M., Menichini, F., De Luca, D., Colica, C. and Menichini, F. (2012). Evaluation of *Citrus aurantifolia* peel and leaves extracts for their chemical composition, antioxidant and anti-cholinesterase activities. *Journal of Science, Food and Agriculture*, 24(12): 1893-18937.

Loomis, T. A. (1983). *Essential of toxicology*. 3rd Ed, Lea and Febiger, Philadelphia. pp. 275-287.

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

Luna, L.G. (1960). *Manual of histologic and special staining technique of the armed forces institute of pathology*. McGraw-Hill, New York, pp. 140-152.

Lyle, S. (2006). *How to Use Citrus Fruit peels in the Home and Garden*. Discovering Fruit and Nuts, pp. 130-142.

MAFF, (1977). *Manual of veterinary parasitological laboratory Techniques*. Technical bulletin No. 18, Hmso, London, pp. 36-37.

Mairiga, A.G., Abubakar, A. K., Mohammed, B. K. (2010). Social and health reasons for lime juice vaginal douching among female sex workers in Borno State, Nigeria. *African Journal of primary health care and family medicine,* 2(1): 1.

Malan, F. S. and van Wyk, J. A. (1992). The packed cell volume and colour of conjunctivae as aids for monitoring *Haemonchus contortus* infestations in sheep. In: *Proceedings of the South Africa Veterinary Association Biennial National Veterinary Congress (Eds: Grahamstown, South Africa,* 7 - 10 September, pp. 139.

Mali, R.G. and Mehta, A.A. (2007). A review of Anthelmintic plants, *Natural Product Radiance,* 7(5): 466-475.

Mangels, A.R., Holden, J.M. and Beecher, G.R. (1993). Carotenoid Content of Fruits and Vegetables, an Evaluation of Analytic Data. *Journal of American Dietetic Association*, 93: 284-296.

Manoj, J. and Rajesh, K. P. (2011). *In Vitro* Anthelmintic Activity of Aqueous and Alcoholic Extracts of *Citrus Medica* Leaves. *Scholar research library, Der Pharmacia Lettre*, 3(1): 396-399.

Manuch, P. (1997). Sheep in the humid tropics-evolution of the fiji sheep. *Proceedings of the Fourth International Veterinary Conference, University of New England. Armidale,* pp. 326-330.

Marie-Magdeline, C., Hoste, H., Mahieu, M. and Varo, A.H. (2009). *In vitro* effects of *Cacurbita moschata* seed extracts on *Haemonchus contortus*. *Veterinary Parasitology*, 161: 99-105.

Martin, R. J. (1997). Mode of action of Anthelmintic drugs. *Veterinary Journal,* 154: 11-34.

Mc Allister, T.A., Annett, C.B., Cockwell, C.L., Olson, M.E., Wang, Y. and Cheeke, P.R. (2001). Studies on the use of *Yucca schidigera* to control giardiosis. *Veterinary Parasitology*, 97: 85–99.

Michael, B., Meinke, P.T. and Shoop, W. (2001). Comparison of ivermectin, doramectin, selamectin, and eleven intermediates in a nematode larval development assay. *Journal of Parasitology*, 87(3): 692–696.

Monteiro, A. M., Wanyangu, S.W., Kariuki, D.P. Brain, R., Jackson, F. and Mckellar, Q.R. (1997). Pharmaceutical quality of anthelmintics sold in Kenya. *Veterinary Record*, 142: 396-398.

Morton, J. (1987). *Mexican Lime*. In: Julia F. M., and Miami, F.L. *Fruits of warm climates*, pp. 168–172.

Murugamani, V., Raju, L., Baskar, V.A.R., Manjir, S.K. and Girija, G.S. (2012). The new method developed for evaluation of anthelmintic activity by house fly worms and compared with conventional earthworm method. *ISRN Pharmacology*, 2012(2012), doi: 10.5402/2012/709860.

Nallely, E., Sandoval, M., Abraham, G., Elizondo-Treviño, E., Garza-González, E., Alvarez, L. and del Rayo, M. (2012). Chemical Composition of Hexane Extract of *Citrus aurantifolia* and Anti-Mycobacterium tuberculosis Activity of Some of Its Constituents. *Molecules*, 17: 11173-11184.

Nelson, B. (2011). Orange fruit peels could be made into biodegradable plastic. *Mother Nature Network*, Retrieved on November 28, 2013.

Ngongeh, L.A. (2004). *Exploitation of extracts from tanninferous forage for the control of gastrointestinal helminth parasites*. Unpublished M.Sc. Thesis submitted to the Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka. pp. 15-16.

Ngongeh, L.A. and Fakae, B.B. (2011). Effect of quebracho tannin drenching on *Heligmosomoides bakeri* infection in mice. *International Journal of Science and Nature*, 2(1): 132-135.

Niezen, J. H., Robertson, A. H., Waghorn, G. C. and Charleston, W. A. G. (1998). Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. *Veterinary Parasitology*, 80: 15-27.

Njoku, C.J. and Asuzu, I.U. (1998). Anthelmintic effects of leaf extract of *Ocimum gratissimum. Phytomedicine*, 5: 485-488.

Nunomura, R.C.S., Silva, E.C.C., Oliveira, D.F., Garcia, A.M., Boeloni, J.N., Nunomura, S.M., and Pohlit, A.M. (2006). *In vitro* studies of the anthelmintic activity of Picrolemma sprucei Hook. f. (Simaroubaceae). *Acta Amazonica*, 36(3): 327 – 330.

Nwachukwu, E., Onovo, O.M. and Ezeama, C.F. (2007). Effect of Lime Juice on the Bacterial Quality of Zobo Drinks Locally Produced in Nigeria. *Research Journal of Microbiology*, 2: 787-791.

Nwauzoma, A.B., Choudhary, M.I., Zulfiqar, A., and Olaiya, C.O. (2013). Biological activity of crude extracts of Citrus species from Nigeria. *Nature Science*, 11(7): 135-139.

Nwude, N. and Ibrahim, M. A. (1980). Plants used in traditional Veterinary practice in Nigeria , *Journal of Veterinary Pharmacology and therapeutics*, 3: 361-372.

Okewole, E. A. and Oduye, O.O. (2001). Experimental studies on hookworm infection. II. Haematological and plasma protein changes following *Ancylostoma caninum* infection in dogs. *Tropical Veterinary*, 19: 23-32.

Okwu, D.E. (2005). Phytochemicals, Vitamins and Mineral Contents of Two Nigerian Medicinal Plants. *International Journal of Molecular Medicine and Advance Sciences*, 1: 375-381.

Okwu, D.E. and Emenike, I.N. (2006). Evaluation of the Phytonutrients and Vitamins Contents of Citrus Fruits. *International Journal Molecular Medicine and Advance Sciences*, 2(1): 1-6.

Okwu, D.E. and Emenike, I.W. (2007). Nutritive Value and Mineral Content of Different Varieties of Citrus Fruits. *Journal of Food Technology*, 5(2): 92-1054.

Okwu, D.E. (2008). Citrus fruits: a rich source of phytochemicals and their roles in human health: a review. *International Journal of Chemical Science*, 6(2): 451-471.

Paolini, V., Bergeaud, J. P., Grisez, C., Prevot, F., Dorchies, P. and Hoste, H. (2003). Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. *Veterinary Parasitology,* 113: 253-261.

Parkins, J.J. and Holmes, P.H. (1989). Effects of gastrointestinal helminth parasites on ruminant nutrition. *Nutrition Research Review*, 2: 227-246.

Pathan, R.K., Papi, R. G., Parveen, P., Tananki, G., and Soujanya, P. (2012). *In vitro* Antimicrobial Activity of *Citrus aurantifolia* and its Phytochemical screening. *Asian Pacific Journal of Tropical Disease,* (2012): 328-331.

Patil, R.J. (2009). *Studies on isolation and characterization of bioactive compounds in lime [citrus aurantifolia (christm) swingle], their antioxidant and anticancer properties* (Ph.D Thesis). submitted to the Department of Crop Physiology. College of Agriculture, Dharwad University of Agricultural Sciences Dharwad. pp. 1-10.

Patil, J.R., Murthy, K.N., Jayaprakasha, G.K., Chetti, M.B. and Patil, B.S. (2009). Bioactive compounds from Mexican lime (*Citrus aurantifolia*) juice induce apoptosis in human pancreatic cells. *Journal of Agricultural Food Chemistry*, 57(22): 10933-10942.

Payne, V. K., Kollins, E.N., Wabo, P.J., Yondo, J., Komtangi, M., Mpoame, M. and Bilong, B. C. F. (2013). *In vitro* comparative effect of extracts of the seeds of *Embelia rowlandii* (Myrsinaceae) on the eggs and L1 and L2 larval stages of the parasitic nematode *Heligmosomoides bakeri* (Nematoda; Heligmosomatidae). *African Journal of Biotechnology*, 12(2): 205-211.

Perry, B. D. and Randolph, T. F. (1999). Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Veterinary Parasitology,* 84: 145-168.

Perry, B. D., Randolph, T. F., McDermott, J. J., Sones, K. R. and Thornton, P. K. (2002). Investing in animal health research to alleviate poverty. *International Livestock Research Institute*, Nairobi, Kenya, pp. 148.

Perumal, S.R., Ignacimuthu, S. and Sen, A. (1998). Screening of 34 Indian medicinal plants for antibacterial properties. *Journal of Ethanopharmacology*, 62: 173–182.

Pessoa, L.M., Morais, S.M., Bevilaqua, C.M.L. and Luciano, J.S. (2002). Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus.Veterinary Parasitology*, 109(2002): 59–63.

Piccinelli, A.L., García, M. M., Armenteros, D.M., Alfonso, M.A., Arevalo, A.C., Campone, L., and Rastrelli, L. (2008). HPLC-PDA-MS and NMR characterization of C-glycosyl flavones in a hydroalcoholic extract of *Citrus aurantifolia* leaves with antiplatelet activity. *Journal of Agricultural Food Chemistry*, 56(5): 1574-81.

Poulose, S. M., Harris, E. D. and Patil, B. S. (2005). *Citrus limonoids* induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *Journal of Nutrition*, 135(4): 870-877.

Pourhossein, Z., Qotbi, A.A., Seidavi, A., Laudadio, V., Centoducati, G. and Tufarelli, V. (2014). Effect of different levels of dietary sweet orange (*Citrus sinensis*) peel extract on humoral immune system responses in broiler chickens. *Animal Science Journal*, doi: 10.1111/asj.12250.

Price, K.R., Johnson, I.N.T. and Fenwick, G.R. (1987). The chemistry and biological significance of saponin in food and feed stuffs: *Research of Food Science*, 26: 27-135.

Prichard, R. (1994). Anthelmintic Resistance. *Veterinary Parasitology*, 54: 259-268.

Rahman, S., Parvez, A.K., Islam, R. and Khan, M.H. (2011). Antibacterial activity of natural spices on multiple drug resistant *Escherichia coli* isolated from drinking water in Bangladesh. *Annual Clinical Microbiology Antimicrobial*, 15(10): 10.

Raihan, M.O., Khalequeuzzaman, M., Brishti, A., Tareq, S.M., Hossain, A. and Rana, S. (2012). Anthelmintic and Antiproliferative activity of aerial parts of *Persicaria hydropiper. Pharmacia Sinica*, 3(1): 104-110.

Raiyemo, D. (2013). Production of citrus fruit in Nigeria: problem and prospects: *Edu Resource World*. Retrieved on November 28, 2013.

Rajarajan, A.T., Vijayasree, V.G., Kenichi, W., Vijaya, S., Narasimman, G. and Sadish, S.K. (2009). Anthelmintic and antimicrobial properties of fruit peels of *Citrus sinensis*. *Pharmacologyonline*, 1: 363-368.

Reddy, J.L., Reshma, D.J., Beena, J. and Spandana, G. (2012). Evaluation of antibacterial and antioxidant activities of the leaf essential oil and leaf extracts of *Citrus aurantifolia*. *Asian Journal of Biochemical and Pharmaceutical Research*, 2(2): 2249 - 4626.

Retsky, K.L., Freeman, M.W. and Frei, B. (1993). Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. Anti- rather than pro-oxidant activity of vitamin C in the presence of transition metal ions. *Journal of Biological Chemistry*, 268 (2): 1304–1309.

Riviere, R.E. and Papich, M.G. (2009). *Veterinary Pharmacology and Therapeutics*. 9th ed. Wiley-Black. pp.1053-1145.

Robertson, S.J. and Martin, R.J. (1993). Levamisole-activated single-channel currents from muscle of the nematode parasite *Ascaris suum. British Journal of Pharmacology*, 108: 70-178.

Robinson, M., Wahid, F. N., Behnke, J. M. and Gilbert, F. S. (1989). Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): dose-dependent expulsion of adult worms. *Parasitology*, 98: 115–124.

Roger, G.D.P. (2002). *Encyclopedia of Medicinal Plants*. Education and Health Library Editorial, pp. 153-154.

Rooprai, H. K., Kandanearatchi, A., Maidment, S. L., Christidou, M., Trillo-Pazos, G., Widmer, W. and Pilkington, G. J. (2001). Evaluation of the effects of swainsonine, captopril, tangeretin and nobiletin on the biological behavior of brain tumor cells *in vitro*. *Neuropathology and Applied Neurobiology*, 27: 29-39.

Roos, M. H. (1997). The role of drugs in the control of parasitic nematode infections: must we do without? *Parasitology*, 114 137-144.

Rosskopf, E.N., Therrien, L.L., Adkins, S.T., Iriarte, F., Foster, J.G., and Kokalis-burelle, N. (2008). Methods of reducing pests and treating gastrointestinal nematode infections. http://www.freshpatents.com. Retrieved on December 13, 2013.

Saeed, M., Iqbal, Z. and Jabbar, A. (2007). Oxfendazole resistance in gastrointestinal nematodes of beetal goats at livestock farms of Punjab (Pakistan). *Acta Veterinaria Brno*, 76: 79 – 85.

San Diego, C.A. (2010). GraphPad Software for windows, USA, http://www.graphpad.com.

Sarojini, N., Anjulata, S.M. and Kanti, C.C. (2011). Phytochemical screening and anthelmintic activity study of *Saraca indica* leave extracts. *International Research Journal of Pharmacy*, 2(5): 194 – 197.

Satrija, F., Nansen, P., Bjorn, H., Murtini, S. and He, S. (1994). Effect of papaya latex against *Ascaris suum* in naturally infected pigs. *Journal of Helminthology,* 68: 343-346.

Seddon, J.M., Ajani, A.U. and Sperduto, R.D. (1994). For the Eye Disease Case-Control Study Group, Dietray Carotenoids, Vitamin A, C, and E and Advanced Age-Related Macular Degeneration. *Journal of America Medical Association,* 271: 1413-1430.

Semenye, P.P., Mbabu, A.N., Mwandoto, B., Nyaribo, F.B., Onim, J.M. and Rurangirwa, F. (1992). Research highlights from the Small Ruminant Collaborative Research Support Program: The dual purpose goat (DPG). In: Small ruminant research and development in Africa. *Proceedings of the first biennial conference of the African small ruminant research network* (Eds: B. Rey, S. H. B. Lebbie and L. Reynolds) International Livestock Centre for Africa (ILCA), Nairobi, Kenya, ILRAD, Nairobi, Kenya, 10 - 14 December 1990, pp. 533 - 543.

Sethpakdee, R. (1992). *Citrus aurantifolia (Christm. and Panzer) Swingle*. In: R.E. Coronel., and E.W., Verheij. (Eds.): Plant Resources of South-East Asia. No. 2: Edible fruits and nuts. Prosea Foundation, Bogor, Indonesia, pp. 126-128.

Shabbir, G., Anwar, F., Sultana, B., Khalid, Z.M., Afzal, M., Khan, M.Q. and Ashrafuzzaman, M. (2011). Antioxidant and antimicrobial attributes and phenolics of different solvent extracts from leaves, flowers and bark of Gold Mohar (*Delonix regia*). *Molecules,* 16: 7302-7319.

Shalaby, N.M.M., Howaida, A., Hanaa, H. A. and Nour, B. (2011). Protective effect of *Citrus sinensis* and *Citrus aurantifolia* against osteoporosis and their phytochemical constituents. *Journal of Medicinal Plants Research*, 5(4): 579-588.

Siddhuraju, P. and Becker, K. (2003). Antioxidant properties of various extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* L.) leaves. *Journal of Agriculture and Food Chemistry*, 51: 2144-2155.

Simon, M.K., Nafanda, W.D. and Obeta, S.S. (2012). *In- vivo* evaluation for anthelmintic effect of alkaloids extracted from the stem bark of *Afzelia africana* in rats. *Journal of Advanced Scientific Research*, 3(1): 100-104.

Singh, B. and Bhat, T. K. (2003). Potential therapeutic applications of some antinutritional plant secondary metabolites. *Journal of Agricultural and Food Chemistry,* 51: 5579-5597.

Smith, W.D., Newlands, G.F.J., Smith, S.K., Pettit, D. and Skuce, P.J. (2003). Metalloendopeptidases from the intestinal brush border of *Haemonchus contortus* as protective antigens for sheep. *Parasite Immunology,* 25: 313-323.

Smyth, J.D. (1994). *Animal Parasitology*. 3rd Ed. Cambridge University Press. Great Britain. pp. 549.

Sofowora, A. (1993). *Medicinal plants and traditional Medicine in Africa*. John Wiley and Sons, NewYork, pp.102.

Sonali, M., Dilip, P. and Vishwas, I. V. (2011). Evaluation of the anthelmintic activity of *Citrus limon* juice sacs. *International Journal of Pharma Professionals*, 2(2): 976-6723.

Soulby, F.J.L. (1982). *Arthropods and protozoa of domesticated animals*. 7th ed, Bailler. Tindal. London, pp. 809.

Souza, A., Datte, Y.J. and Abo, K.J.C. (2002). Effet myostimulant de l'extrait aqueux de *Citrus aurantifolia* sur l'activité contractile du muscle lisse :le taenia coli de cobaye. *Review of Medicine and Pharmacy of Africa,* 16: 103-112.

Souza, A., Lamidi, M., Ibrahim, B., Aworet. S.R.R., Boukandou, M. and M'Batchi, B. (2011). Antihypertensive effect of an aqueous extract of *Citrus aurantifolia* (Rutaceae) (Christm.) Swingle, on the arterial blood pressure of mammal. *International Research of Pharmacy and Pharmacology*, 1(7): 142-148.

Spurlock, G. M. (1943). Observations on host-parasite relations between laboratory mice and *Nematospiroides dubius*. *Journal of Parasitology*, 29: 303–311.

Squires, J.M., Foster, J., Lindsay, D., Caudell, D. and Zajac, A. (2010). Efficacy of an orange oil emulsion as an anthelmintic against *Haemonchus contortus* in gerbils (*Meriones unguiculatus*) and in sheep. *Veterinary Parasitology*, 172: 95–99.

Strohle, A. and Hahn, A. (2009). Vitamin C and immune function. *Med Monatsschr Pharm*, 32(2): 49-54.

Suleiman, M.M. (2002). *Anthelmintic screening of extracts of Terminalia avicennoides and Xylopia aethiopica in rats experimentally infected with Nippostrongylus brasiliensis*. Unpublished M.Sc. Thesis submitted to the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. pp. 21-74.

Suleiman, M. M., Mamman, M., Aliu, Y.O. and Ajanusi, J.O. (2005). Anthelmintic activity of the crude methanol extract of *Xylopia aethiopica* against *Nippostrongylus brasiliensis* in rats. *Veterinary arhiv*, 75: 487-495.

Sultana, B., Anwar, F. and Przybylski, R. (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica,* and *Eugenia jambolana* Lam Trees. *Food Chemistry,* 104: 1106-1114.

Sykes, A. R. and Coop, R. L. (2001). Interaction between nutrition and gastrointestinal parasitism in sheep. *New Zealand Veterinary Journal,* 49: 222-226.

Tagboto, S. and Townson, S. (2001). Antiparasitic properties of medicinal plants and other naturally occurring products. *Advance Parasitology,* 50: 199–295.

Taiwo, T.A. (2005). *Production of Fruits, Vegetables, Grains, Legumes.Root crops in Nigeria; Problems and Prospects*. University Press, Vol. 1, pp. 9.

Tare, H.L., Gore, M.S., Deore, S.R., Bidkar, J.S. and Dama, G.Y. (2011). Comparative helmintholytic potential of extracts obtained from *Cymbopogon citratus* and *Wrightia tinctoria* leaves. *International Journal of Pharmaceutical and Biological Sciences*, 2(1): 323-327.

Tariq, K.A., Chishti, M.Z., Ahmad, F. and Shawl, A.S. (2009). Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. *Veterinary Parasitology*, 160: 83–88.

Telford, G., Wheeler, D.J., Appleby, P., Bowen, J.G. and Pritchard, D.I. (1998). *Heligmosomoides polygyrus* immunomodulatory factor (IMF), targets T-lymphocytes. *Parasite Immunology*, 20: 601-611.

Thamsborg, S. M., Roepstorff, A. and Larsen, M. (1999). Integrated and biological control of parasites in organic and conventional production systems. *Veterinary Parasitology*, 84: 169-186.

Thompson, D. P. and Geary, G.T. (1995). *The structure and function of helminth surfaces*. In: J.J. Marr., Biochemistry and Molecular Biology of Parasites. 1st ed. Academic Press, New York, pp. 203-232.

Tiwari, P., Bimlesh, K., Mandeep, K., Gurpreet, K. and Harleen, K. (2011). Phytochemical screening and Extraction: A Review *Internationale Pharmaceutica Sciencia*, 1:1.

Trease and Evans (2007). *Pharmacognosy*. Springer publication, pp: 153-157.

USDA. (2013). Agricultural Research Service, United States Department of Agriculture. National Nutrient Database for Standard Reference, Release 26 Software v.1.3.1. Retrieved December 13, 2013.

Vagionas, K., Graikou, K., Chinou, I.B., Runyoro, D. and Ngassapa, O. (2007). Chemical analysis and antimicrobial activity of essential oils from the aromatic plants *Artemisia afrajacq* and *Leonotiso cymifolia* (Burm.F.) growing in Tanzania. *Journal of Essential Oil Research*, 482.

van Houtert, M.F.J. and Sykes, A.R. (1996). Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. *International Journal for Parasitology*, 26: 1151-1167.

van Wyk, J. A. and Bath, G. F. (2002). The FAMACHA© system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. *Veterinary Research*, 33: 509-529.

van Wyk, J. A., Malan, F.S., van Rensburg, L.J., Oberem, P.T. and Allan, M.J. (1997). Quality control in generic anthelmintics: is it adequate? *Veterinary Parasitology*, 72: 157–165.

van Wyk, J. A., Bath, G. F. and Malan, F. S. (1998). The need for alternative methods to control nematode parasites of ruminant livestock in South Africa. *World Animal Review*, 91.

Vatta, A. F., Krecek, R. C., Letty, B. A., van der Linde, M. J., Motswatswe, P. W. and Hansen, J. W. (2002). Effect of nematode burden as assessed by means of faecal egg counts on body condition in goats farmed under resource-poor conditions in South Africa. *Veterinary Parasitology*, 108: 247-254.

Wabo Poné J, Bilong, B.C.F. and Mpoame, M. (2010). *In vitro* nematicidal activity of extracts of *Canthium mannii* (Rubiaceae) on different life cycle stages of *Heligmosomoides polygyrus* (*Nematoda, Heligmosomatidae*). *Journal of Helmenthology*, 84:156-165.

Wabo, P.J., Yondo, J., Fossi, T.O., Marie, C. K., Bilong, B. C. F. and Mpoame, M. (2011). The *in vitro* effects of *Chenopodium ambrosioides* (Chenopodiaceae) extracts on the parasitic nematode *Heligmosomoides bakeri* (*Nematoda, Heligmosomatidae*). *Journal of Pharmacognosy and Phytotherapy*, 3(4): 56-62.

Wabo, P.J., Payne, V.K., Mbogning, T.G., Komtangi, M. C., Yondo, J., Ngangout, A.M., and Bilong, B.C.F. (2013). *In vitro* anthelminthic efficacy of *Dichrocephala integrifolia* (Asteraceae) extracts on the gastro-intestinal nematode parasite of mice: *Heligmosomoides bakeri* (*Nematoda, Heligmosomatidae*). *Asian Pacific Journal of Tropical Biomedicine*, 3(2): 100-104.

Waller, P.J. (1987). Anthelmintic resistance and future for roundworm control. *Veterinary Parasitology*, 15(2): 177-191.

Waller, P. J. (1997). Nematode parasite control of livestock in the tropics/subtropics: the need for novel approaches. *International Journal of Parasitology*, 27: 1193-1201.

Waller, P.J. (1999). International approaches to the concept of integrated control of nematode parasites of livestock. *International Journal of Parasitology*, 29: 155-164.

Wang, Y.C., Chuang, Y.C. and Ku, Y.H. (2007). Quantitation of bioactive compounds in citrus fruits cultivated in Taiwan. *Food Chemistry*, 102(4), 1163-1171.

Wasswa, P. and Olila, D. (2006). The *in-vitro* Ascaricidal activity of selected indigenous Medicinal Plants used in Ethno Veterinary Practices in Uganda. *African Journal of Traditional Complementary and Alternative Medicines*, 3(2): 94 – 103.

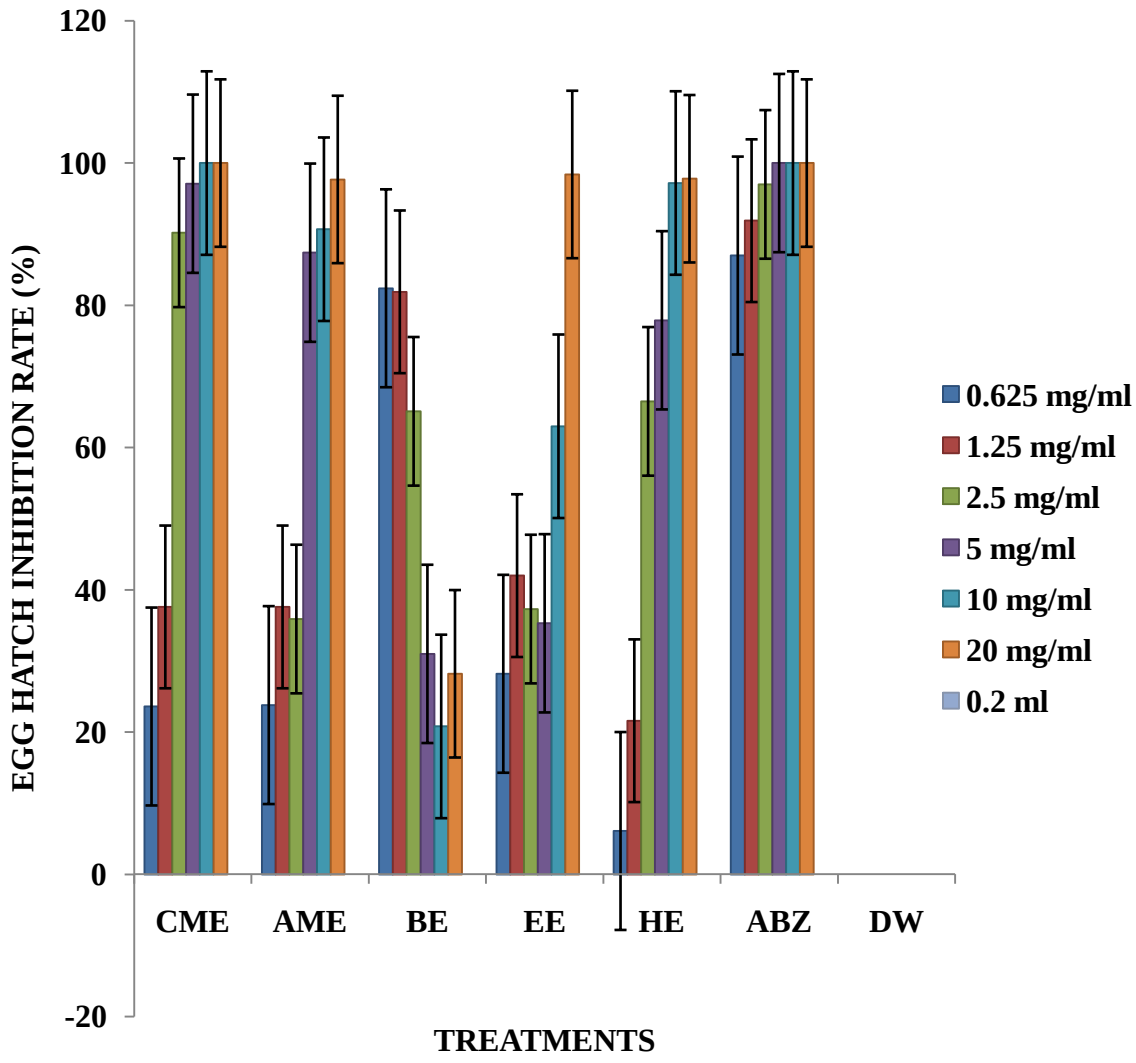
Watt, J.M. and Breyer-Brandwijk, M.G. (1962). *Medicinal and poisonous plants of Southern and Eastern Africa.* 2nd ed. E and S Livingstone Ltd, Edinburgh and London, pp. 1457.

Yano, M., Kawaii, S., Tomono, Y., Katase, E. and Ogawa, K. (1999). Quantification of Flavonoid Constituent in Citrus Fruits. *Journal of Agricultural Food Chemistry*, 47: 3565-3571.

APPENDICES

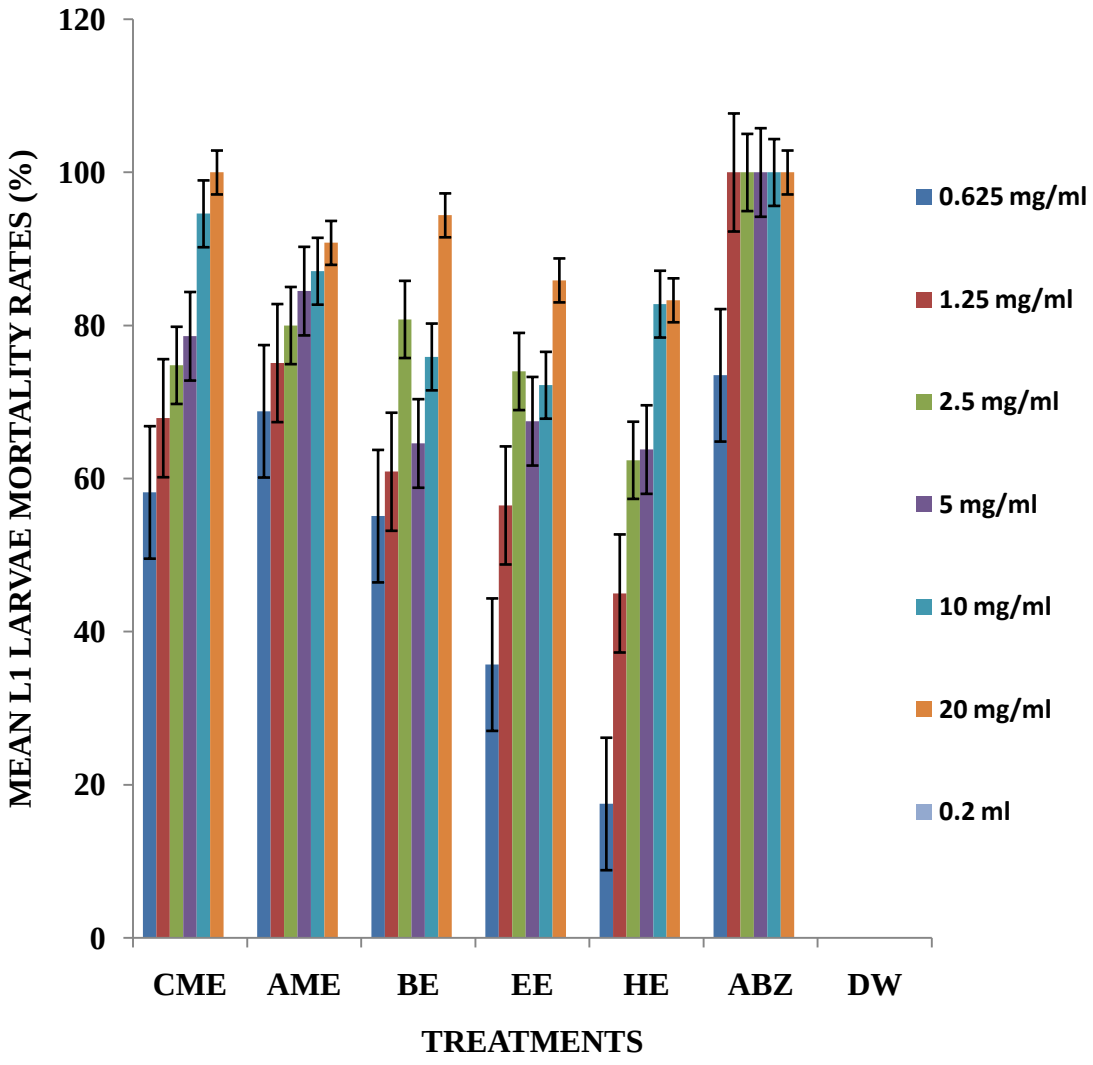
APPENDIX I

Inhibitory effects of different concentrations (0.625 – 20 mg/ml) of crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE), ethylacetate extract (EE), hexane extract (HE) of *C. aurantifolia* peels and albendazole (ABZ) (0.0625 – 2 mg) on the egg hatching of *H. bakeri* after 48 hours of incubation. Values are mean (± S.D).



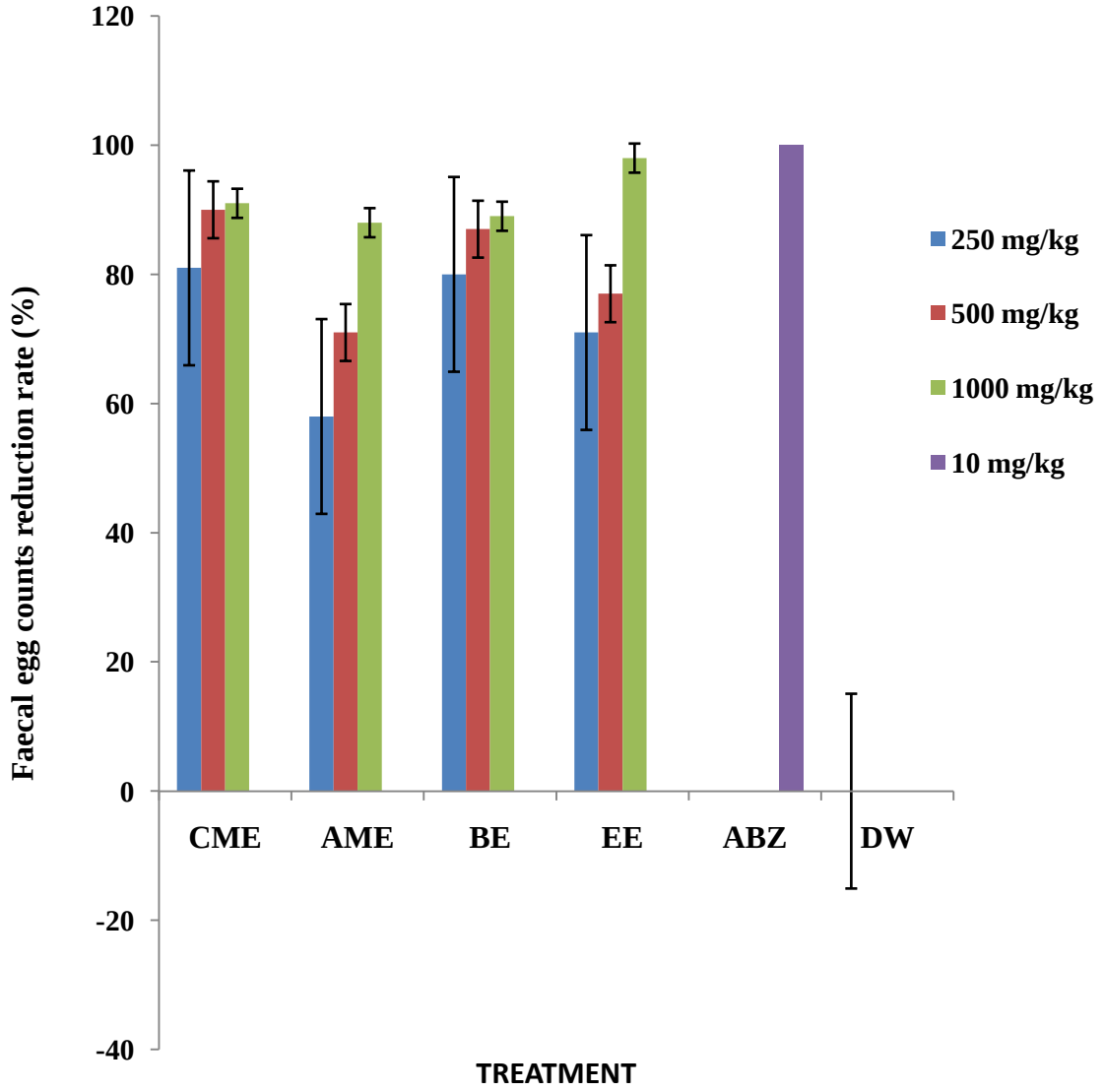
APPENDIX II

Effects of different concentrations (0.625 – 20 mg/ml) of the crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE), ethylacetate extract (EE), hexane extract (HE) of *C. aurantifolia* peels and albendazole (ABZ) (0.0625 – 2.0 mg/ml) on the L_1 Larvae of *H. bakeri* after 24 hours of incubation at 25°C. Values are mean (± SD).



APPENDIX III

Mean faecal egg count reduction rate in mice experimentally infected with *H.bakeri* and treated with crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BE) and ethylacetate extract (EE) of *C. aurantifolia* fruit peels and albendazole (ABZ).



APPENDIX IV

Composition of Reagents Used for Phytochemical Screening and TLC Analysis

Reagent	Composition	Proportion
Ammonia solution	Ammonia	10 ml
	Water	90 ml
Dragendoff's 'A`	Basic Bismuth-Nitrate	1.7 g
	Tartaric acid	20 g
	Distilled water	80 ml
Dragendoff's 'B`	Potassium iodide	16 g
	Distilled water	40 ml
Dragendoff spray	Stock solution A and B (1:1)	5 ml
	Tartaric acid	10 g
	Distilled water	50 ml
Fehling's solution A	Copper sulphate	34.64 g
	Sulphuric acid	0.5 ml
	Distilled water	500 ml
Fehling's solution B	Sodium potassium tartrat	176 g
	Sodium hydroxide	77 g
	Distilled water	100 ml
Ferric chloride solution	Ferric chloride	5 g
	Distilled water	100 ml
Mayer's reagent	Mercuric chloride	1.36 g
	Water	60 ml
	Potassium iodide	5 g
	Water	20 ml
Wagner's reagent	Iodine	2 g
	Potassium iodide	3 g
	Water	100 ml
Molisch's reagent	1-Naphtol	1 g
	95%-ethanol	100 ml
Sodium hydroxide solution	Sodium hydroxide	10 g
	Distilled water	100 ml