

**PHYTOCHEMICAL COMPOSITION AND ANTHELMINTHIC EFFECTS OF
ESSENTIAL OILS FROM THREE NIGERIAN CITRUS VARIETIES ON
*ASCARIDIA GALLI***

BY

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DECLARATION

The Research work in this Thesis entitled PHYTOCHEMICAL COMPOSITION AND ANTHELMINTHIC EFFECTS OF ESSENTIAL OILS FROM THREE NIGERIAN CITRUS VARIETIES ON *ASCARIDIA GALLI* has been carried out by me in the Department of Biochemistry, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Shaibu Isaac Eleojo

Name of student

Signature

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CERTIFICATION

This project work entitled PHYTOCHEMICAL COMPOSITION AND ANTHELMINTHIC EFFECTS OF ESSENTIAL OILS FROM THREE NIGERIAN CITRUS VARIETIES ON *ASCARIDIA GALLI* by Isaac Elejo SHAIBU meets the regulations governing the award of Master of Science degree of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To God Almighty and to Loving memory of my Father, Mr. Shaibu Jeremiah Abimaje
Who against all odds wanted his children to be educated but never live to see his dreams
come true, we love you father and we will do everything possible to keep your dreams
alive.

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Table of Content

Titled page.....	i
Declaration.....	ii
Certification.....	iii
Dedication.....	iv
Acknowledgement.....	v
Table of Contents.....	vi
List of Figures	ix
List of Tables	x
List of Plates.....	xi
List of Abbreviation	xii
List of Appendices	xiii
Abstract.....	xiv

CHAPTER ONE

1.0 INTRODUCTION	1
1.1 Statement of Research Problem	3
1.2 Justification	4
1.3 Aims and Objectives	5
1.3.1 General Aim	5
1.3.2 Specific Objectives	5

CHAPTER TWO

2.0 LITERATURE REVIEW	6
2.1 Citrus Fruits	6
2.2 Essential Oils from Citrus Peels	7
2.2.1 Uses of citrus peels essential oils in plants.....	9
2.2.2 Extraction of citrus peel oil.....	11
2.2.3 Nature of essential oil.....	11
2.2.4 Quality of essential oils.....	12
2.3. Constituents of Essential Oils	12
2.3.1 Chemical composition of citrus peel oils.....	12
2.3.2 Polyphenols.....	15
2.3.3 Flavonoids.....	16
2.3.4 Terpenoids.....	17
2.3.5 Alkaloids.....	17
2.3.6 Tannins.....	18
2.4 Mechanism of Action of Essential Oils	19
2.5 Therapeutic properties of citrus peels essential oil	20
2.5.1 Antihelminthic properties.....	20
2.5.2 Anti-microbial activities of essential oils	20

2.5.3 Anti-protozoan activity.....	22
2.5.4 Antiviral activity.....	22
2.5.5 Insecticidal activity.....	23
2.6 Challenges inthe used of citrus peels essential oil.....	24
2.6.1 Changes of composition during storage.....	25
2.7Prospects in Citrus Essential Oil.....	27
2.8.Poultry Husbandry.....	30
2.8.1 Host.....	30
2.8.2 Ascariidosis.....	29
2.8.3 Effects of parasitic infections on poultry husbandry.....	30
2.9. Helminths.....	31
2.9.1 <i>Ascaridia galli</i>	31
2.9.2Scientific classification.....	31
2.9.3 Morphology.....	32
2.9.4 Occurrence.....	33
2.9.5 Life cycle.....	33
2.9.6 Pathogenesis and clinical symptoms of ascariidosis.....	35
2.9.7 Economic value.....	36
2.9.8 Prevalence of <i>Ascaridia galli</i>	37
2.9.9 Reviews of some indigeneous anthelminthics.....	38
2.9.10Some examples of indigeneous anthelminths.....	40
2.9.11 Prospective benefit from <i>Ascaridia galli</i> infection.....	42
CHAPTER THREE	
3.0 MATERIALS AND METHODS.....	44
3.1 Materials.....	44
3.1.1 Chemicals and reagents:.....	44
3.1.2 Equipments.....	44
3.1.3 Collection and identification, Preparation of <i>Ascaridia galli</i> eggs and larvae.....	44
3.2Methods.....	45
3.2.1 Experimental design.....	45
3.2.2 Collection and identification of citrus peels.....	46
3.2.3 Extraction of essential oil from orange peels.....	46
3.2.4 Citrus peels N-hexane extraction.....	46
3.2.5 Anthelminthic assay.....	47
3.2.6 Grouping.....	48
3.2.7 Quantitative phytochemical determination.....	50
3.2.8 Determination of total phenolic content (TPC).....	52
3.2.9 Gas chromatography/mass spectroscopy.....	52
3.2.10 Fourier transform infra-red spectrophotometer (FTIR) analysis.....	53
3.2.11 Statististical Analysis of result.....	54

CHAPTER FOUR

4.0 RESULTS	55
4.1 Percentage Yield Essential Oils from Citrus Peels	55
4.2 Phytochemical Analysis	57
4.2.1 Quantitative phytochemical analysis.....	57
4.2.2 Total polyphenol content.....	59
4.2.1 Quantitative phytochemical analysis.....	55
4.2.2 Total polyphenol content.....	57
4.3 Citrus Peels Essential Oil Analysis	61
4.3.1 Gas chromatography-microscopy analysis of chemical composition of essential oils from citrus peels.....	61
4.3.3 Fourier transform infrared (FTIR) analysis.....	78
4.4 Anthelmintics Effects of Essential Oils from <i>Citrus aurantifolia</i>, <i>Citrus limon</i>, <i>Citrus sinensis</i> Fruit Peels	90
4.4.1 Lethal concentrations (LC_{50} and LC_{90}).....	90
4.4.2 Ovicidal effects of individual citrus essential oil on hatchability of <i>Ascaridia galli</i> eggs.....	93
4.4.3 Combine ovicidal effects of individual samples on hatchability of <i>Ascaridia galli</i> eggs.....	99
4.4.4 Larvicidal effects of individual citrus essential oil on the larvae of <i>Ascaridia galli</i>	101
4.4.5 Combine Larvicidal effects of citrus essential oil on the larvae of <i>Ascaridia galli</i>	107
4.4.6 Microscopic view of anthelmintic activities of Citrus Essential oil on eggs of <i>Ascaridia galli</i>	109

CHAPTER FIVE

5.0 DISCUSSION	111
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CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS	120
6.1 Summary	120
6.2 Conclusion	123
6.3 Recommendations	123
References	124
APPENDICES	165

LIST OF FIGURES

Figure 4.1 Total Polyphenol Content of Essential Oils of <i>Citrus aurantifolia</i> , <i>Citrus.limon</i> and <i>Citrus.sinensis</i>	60
Figure 4.2. Ovicidal activities of essential oil of <i>Citrus sinensis</i> Peels on egg of <i>Ascaridia galli</i>	94
Figure 4.3. Ovicidal activities of essential oil of <i>Citrus aurantifolia</i> peels on egg of <i>Ascaridia galli</i>	96
Figure 4.4. Ovicidal activities of essential oil of <i>Citrus limon</i> peels on egg of <i>Ascaridia galli</i>	98
Figure 4.5 Ovicidal activities of essential oil of <i>Citrus sinensis</i> , <i>Citrus aurantifolia</i> , <i>Citrus limon</i> peels and peperazine citrate against the eggs of <i>Ascaridia gali</i>	100
Figure 4. 6 Larvacidal activities of essential oil of some <i>Citrus sinensis</i> peels on the larvae of <i>Ascaridia galli</i>	102
Figure 4.7. Larvacidal activities of essential oil of <i>Citrus aurantifolia</i> peels on the larvae of <i>Ascaridia galli</i>	104
Figure 4. 8. Larvacidal activities of essential oil of <i>Citrus limon</i> peels on the larvae of <i>Ascaridia galli</i>	106
Figure 4.9Larvacidal activities of essential oils from <i>Citrus sinensis</i> , <i>Citrus aurantifolia</i> , <i>Citrus limon</i> peels and peperazine citrate against the L3 larvae of <i>Ascaridia galli</i>	108

LIST OF TABLES

Table 4. 1: Percentage Yield of Three Citrus Peels Essential Oils.....	56
Table 4.2 Quantitative Phytochemical Constituents in <i>Citrus aurantifolia</i> , <i>Citrus.limon</i> and <i>Citrus.sinensis</i> Fruit Peels.....	58
Table 4.3: Chemical Composition of <i>Citrus sinensis</i> -Hydrodistilled Fraction.....	62
Table 4.4: Chemical composition of <i>Citrus sinensis</i> -N-Hexane fraction.....	64
Table 4.5: Chemical composition of <i>Citrus auriantifolia</i> Hydrodistilled fraction...	66
Table 4.6: Chemical composition of <i>Citrus auriantifolia</i> N-hexane fraction.....	68
Table 4.7: Chemical composition of <i>Citrus limon</i> hydrodistilled fraction.....	70
Table 4.8: Chemical composition of <i>Citrus limon</i> N-hexane fraction.....	72
Table 4.9 Comparative Analysis of Chemical Composition of Hydrodistilled and N- Hexane Fractions of Essential Oil from Three Citrus Peels.....	75
Table 4.10: Functional groups in Essential oil of <i>Citrus sinensis</i> Hydrodistilled Fraction.....	79
Table 4.11: Functional groups in Essential oil of <i>Citrus sinensis</i> N-hexane Fraction	81
Table 4.12: Functional groups in Essential oil of <i>Citrus aurantifolia</i> Hydrodistilled Fraction.....	83
Table 4.13: Functional groups in essential oil of <i>Citrus aurantifolia</i> N-hexane Fraction.....	85
Table 4.14: Functional groups in essential oil of <i>Citrus limon</i> Hydrodistilled Fraction.....	87
Table 4.15:Functional groups in Essential Oil of <i>Citrus Limon</i> N-hexane Fraction.....	89
Table 4. 16: Lethal concentrations at 50 and 90 percent of essential oils from three species of <i>Citrus</i> peels and peperazine citrate against <i>Ascaridia galli</i> larvae.....	91
Table 4.17: Lethal concentration at 50 and 90 percent of essential oils from three species of peels and peprazine citrate against eggs of <i>Ascaridia galli</i>	92

LIST OF PLATES

Plate 2.1 <i>Ascaridia galli</i>	33
Plate 2. 2 Life cycle of <i>Ascaridia galli</i>	35
Plate 4.1: Microscopic views effect of Essential Oils <i>C.sinensis</i> , <i>C.aurantifolia</i> , <i>C.limon</i> on Eggs and larvae of <i>Ascaridia galli</i> before and after administration....	110

LIST OF ABBREVIATIONS

<i>A. galli</i> :	<i>Ascaridia galli</i>
<i>C. aurantifolia</i> .H:	<i>Citrus aurantifolia</i> -hydrodistilled fraction
<i>C. aurantifolia</i> .N:	<i>Citrus aurantifolia</i> -N-hexane fraction
<i>C. limon</i> H:	<i>Citrus limon</i> -hydrodistilled fraction
<i>C. limon</i> N:	<i>Citrus limon</i> N-hexane fraction
<i>C. sinensis</i> H:	<i>Citrus sinensis</i> -hydrodistilled fraction
<i>C. sinensis</i> N:	<i>Citrus sinensis</i> -N-hexane fraction
DEC:	Diethylcarbamazine
DMSO:	Dimethyl Sulfoxide
EC:	Experimental Composition
FTIR:	Fourier Transformed Infra Red spectroscopy
GC-MS:	Chromatography-Mass Spectrometry
GCO:	Gas Chromatography-Olfactometry
HOCl:	Hypochlorous Acid
HPLC:	High-Performance Liquid Chromatography
LC:	Lethal Concentration
NC:	Normal Control,
NDMA:	N-Nitrosodimethylamine
NIR:	Near Infrared
NMR:	Nuclear Magnetic Resonance
PBS:	phosphate buffer saline
SD:	Standard Drug
TLC:	Thin Layer Chromatography
TPC :	Total Phenolic Content
WHO:	World Health Organisation.

LIST OF APPENDICES

Appendix 1: Ovicidal effects of essential oils of <i>Citrus sinensis</i> , <i>Citrus aurantifolia</i> , <i>Citrus limon</i> peels and peperazine citrate against the eggs of <i>Ascaridia galli</i>	165
Appendix 2: Larvicidal effects of essential oils of <i>Citrus sinensis</i> , <i>Citrus aurantifolia</i> , <i>Citrus limon</i> peels and peperazine citrate against the L ₃ larvae of <i>Ascaridia galli</i>	166
Appendix 3: GC-MS Chromatogram of essential oil from <i>Citrus sinensis</i> Hydrodistilled fraction showing various components	167
Appendix 4: GC-MS Chromatogram of essential oil from <i>Citrus sinensis</i> N-Hexane fraction showing various components	168
Appendix 5: GC-MS Chromatogram of essential oil from <i>Citrus aurantifolia</i> Hydrodistilled fraction showing various components	169
Appendix 6: GC-MS Chromatogram of essential oil from <i>Citrus aurantifolia</i> N-Hexane fraction showing various components	170
Appendix 7: GC-MS Chromatogram of essential oil from <i>Citrus limon</i> Hydrodistilled fraction showing various components	171
Appendix 8: GC-MS Chromatogram of essential oil from <i>Citrus limon</i> N-Hexane fraction showing various components	172
Appendix 9: Fourier Transformed Infrared Spectroscopy Chromatogram of functional groups in essential oil of <i>Citrus sinensis</i> Hydrodistilled fraction.....	173
Appendix 10: Fourier Transformed Infrared Spectroscopy(FTIR) Chromatogram of functional groups in essential oil of <i>Citrus sinensis</i> N-Hexane fraction.....	174
Appendix 11: Fourier Transformed Infrared Spectroscopy (FTIR) Chromatogram of functional groups in essential oil of <i>Citrus aurantifolia</i> Hydrodistilled fraction	<u>175</u>
Appendix 12: Fourier Transformed Infrared Spectroscopy (FTIR) Chromatogram of functional groups in essential oil of <i>Citrus aurantifolia</i> N-Hexane fraction.....	176
Appendix 13: Fourier Transformed Infrared Spectroscopy (FTIR) Chromatogram of functional groups in essential oil of <i>Citrus limon</i> Hydrodistilled fraction.....	<u>177</u>
Appendix 14: Fourier Transformed Infrared Spectroscopy (FTIR) Chromatogram of functional groups in essential oil of <i>Citrus limon</i> N- Hexane fraction.....	178
Appendix 15: Standard Curve of Gallic acid.....	179
Appendix 16: Standard curve for probit analysis.....	180
Appendix 17: General Preparation Protocol of Phosphate Buffer Saline.....	181

ABSTRACT

The use of plant materials as source of remedy to diseases and health problems like parasitic infections is a traditional practice still in wide use throughout developing countries, which formed the basis of this research aimed at studying the Anthelmintic potentials of Essential Oils from three citrus varieties: - *Citrus limon*, *Citrus aurantifolia* and *Citrus sinensis* on *Ascaridia galli* was investigated. Essential oils from these citrus peels contain different phytochemicals. Quantitative phytochemical analysis of peels oils in the different Citrus varieties revealed:- *Citrus aurantifolia*; Alkaloids (0.26 ± 0.04 $\mu\text{g}/100\text{g}$), flavonoids (0.24 ± 0.04 $\mu\text{g}/100\text{g}$), tannins (0.06 ± 0.02 $\mu\text{g}/100\text{g}$), saponins (0.13 ± 0.03 $\mu\text{g}/100\text{g}$) and steroids (0.23 ± 0.05 $\mu\text{g}/100\text{g}$), *Citrus limon*; alkaloids (0.21 ± 0.03 $\mu\text{g}/100\text{g}$), flavonoids (0.27 ± 0.03 $\mu\text{g}/100\text{g}$), tannins (0.06 ± 0.03 $\mu\text{g}/100\text{g}$), saponins (0.21 ± 0.05 $\mu\text{g}/100\text{g}$) and steroids (0.25 ± 0.04 $\mu\text{g}/100\text{g}$) and *Citrus sinensis* alkaloids (0.32 ± 0.06 $\mu\text{g}/100\text{g}$), flavonoids (0.15 ± 0.04 $\mu\text{g}/100\text{g}$), tannins (0.10 ± 0.01 $\mu\text{g}/100\text{g}$), saponins (0.11 ± 0.03 $\mu\text{g}/100\text{g}$) and steroids (0.21 ± 0.01 $\mu\text{g}/100\text{g}$) at ($P \leq 0.05$) significance. The GC-MS analysis of essential oils from the three Citrus varieties revealed proposed components from both fractions:- hydrodistilled fractions gave the following numbers of compounds {*Citrus sinensis* (Thirteen), *Citrus aurantifolia* (Fourteen) and *Citrus limon* (Eighteen)} while N-hexane gave {*Citrus sinensis* fractions (Ten), *Citrus aurantifolia* (Eighteen) and *Citrus limon* (Twelve)} respectively. Limonene, β -Myrcene, α -Pinene, β -Pinene, p-menth-1-en-8-ol, β -Linalool, β -Citronellol, cis-Geraniol, Trans-Geraniol, α -Farnesene, β -Farnesene, γ -Elemene are the most prominent. Limonene is both prominent and common component in the three species. Fourier Transformed Infrared Spectroscopy (FTIR) result from both fractions of the three Citrus varieties shows similar functional groups: Alkyl halide, Alkenes, Ethers, Alcohol, Alkyls, Aromatic compounds, Amides, Acyl chlorides, Aldehydes, Nitriles, Carboxylic acids, Alkanes and Amines. Estimation

revealed least LC₅₀ and LC₉₀ for larvicidal activities in *C. limon* (0.066 µg/100g) and *C. aurantifolia* (1.394 µg/100g) respectively for hydrodistilled fraction and *C. sinensis* (0.403 µg/100g) and *C. aurantifolia* (0.644) respectively for N-hexane fractions. For most effective ovicidal activities, the least LC₅₀ and LC₉₀ were obtained in *C. sinensis* (0.089 µg/100g) and *C. limon* (0.712 µg/100g) respectively for hydrodistilled fraction and for N-hexane fractions as *C. limon* (0.147 µg/100g) and *C. limon* (30.750µg/100g) respectively. *In-vitro* anthelmintic study of hydrodistilled and N-hexane fractions of Citrus peels essential oils on larvae and eggs of *Ascaridia galli*, demonstrated a dose dependant anthelmintic activities compared with Piperazine citrate. Essential oils were applied at three concentrations. 0.1, 0.3 and 0.5µg/ ml in phosphate buffer saline plus 80% of DMSO as emulsifier. The best dose dependant effect on larvae mortality and prevention of egg hatchability at 50% and 90% as LC₅₀ and LC₉₀ (µg/ml) of the three Citrus peels Oils were recorded. These results show that essential oils extracted from *Citrus sinensis*, *Citrus aurantifolia* and *Citrus limon* L have anthelmintic properties on both larvae and ova of *Ascaridia galli*.

CHAPTER ONE

1.0 INTRODUCTION

Essential oils are volatile odouriferous oils occurring in buds, flowers, leaves, fruits, seeds and roots, of plants. They represent the total odour principle of any single botanical species (Stashenko *et al.*, 2002). Essential oils have been used commercially as aromas in fragrances and perfumes. They are also used as flavouring in food additives and as pharmaceuticals as well as insecticides. They have received much attention due to their multi-functions as antihelminth, antimicrobial, antifungal, antitumor and insecticidal agents (Franzios *et al.*, 1997). In recent times, there has been a growing interest in research on citrus due to the sweet refreshing nature of the juice and the result of increased knowledge of their nutritional and medicinal values. Consumption of citrus fruits generates peels as wastes that could bring about environmental pollution if not properly handled (Okwu and Emenike, 2006).

The citrus essential oils have been identified in different parts of the fruit much in fruit flavedo (outside of orange) and are generally isolated by some physical process (by expression, solvent extraction or distillation) (Heath, 1981; Handa *et al.*, 2008). They are mostly volatile and made up of wide variety of organic compounds of many different functional groups and molecular structures. Other compounds also present in essential oils are alcohols, aldehydes, acids and other aromatic compounds (Caccioni *et al.*, 1998; Stashenko *et al.*, 2002).

Essential oils exhibit different modes of action against micro organisms, such as interference with the phospholipids bilayer of the cell membrane or disturbance of the cytoplasmic membrane, which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular

energy and synthesis of structural components, and destruction or inactivation of genetic material, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents these make them best-known substances tested against micro organisms (Kotzekidou *et al.*, 2008).

Parasitism presents main threat to the indigenous poultry production and cause heavy economic losses in the production of meat and eggs (Soulsby, 1982). Helminthiasis is considered one of the most common diseases that affect free-range backyard chickens (Permin *et al.*, 1997).

Ascaridia galli is an intestinal helminth parasite that belongs to the superfamily Ascarioidea within the phylum Nematoda. *Ascaridia galli* is ubiquitous and pathogenic species essentially parasitic in birds the most, The adult worms live in the lumen of the intestine, but are occasionally also found in the crop, gizzard and rarely in the oviduct or body cavity (Yamaguti, 1961) and most prevalent in fowl, particularly in chicken (Permin and Hansen, 2003). The body is semitransparent, cylindrical and has a creamy-white colour with the entire body covered with a thick cuticle, which is striated transversely throughout the length of the body. Like all other nematodes, *Ascaridia galli* is dioecious with distinct sexual dimorphism (Hassanain *et al.*, 2009). The life cycle of *Ascaridia galli* is direct and involves a single host (Permin, 1997).

Economic losses are usually associated with *Ascaridia galli* infections because of treatment cost, decreased feed efficiency and impaired performance (Ruff, 1999; Martín- Pacho *et al.*, 2005). *Ascaridia galli* has ability to act as a vector of other diseases (Chadfield *et al.*, 2001; Permin *et al.*, 2006). Different approaches were employed to sustainable control of *Ascaridia galli*, (Gauly *et al.*, 2002; Abdelqader *et al.*, 2007; Kaufmann *et al.*, 2011; Braga *et al.*, 2011), among which is the use of plants

with anti-parasitic properties as well as the use of traditional herbal remedies (Siamba *et al.*, 2007; Lalchhandama *et al.*, 2009)

The traditional use of aromatic plants for their anthelmintics or pesticides properties is a local practice in developing countries. A practice that informed the use of essential oils from Lemon orange (*Citrus limon*), Lime orange (*Citrus aurantifolia*) and Sweet orange (*Citrus sinensis*) for their larvicidal and ovicidal potential on *Ascaridia galli* and the characterisation of the components of the essential oil in these orange peels.

1.1 Statement of Research Problem

Currently in citrus industry, emphasis are laid only on orange fruits harnessed which are either marketed fresh or as processed (canned) juice, while fruit peels produced in great quantities during the process are mainly discarded as waste (Kubo *et al.*, 2005; Wu *et al.*, 2007). Nigeria produces 0.3 metric tonnes of the 36.0 metric tonnes world orange peel waste and has the potential to produce more, leading to environmental pollution due to waste generated from citrus fruits consumption and processing (Odbanjo and Sangodoyin, 2002).

Helminthiasis a neglected tropical disease of grazing animals is of especial occurrence in developing countries due to improper handling and control activities (Lateef *et al.*, 2003). Although synthetic chemicals have been used extensively for controlling parasitic infections; these synthetic anthelmintics are costly on one hand and often are not available to the farmers in rural areas (Henrioud, 2011).

Development of resistance in helminthes to various anthelmintic compounds (Henrioud, 2011).

1.2 Justification

Concerns over food safety and health issues has resulted in a paradigm shift in consumer preferences to chickens raised in a more humane environment, with minimal antibiotics or chemical-based feed additives (Aagnet.org).

The increasing demand on poultry products from free-range and deep-litter production systems is restricted by the heavy burden of different poultry helminthes (Permin *et al.*, 1999; Fossum *et al.*, 2009). Large amounts of Citrus processing waste are left after the processing of citrus fruits for juice and jam production (Wilkins *et al.*, 2007).

Parasitic helminths continue to cause considerable morbidity and mortality in humans and their domestic livestock, translating into substantial socio economic losses. (Carod-Artal, 2008; King and Dangerfield-Cha, 2008),

Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications (Bakkali *et al.*, 2008). The use of orange essential oil emulsions has been reported to provide effective control against gastrointestinal nematodes in ruminants *Haemonchus contortus* in sheep (Egualde *et al.*, 2007; Rosskopf *et al.*, 2008; Gbolade and Adeyemi 2008; Sujon *et al.*, 2008; Ademola *et al.*, 2010; Squires *et al.*, 2010).

Persistence of Chemical residue and toxicity are nagging problems associated with the use of synthetic chemicals for control of worms. These, have prompted the search for medicinally important plants for their 'anthelmintic activity' besides the too much use of synthetics in the world-over (Lateef *et al.*, 2003).The use of essential oils is environmentally friendly and biodegradable, less hazardous and handy (Nery *et al.*, 2010).The use of citrus peels essential oils for nematode control will not only provides alternatives to synthetic chemotherapy but also reduces impact on environment.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this work is to study the phytochemical composition and anthelmintic potentials of Essential Oils from *Citrus limon*, *Citrus aurantifolia* and *Citrus sinensis* on *Ascaridia galli*, as natural product recovered from citrus waste.

1.3.2 Specific objectives.

The aim of this work was achieved through the following objectives:-

1. To carry out quantitative phytochemicals analysis on the three citrus varieties
2. To determine the essential oil constituents and their functional groups using (Gas Chromatography-Mass Spectroscopy (GC-MS); Fourier Transformed Infra Red spectroscopy (FTIR).
3. To determine the lethal concentration (LC₅₀ and LC₉₀) of essential oils from citrus peels on the helminth Larvae and ova potentials.
4. To determine anthelmintic (Larvicidal and Ovicidal) potentials of the essential oils from peels of *C. sinensis*, *C. aurantifolia*, and *C.limon* on the *A.galli*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Citrus Fruits

Citrus fruits, which belong to the family of Rutaceae, are one of the main fruit tree crops grown throughout the world. Although sweet orange (*Citrus sinensis*) is the major fruit in this group accounting for about 70% of citrus output. The group also encompasses small citrus fruits such as tangerine tree (*Citrus reticulata*), grapefruit tree (*Citrus vitis*), lime tree (*Citrus aurantifolia*) and lemon tree (*Citrus limonum*) (Cowan, 1999)

The exact origin of citrus fruits is not clearly identified, although most researchers place its origin to be South East Asia. Later, the citrus fruits were transported to America by the Spaniards, specifically to Mexico, Florida, Brazil and California, where we currently find the largest orange orchards in the world (Kumar *et al.*, 2010).

Citrus fruits are among the most popular fruits nowadays and have a very long history of production and use. However, within the past century, industrial technologies began to develop in order to convert citrus fruits into commercial products (Swisher and Swisher, 1977). In many parts of the world, citrus fruits have remained part of human diet for ages. In recent times, there has been a growing interest in research on citrus due to the sweet refreshing nature of the juice and the result of increased knowledge of their nutritional and medicinal values. The consumption of citrus fruits generates peels as wastes that could bring about environmental pollution if not properly handled (Okwu and Emenike, 2006).

Each year, millions of tons of citrus fruits are delivered to factories for processing and juice production. Historically, the oldest citrus product is the oil. In ancient Sicily,

where early Italian citrus industry were introduced, lemons were primarily grown for production of lemon oil, and juice was treated as a waste product until its later use for citric acid recovery. The early use of lemon and orange oils was mainly in perfumery and pharmaceuticals (Swisher and Swisher, 1977). With rapid development of science and technology, more areas of use of citrus oils were found, for which more detailed information on chemical composition and properties were required. The modern perfume and flavour industries have benefited from further research on citrus peel oil and essence. Besides, the yield of citrus seed oil has increased since citrus seeds were discovered as a new source of edible oil. Citrus has proven to be a very good option for the oil and essence production (Swisher and Swisher, 1977).

2.2 Essential Oils from Citrus Peels

Essential oils are volatile odouriferous oils occurring in buds, flowers, leaves, fruits, seeds, roots or rhizomes, of plants and they represent the total odour principle of any single botanical species (Stashenko *et al.*, 1996). Essential oils are aromatic oily liquids, obtained from vegetative parts, twigs, barks, woods and roots of various plants. The term “essential” is derived from “essence”, which means smell or taste and relates to the property of these substances providing specific flavours (Calsamiglia *et al.*, 2006). They are complex mixtures of different organic compounds, which possess diverse potentials (Ouattrra *et al.*, 1977), and are distributed throughout the plant kingdom occurring in about 60 angiosperm families. The important families are Lamiaceae, Rutaceae, Geraniaceae, Apiaceae, Aspetaceae, Lauraceae, Fabaceae and Poaceae. Essential oils have a tendency to evaporate on exposure to air even at ambient conditions and are therefore referred to as volatile oils or ethereal oils. They mostly contribute to the odouriferous constituents or ‘*essences*’ of the aromatic plants and are abundantly used in enhancing the aroma of some spices (Martinez *et al.*, 2008). Other

purposes include flavouring, perfuming (Lawless, 1995; Jones *et al.*, 1997) food preservation (Mishra and Dubey 1994; Faid *et al.*, 1995; Buttner *et al.*, 1996), alternative medicine and natural therapy (Reynolds, 1996; Lis-Balchin *et al.*, 1998).

Essential oils target microbial strains both *in vivo* and *in vitro* (Hilli *et al.*, 1997) and possess anti-cancer and antioxidant properties (Mimica-Dukic *et al.*, 2004; Burt, 2004; Sousa *et al.*, 2004; Burt *et al.*, 2005; Sylvestre *et al.*, 2006).

Essential oils are secreted either directly by the plant protoplasm or by the hydrolysis of some glycosides and structures, such Plant structures directly associated with the secretion of essential oils include: Glandular hairs (Lamiaceae e.g. *Lavandula angustifolia*), Oil tubes (or vittae) (Apiaceae eg. *Foeniculum vulgare*, and Pimpinella anisum (Aniseed), modified parenchymal cells (Piperaceae e.g. *Piper nigrum* - Black pepper) and Schizogenous or lysigenous passages (Rutaceae e.g. *Pinus palustris* - Pine oil). Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour (Firn, 2010).

The citrus essential oils have been identified in different parts of the plant (much in fruit flavedo) and are generally isolated by some physical process (by expression, solvent extraction or distillation) (Heath, 1981; Handa *et al.*, 2008). They are mostly volatile and made up of wide variety of organic compounds of many different functional groups and molecular structures. Other compounds also present in essential oils are alcohols, aldehydes, acids and other aromatic compounds (Stashenko *et al.*, 1996; Caccioni *et al.*, 1998). Some Plant species that have insecticidal and antibiotic properties have long been use for a variety of ethnobotanical purposes. Different types of aromatic plant preparations such as powders, solvent extracts, essential oils and whole plants are being

investigated for their antibiotics activity including their action as fumigants, anthelmintics, repellants, antifungal, anti-feedants antimicrobial, antitumor, and insect growth regulators(Weaver and Subramanyam, 2000).The use of aromatic plants as a source of pesticides or repellants is a traditional practice still in wide use throughout developing countries (Maia and Moore, 2011).

2.2.1 Uses of citrus peels essential oil in plants

The applications of citrus oils are versatile and in many domains. The aromatic characteristics of essential oils provide various functions for the plants including (i) Attracting or repelling insects, (ii) Protecting themselves from heat or cold; (iii) Utilizing chemical constituents in the oil as defence materials and (iv) as defense molecules for plants by inhibiting the growth of microbes and can kill them.As a result of their freshness, lightness, and fine fruity aroma, citrus peel oils and essences are widely used in the food and beverage industries as well as in some non food applications (Swisher and Swisher, 1977). Citrus peel oils may also be used for their antioxidative, antitumor, and radical scavenging activities. The radical-scavenging ability of citrus peel oil may help prevent free radical-induced and various chronic diseases (Calabrese *et al.*, 1999; Choi *et al.*, 2000). Monoterpenes from volatile components and polymethoxylated flavones from non volatile residues have been reported to be effective inhibitors of tumor cell growth, implying that citrus peel oils may be good cancer preventive food additives (Chen *et al.*, 1997; Crowell, 1999; Palazzolo *et al.*, 2013).

Furthermore, citrus peel oils are useful to alleviate pain from burnt skin (Brown *et al.*, 1999). Demonstrating anxiolytic and sedative effect, they could also be used in primary medical care against insomnia, anxiety, and epilepsy (Carvalho-Freitas *et al.*, 2002).

Mostly terpenoids are known for odours while some are pigments; they are highly volatile at room temperature, important for aroma therapy and effective against *Haemophilus influenzae*, *Streptococcus pneumoniae*, *pyogenes* and *Staphylococcus aureus* (Singh *et al.*, 1995).

Essential oils show antimicrobial activity in liquid and solid media (Gocho, 1991; Gutierrez *et al.*, 2009), and diffuse in the agar to inhibit the growth of bacteria (Inouye *et al.*, 1983; Goi, 1985). Spices and herbs have been known for antimicrobial activity for centuries (Bagamboula *et al.*, 2003).

The insecticidal property and antimicrobial activity of citrus peel oils have been reported. The oil can repel moth, mosquito, cockroach, domestica, and housefly (Omer, *et al.*, 1997; Ezeonu *et al.*, 2001). It also inhibits the growth of microbes such as fungi and salmonellae, with monoterpenes being the major compounds that account for pathogenic fungi inhibition (Caccioni *et al.*, 1998; Vargas *et al.*, 1999; Parish *et al.*, 2003).

Essential oils have received much attention due to their multi-functional nature (Franzios *et al.*, 1997), a characteristic attributed to number of diverse terpenoids in essential oils (Gurib-Fakim, 2006). Citrus seed oil is mainly used after refining as edible oil and a source of essential fatty acids, widely used in margarines, shortenings, salad dressings and cooking oils. Crude citrus seed oils are useful in the preparation of fatty acid derivatives, soap and detergent, and for the treatment of leather and textile (Swisher and Swisher, 1977).

The applications of cold-pressed peel oils in food and beverage are mainly in the soft drinks, Sherbet, confectionery, bakery, and household extracts (Swisher and Swisher, 1977). In addition, they can act as reducing agents of peroxidase activity in leafy

vegetables and antioxidants for edible oils, such as olive oil, to improve their sensory properties (Charai *et al.*, 1999; Ponce *et al.*, 2004). Moreover, they are effective inhibitors for the formation of N-nitrosodimethylamine (NDMA), a known carcinogen that may occur during production and storage of food (Sawamura *et al.*, 1999). Citrus peel oils are also added as flavouring agents to pharmaceuticals and herbal medicines in order to mask their unpleasant tastes (Lota *et al.*, 2002).

2.2.2 Extraction of citrus peel oil

Citrus peel oils are of very complex composition, contained in oval, balloon-shaped oil sacs, or vesicles, located in the outer rind, or flavedo, of the fruit (Usai *et al.*, 1992). Essential oils can be isolated as single or in combination without any change in their chemical composition and are obtained by distillation of leaves, fruits, flowers, stem, flower bud or peel and roots in Cleavenger apparatus (Cosentino *et al.*, 2003). They are also obtained by fractionation or rectification and steam distillation. Other methods used are effleurage and solvent extraction. The oil is usually extracted by mechanical separation or hydrodistilled. Mechanical separation, known as cold-pressing of peel oils, does not use heat in order to avoid loss of volatile components. Swisher and Swisher (1977) described three general commercial methods that are widely used in citrus industry to extract crude oils from fruit peels:

1. Oil recovery from peel after juice extraction
2. Simultaneous extraction of juice and oil emulsion from whole fruit
3. Recovery of oil from the peel flavedo after removal from the whole fruit by abrasion or shaving.

Citrus peel oils for small scale use may be obtained by hand-pressing. Fruits are sliced, and mesocarp and albedo layers are peeled from the flavedo before handpressing. The total final oil extract is about 1% of the flavedo by weight (Choi, 2003).

2.2.3 Nature of essential oil

Typically, these oils are liquid at room temperature and get easily transformed from a liquid to a gaseous state at room or slightly higher temperature without undergoing decomposition. The amount of essential oil found in most plants is 1 to 2%, but can contain amounts ranging from 0.01 to 10%. For example, orange tree produce different composition of oils in their blossoms, citrus fruits, and/or leaves. In certain plants, one main essential oil constituent may predominate while in others it is a cocktail of various terpenes (Choi, 2003).

Most essential oils comprise of monoterpenes - compounds that contain ten (10) carbon atoms often arranged in a ring or in acyclic form, as well as sesquiterpenes which are hydrocarbons comprising of fifteen (15) carbon atoms. Higher terpenes may also be present as minor constituents. The most predominant groups are cyclic compounds with saturated or unsaturated hexacyclic or an aromatic system. However, intraspecific variability in chemical composition does exist, which is relative to ecotypic variations and chemotypic races or populations (Andrés *et al.*, 2012). The final oil extract is a liquid with its colour varying depending on the species of the fruit: - lemon oil is pale yellow to pale greenish-yellow, lime oil is colorless to pale yellow and sweet orange oil is yellow to reddish-yellow therefore, different citrus peel oils have different physicochemical properties (Bauer *et al.*, 1990)

2.2.4 Quality of essential oils

Citrus peel oils have a lower price in the market place except for cold-pressed oil which is recovered from peels by steam distillation. This oil possesses an odour and flavour that is generally inferior to that of the cold-pressed oil (Swisher and Swisher, 1977).

2.3. Constituents of Essential Oils

2.3.1 Chemical composition of citrus peel oils

Chemical constituents of essential oils, lectins, polypeptides alkaloids, phenols, quinones, flavones, flavonoids, terpenoids, tannins and coumerins isolated from different plant species, which were found active against a wide variety of pathogens. Some other derivatives of essential oils are triterpenes, tetraterpenes and hemiterpenes (aromatic substances) few are phenols or their oxygen substituted derivatives (Das et al 2010) and found effective against various pathogens (Mahesh, and Satish 2008).

Different methods are been adopted to analyze the chemical composition of citrus oils as well as their odour activities, including Gas Chromatography-Mass Spectrometry (GC-MS) (Song *et al.*, 2000; Mondello *et al.*, 2003), Gas Chromatography-Olfactometry (GCO) (Choi, 2003), Nuclear Magnetic Resonance (NMR) (Lota *et al.*, 2002), Near Infrared (NIR) (Steuer *et al.*, 2001), High-Performance Liquid Chromatography (HPLC) (Buiarelli *et al.*, 1996) thin layer chromatography (TLC) (El-Adawy *et al.*, 1999) and other chromatographic procedures. Improvement of the available techniques has given rise to more precise and detailed data for qualitative and quantitative determination of citrus oils. Accordingly, 55 volatile components in lemon oil, 62 in lime oil (Lota *et al.*, 2002). 79 in mandarin oil (Verzera *et al.*, 2000), 88 in tangerine oil (Feger *et al.*, 2003) and some more components in hybrid citrus oils have been identified (Choi and Sawamura, 2000).

Citrus peel oils are characterized by complex mixtures containing mostly terpenes as well as oxygen-containing compounds (Veriotti and Sacks, 2002). Volatile components account for more than 90% of the oil mass, whereas nonvolatile residues are present in very small amounts. Most citrus peel oils exist almost exclusively in form of

monoterpenes, sesquiterpenes, and other aliphatic hydrocarbons. The hydrocarbon contents in Vietnamese pummelo, orange, tangerine, and lime, respectively, exceeded 98.7%, 97.6%, 98.6%, and 95.4% (Tu *et al.*, 2002). Among these, monoterpenes were dominant. Limonene, a monoterpene often used as a functional index of ripeness, was the major component of all citrus peel oils, followed by β -pinene in lemon oil (Vekiari *et al.*, 2001) while γ -terpinene are found in tangerine and lime oils (Lota *et al.*, 2002), myrcene in sweet orange oil, and p -cymene in mandarin oil (Veriotti and Sacks, 2002). The compounds α -pinene, sabinene, β -phellandrene, and terpenolene were present at lower levels. A few sesquiterpenes were also found in very small amounts but made appreciable contributions to flavour and odour; these included trans- β -farnesene, trans- α -bergamotene, trans-caryophyllene, germacrene A to D, and β -bisobolene (Feger *et al.*, 2001; Lota *et al.*, 2002).

Although terpene hydrocarbons, especially monoterpenes, are the most abundant constituents of citrus peels oil, they serve only as a flavour carrier and contribute little to flavour on their own (Shen *et al.*, 2002). These terpenoid hydrocarbons are usually removed by deterpenation in order to increase the concentration of flavour and fragrance compounds. Furthermore, unsaturated hydrocarbons (terpenes) are unstable to heat and light, and may oxidize rapidly to produce undesirable off-flavour compounds that adversely affect the desirable aroma of products (Sato *et al.*, 1995). Therefore, concentrated and deterpenated oils have become popular in the citrus oil market. Oxygenated compounds, mainly oxygenated terpenes, rather than terpene hydrocarbons, have been found to be responsible for the characteristic odour and flavour of citrus fruits, although they occur in relatively small amounts. When the hydrocarbon fraction is removed from the oil, the oxygenated fraction becomes more odourous due to a higher concentration (Tu *et al.*, 2002) Characterized by quantitative abundance in

aldehydes and a relatively wide variety of alcohols, oxygenated fraction includes aldehydes, alcohols, ketones, esters, oxides, acids, and trace amounts of fugenol methyl ether (Choi and Sawamura, 2000). Geranial and neral are the major aldehydes, both of which account for the fresh floral and citrus-like character of lemon and lime oils (Vekiari *et al.*, 2001; Chisholm *et al.*, 2003). Citronellal has a green-citrusy odour rather than a sweet and fruity odour (Choi and Sawamura, 2000). In addition, many simple aliphatic aldehydes, such as octanal, decanal, and dodecanal, impart a characteristic aroma to citrus peel oils (Verzera *et al.*, 2000; Chisholm *et al.*, 2003). Among alcohols, monoterpene alcohols such as linalool, followed by octanol and α -terpineol, are most predominant (Choi and Sawamura, 2000). Nerol and geraniol are also found in high levels. Among these, linalool and octanol are regarded as the most odour-active compounds in such citrus as Hyuganatsu (*Citrus tamurana*) (Choi *et al.*, 2001). Ketones, esters, oxides, and acids are less represented, but make appreciable contributions to flavour. Nootkatone is an important flavour compound of grapefruit oils (Sun and Petracek, 1999), neryl acetate, geranyl acetate, and bornyl acetate have been used as sweeteners, and linalool oxide provides a powerful sweet odour (Choi and Sawamura, 2000)

From Composition of volatiles in different orange oils shown, it is evident that most of the constituents belong to the terpene family and may be arranged into two groups, terpene hydrocarbons (terpenes and sesquiterpenes) and oxygenated terpene products (Vekiari *et al.*, 2001). Aside from the volatile components, there are small amounts (2–15%) of nonvolatile residues in citrus peel oils that possess antioxidative property; these include coumarins, psoralens, and polymethoxylated flavones (Chouchi and Barth, 1994; Dugo *et al.*, 1996; Stremple, 1998; Andrea *et al.*, 2003; Njoroge *et al.*, 2003).

2.3.2 Polyphenols

Phenolic compounds contain hydroxyl group which acts as a transmembrane carrier of monovalent cation and protons (Ultee *et al.*, 2002). Phenols due to selection of high acidity and dislocated electron system release proton from hydroxyl group. Undissociated covalent ions diffuse out, come across the cytoplasmic membrane and dissociate to release proton in microbial cells, while terpenoids and phenylpropanoids interact with the bacterial cell membranes (Griffin *et al.*, 1999; Davidson and Naidu, 2000; Dorman and Deans, 2000). This activity may be due to the hydrophobic nature of cyclic hydrocarbon which allows them to interact with bacterial cell membrane and occupy space between fatty acid chains and accumulate in lipid bilayer. This interaction leads to some important conformational changes in the membrane structure, affecting their stability and resulting in expansion and formation of pore and fluidification of bacterial cells (Griffin *et al.*, 1999). Thus bacterial cells face leakage of ions across the cell membrane and lose trans-membrane ionic gradient very fast. Bacterial cells however restore the ionic disturbances and counter balance by using ionic pumps that check the cell death but take a large portion of energy and make cell growth very slow (Griffin *et al.*, 1999, Ultee *et al.*, 1999; Cox *et al.*, 2001).

Phenolic components of essential oils also interact with chemical groups of proteins and enzymes (Juven *etal.*, 1994), and most do so with protein through hydrogen bridges and ionic or hydrophobic interactions while nonphenolic compounds interact with other functional groups. Cinnamaldehyde, a natural constituent of Cinnamon activates the bacterial nucleic acids and proteins by alkylation or formation of cross bridges (Presscott *et al.*, 2004). It also inhibits RNA, DNA and protein synthesis in bacterial cells (Feldberg *et al.*, 1988). It is also possible that essential oils might coagulate some cell constituents and cause denaturation by ionic release. The sulfhydryl groups in

active components of essential oils may interact more favorably against the bacterial cells. Thus garlic oil is more suitable than any other essential oil to kill both Gram negative and Gram positive bacteria (Reuter *et al.*, 1996). Few other chemical groups such as ketone, aldehyde and acid may also interact in a better way with bacterial cells and its components and show high antimicrobial activity but their major mode of action is unknown. Few oil components in combination with others might show synergistic effect against microbes. Oxidized phenolic compounds are more toxic to bacterial cells. (Reuter *et al.*, 1996).

2.3.3 Flavonoids

Flavonoids occur as C₆-C₃ unit linked to an aromatic ring. These are hydroxylated phenolic substances, synthesized in plants in response to microbial infection. Flavonoids are highly active against viruses. These are phenolic structures, which contain carboxyl group. Flavonoids are formed after addition of a 3-hydroxyl group. Mode of action of both flavones and flavanoids is membrane disruption and enzymatic bindings, which depends on hydroxylation due to presence of hydroxyl groups on their rings.

Flavonoids lacking hydroxyl group, but effectively disrupt the microbial membrane. However, more hydroxylated phenolic compounds act on the membrane surface due to the presence of hydroxyl group. Flavonoids are highly effective antimicrobial substances and inhibit the growth of a variety of micro-organisms. These form complexes with extracellular and soluble bacterial proteins and also bind to bacterial cell walls thus, inhibit the growth of *bacteria* and a number of viruses.

2.3.4 Terpenoids

These are most diversified group of secondary plant metabolites, derived from a basic structure of 5 carbons (C₅H₈) commonly known as isoprenoid unit. Terpenoids are

classified on the basis of isoprenoid units. The mechanism of action of terpenoids is not well understood but it is believed to be involved in interaction with the cell membrane and accumulate in the lipidic bilayer of bacterial membrane causing membrane disruption due to their lipophilic activity (Prabuseenivasan *et al.*, 2006; Liu *et al.*, 2007). The purified terpenoid constituents of essential oils are moderately toxic to mammals but, with few exceptions, the oils themselves or products based on oils are mostly nontoxic to mammals except cat (Isman, 2000), therefore, justifying their placement under “green pesticides”. Owing to their volatility, essential oils have limited persistence under field conditions (Isman, 2000),

2.3.5 Alkaloids

Another group of plant derived heterocyclic nitrogen compounds are alkaloids, mostly used in medicine for treating various ailments (Fessenden and Fessenden 1982), is also used for the treatment of bacterial and viral diseases commonly isolated from the plants and have shown antimicrobial properties (Omulokoli *et al.*, 1997).

2.3.6 Tannins

Tannin is a group of polymeric phenolic substances used for the tanning of leather and precipitation of gelatin. These possess astringent properties and are present in wood, bark, leaves, fruits and roots. These are very strong antimicrobial agents. Tannins act by penetration through membrane, adhesion, competitive inhibition of enzymes and binding to cellular envelopes. Tannins inhibit growth of larvae, effect moulting in insects and are inhibitors of reverse transcriptase enzyme. There are two categories of tannins, hydroxylizable (gallic acid) and condensed (proanthocyanidins).

2.4 Mechanism of Actions of Essential Oils

Essential oils are highly potent phytochemicals which with high antimicrobial potential in comparison to synthetic drugs as proved by low minimum inhibitory concentration (MIC) values obtained in *vitro*. The rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Kostyukovsky *et al.*, 2002) by some oils and with GABA-gated chloride channels by others (Priestley *et al.*, 2003).

Further, these natural products can stop genetic transformation in microbes. These phyto-chemicals are active against more than one microbial strain while antibiotics are not. These are either water or buffer soluble, show least residual effect in the body, and do not show molecular catalysis and cross reactivity. These phytochemicals possess mixed functional groups and are too complex in their structure; therefore microbes cannot develop resistance easily. Essential oils are multicomponent plant products and contain different chemical groups in different compounds such as pinene, camphor, limonene, linalool, pyragalol and chamaecynone. All such componets show diverse action mechanism. Essential oils in eukaryotic cells act as prooxidants affect cell membrane permeability and inhibit the function of cell organelles such as mitochondria. An important characteristic of essential oils and its components is their binding with lipids of bacterial cell membrane and mitochondrial cell structures making them to compromise the integrity of cell membrane and become more permeable for water (Knobloch *et al.*, 1986).

2.5 Therapeutic Properties of Citrus Peels Essential Oil

2.5.1 Antihelminthic properties

Artemisia absinthium (L.) essential oil is traditionally used as anthelmintic, (Abad *et al.*, 2012) and antiseptic herbal drug (Abebe, 2002). Besides this, *Lippiasidoides* essential oil works against sheep gastrointestinal nematodes at 200µg/kg (Camurca-Vasconcelos *et al.*, 2007). *Croton zehntneri* and *Lippia sidoides* essential oils and their major constituents such as anethole and thymol were found effective against eggs and larvae of the sheep gastrointestinal nematode, *Haemonchus contortus* (Camurca-Vasconcelos *et al.*, 2007). Both the oils showed ovicidal activity against intestinal nematodes of mice at 800mg/kg dose. Few commercial essential oils showed nematicidal activity against pine wood nematode, *Bursaphelenchus xylophilus* (Kim *et al.*, 2008). Essential oils from Coriander (*Coriandrum sativum*), oriental sweet gum (*Liquidambar orientate*), and Valerian (*Valeriana walliachi*) (Kim *et al.*, 2008) were effective against *B. xylophilus*. Important compounds such as benzaldehyde, transcinamyl alcohol, cis-asarone, octanol, nonanol, decanol, trans-2-decanol, undecanol and trans- 2-decen-1-ol, from these essential oils had strong nematicidal activity, were effective against levamisole, avermectin and milbemycin resistant helminthes.

2.5.2 Anti-microbial activities of essential oils

Essential oils from higher and aromatic plants have shown growth inhibitory potential against microbes due to the presence of certain secondary metabolites (Karaman *et al.*, 2001). People in different parts of the world traditionally use essential oils and their components for various microbial infections related to skin, fever, gut and respiratory tract. Clove, rosemary and lavender oils have shown strong antibacterial and antifungal properties (Quale *et al.*, 1996; Chang *et al.*, 2001; Wilkinson and Cavanagh, 2002, Prabuseenivasan *et al.*, 2006). Cinnamon oil possesses anti-diabetic and anti-

inflammatory activity (Mitra *et al.*, 2000), while lemon and peppermint show anticancer activity (Imai *et al.*, 2001). Plant essential oils have been used traditionally for eradication of respiratory tract infection and as ethical medicines by the tribals (Federspil *et al.*, 1997). They are also been used in inhalation therapy for chronic bronchitis, sinusitis (Boyd and Sheppard, 1970) and respiratory tract myocoses, (Singh *et al.*, 1995). In aromatherapy, inhaled essential oil vapours augment the out put of respiratory tract fluid and maintain the ventilation and drainage of the sinuses (Burrow *et al.*, 1983). Essential oils also restore tracheal choking (Shubina, 1990) and reduce the intensity of asthma attack (Frohlich, 1968). Oils of *Limon* and Limeleaves were effective against infectious bacterial strains(Lota *et al.*, 2001).

Sesame seed oil from *Sesame radiatum* and *S. indicum* were used for wound healing, the seeds as staple food by local population in Nigeria and the oil roasted as animal feed. It is cultivated by local subsistence farmers (Akpan-Iwo *et al.*, 2006).

Essential oils from the Rhizome of *Hedychium* species (Zingerberaceae) possess bactericidal and fungicidal activity. Oil is also used for the treatment of stomach ailments and infections. Oil of *H. coronarium* is used for the treatment of swelling and inflammation in Tumor (Johnson, 1999).

Garlic is traditionally used as natural medicine for the treatment of various diseases (Reuter *et al.*, 1996; Koch, 1996). Its oil and powder were effective against bacterial pathogens by virtue of systemic distribution in the small intestine (Koch, 1996). Garlic and its compounds are safer than antibiotics in eradicating the infection caused by *Helicobacter pylori* (Labez and Brosch, 1994). Most of the commercial Garlic preparations, available in the market, contain garlic oil and allicin. Allicin derivatives allyl and methyl sulphide showed very high antimicrobial potential and were effective against stomach cancer (Lawson, 1998).

Chile peppers contain provitamins A, E and several B, used in flavouring food materials (Bosland, 1994). Its constituents Capsaicin (terpenoid) showed biological activity against disease causing pathogens in humans (Virus and Gebhart, 1979). It is used as an analgesic (Corbeau *et al.*, 1994) and shows bacteriocidal activity against *H.pylori* (Jones *et al.*, 1997). Diterpenes from spices act as broad spectrum antibacterial and (Batista *et al.*, 1994) antifungal agents (Ayafor *et al.*, 1994). Essential oils from different *Mentha* species exhibited strong activity against *E. coli* (Mimica-Dukic *et al.*, 2003).

2.5.3 Anti-protozoan activity

Essential oils were also found active against protozoan pathogens (Trypanosomatids) causing leishmaniasis, chagas disease and malaria in humans. Some important trypanosomatids are Crithidia, Blastocrithidia and Herpetomonas, Monoxenous protozoans, which usually occur in insect, hosts (Wallace, 1966). Essential oils from *C. citratus* showed inhibitory effect on *C. deanei* at 100µg/ml (Pedroso *et al.*, 2006). *Ocimumgratissimum* showed inhibitory effect on *Herpetomonassamuelpessoai* (Holetz *et al.*, 2003) and *Kola aciminata* against *Trypanosoma brucei* (Kubata *et al.*, 2005). Eugenol rich essential oil from *O. gratissimum* showed anti-leishmanial activity at a very low dose (Ueda-Nakamura *et al.*, 2006).

2.5.4 Antiviral activity

Artimesia arborescence essential oils showed cytotoxicity against HSV-1 and HSV-2 viruses at 2.4 and 4.1 µg/ml. This plant is also used for the treatment of malaria, hepatitis, cancer, inflammation, and fungal and bacterial infections (Ma *et al.*, 2001). Sandalwood oil (*Santalum album*) also works against HSV-1 and HSV-2 (Benencia, 1999). Tea tree oil (*Melaleuca alternifolia*) was effective against TMV virus at a low concentration, while Eucalyptus oil (*Eucalyptus gliovulus*), Manuka oil (*Leptospermum*

scoparium) and Peppermint oils are very effective against Herpes simplex virus-1 and 2 at low concentrations. Thyme, a natural plant was very effective against HSV type 1 and Ginger (*Zingiber officinale*) and Santolina (*Santolina insularis*) were active against N HSV viruses. *Artemisia arborescens* showed virucidal effects as determined by plaque reduction assay. Besides, essential oils from this plant have also shown antiviral activity against HSV-1 (Saddi *et al.*, 2007).

2.5.6 Insecticidal activity

Essential oils have shown high insecticidal activity against field crop pests (Isman *et al.*, 2001), stored grain (Tripathi *et al.*, 2000; Verma *et al.*, 2000) and household insect pests (Singh *et al.*, 2000). Many of these essential oils have shown oviposition inhibition in insects (Tripathi *et al.*, 2000b). Besides this, toxic effect of d-limonene, linalool and terpenols constituents of essential oils has been determined (Weaver *et al.*, 1991, 1995). Essential oils from common spices have shown toxic, anti-feedent, growth inhibitory activity, repellent and oviposition inhibitory activity against insect pests of stored grains (Vale *et al.*, 1999; Upadhyay *et al.*, 2007).

Essential oils from some African plants have shown fumigant toxicity against *Anopheles gambiae* (Omolo *et al.*, 2005). Thymol, a constituent of thyme essential oils binds GABA receptor of human and house fly to act as modulator (Priestley *et al.*, 2003). Volatile compound diallyl disulphide from neem has shown potent toxic, fumigant and feeding deterrent activity against stored grain pests, *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) (Koul, 2004).

Essential oils elicit negative responses in stored grain pests and the insects keep away from the treatment area. Further, essential oils act as repellent, after evaporation in the medium, deter insects from feeding and cause high mortality in insects. Besides volatile oils impose negative orientation responses in insects and inhibit egg deposition on the

surface. Contrary to this, n-alkenes from *Ostrinia nubilalis* (Hubner) (Lupoli *et al.*, 1990; Udayagiri and Mason, 1995) induce oviposition, but pentane extracts deter females from oviposition (Konstantopoulou *et al.*, 2004).

2.6 Challenges in the used of citrus peels essential oil

Despite increasing applications of citrus oils, certain challenges related to potential health-damaging properties and contamination of citrus oils should not be ignored.

Citrus peel oil, such as bergamot oil, has been reported to show potential health hazards. Bergamot oil has a pleasant refreshing scent, and had been used widely in cosmetics until it was restricted in most countries a few years ago because of certain adverse effects, primarily phototoxicity and Berloque dermatitis (Kaddu *et al.*, 2001). More recently, there seems to be increasing application of bergamot oil in aromatherapy.

However, as reported by Kaddu *et al.*, (2001), Bergamot oil possesses potential phototoxic and photomutagenic properties, indicating that special attention should be paid in using psoralen-containing aromatherapy oils such as bergamot oil. Aside from potential adverse health effects, contamination is another problem that should be considered in production and application of citrus oils. Citrus oils may be contaminated with plastic materials employed in production process or storage. Chloroparaffins, phthalate esters, and phosphorylated plasticizers are the major contaminants extracted from plastic components by citrus oils as the oil- water emulsions pass through various production phases (Bella *et al.*,2001). Phthalate esters have a wide range of activities and may be hepatotoxic, carcinogenic, and possibly damaging to the gastrointestinal tract (Bella *et al.*, 1999). Chloroparaffins, another class of plasticizers, are toxic by ingestion and subcutaneous route and are classified as being potentially carcinogenic to humans (Bella *et al.*, 2000).

Another contamination of citrus peel oils comes from chlorine-treated water used in the oil recovery process and sanitizers used in postharvest handling and process equipment cleaning, which serve as a potential source of hypochlorous acid (HOCl) (Weiss *et al.*, 2003). HOCl can react with a variety of terpenes similar to d-limonene in structure, including limonene, α -pinene, and α -terpineol, resulting in the formation of terpene chlorohydrins (Weiss *et al.*, 2003).

Pesticide residues may contaminate citrus peel oils since cultivation of citrus crops commonly involves the use of chemicals such as fertilizers and pesticides. Citrus peel oils, extracted from citrus peels have been discovered to contain a higher concentration of pesticide residues than the fruits, due to the direct contact of the peels with pesticides (Saitta *et al.*, 2000). Regulations are increasingly stricter in terms of residual levels of pesticides because of the application of citrus oils in food, pharmaceutical, and cosmetic industries (Barrek, *et al.*, 2003).

2.6.1 Changes of composition during storage

Citrus peel oils have been used widely in beverages, cosmetics, pharmaceuticals, and perfumery industry, whereas seed oils are used in cooking and for treatment of leather and textile. The quality, freshness, and uniqueness of citrus oils are major considerations pertaining to their value and applications (Njoroge, *et al.*, 2009). However, large amounts of volatile components, as well as unsaturated compounds, render the oils unstable and prone to change with time and storage conditions.

Most of the qualitative changes of citrus peel oil during storage occur due to evaporation, oxidation, polymerization, rearrangement, and cyclization of some labile constituents in the presence of heat, light, oxygen, moisture, and catalysts results in the loss of volatile components and formation of off-flavour artifacts (Schieberle and Grosch, 1991; Choi and Sawamura, 2002). This major deterioration in citrus peel oil

occurs when stored at 20⁰C, with a notable decrease in monoterpenes and an increase in oxygenated compounds (Njoroge *et al.*, 2003). Monoterpenes may undergo oxidation and, hence, formation of oxygenated terpenes, rearrangement and cyclization into sesquiterpenes, polymerization into artifacts, and evaporation that causes loss of these components as well. As reported by Njoroge *et al.*, (2003). The total percentage of monoterpenes decreased progressively from 98.0 to 66.4 in Daidai (*Citrus aurantium*) peel oil after 12 months of storage indicating loss of freshness of the oils, which might be explained by dehydrogenation and rearrangement of α - and γ -terpinene, and hydrogenation and double-bond rearrangement of limonene as well (Schieberle and Lebensm.,1988; Njoroge *et al.*, 1996).

The content of oxygenated compounds increased considerably during storage (Choi and Sawamura, 2002). Monoterpene alcohols, as oxidized products of monoterpenes, showed the most significant increase. In contrast, linalool (both *cis* and *Trans*) was found to decrease at 20_C after 12 months of storage (Schieberle and Lebensm. 1988; Choi and Sawamura., 2002). Esters, such as linalyl and geranyl acetates, and most oxides also increased significantly. Besides, artifacts were formed in citrus peel oil upon prolonged storage at higher temperatures in the forms of alcohol, carbonyl compound, ester, epoxide, and hydrocarbon (Njoroge, *et al.*, 2003).

These compositional changes usually negatively influence the odour and flavour of citrus peel oils by generating off-flavour products. It has been shown that nonvolatile residues of citrus peel oil contain some compounds that exhibit antioxidative activities, among which permethoxylated flavones, dehydroabietic acid (Vargas, *et al.*, 1999). Coumarins and psoralens have been identified (Njoroge, *et al.*, 2003). In this respect, cold-pressed citrus peel oil is more stable than distilled oil and essence oil, in which

most of the natural antioxidants present are left behind when the oil is distilled (Swisher and Swisher, 1977).

Encapsulation is a technique frequently used in the storage of citrus peel oils and essences, which isolates the oils from the atmospheric oxygen, moisture, temperature, and light, and hence minimizes the oxidation of oil and reduces the release of volatile flavour compounds (Edris and Bergnstahl, 2001).

2.7 Prospects in Citrus Essential Oil

In fact, anthelmintics derived from plant essential oils were shown to have several important benefits. Due to their volatile nature, there is a much lower level of risk to the environment than with current synthetic anthelmintics. Predator, parasitoid and pollinator insect populations were less impacted because of the minimal residual activity, making essential-oil-based anthelmintics compatible with integrated pest management programs. It is also obvious that resistance will develop more slowly to essential-oil based anthelmintics owing to the complex mixtures of constituents that characterize many of these oils. Ultimately, it is in developing countries where the source plants are endemic that these anthelmintics may ultimately have their greatest impact in integrated pest management strategy. It is expected that these anthelmintics will find their greatest commercial application in urban pest control, public health, veterinary health, vector control vis-à-vis human health and in protection of livestock (Jain, and Verma, 2012)

2.8 Poultry Husbandry

2.8.1 Host

Domestic chickens are still very common in the backyards of most rural people. The native chicken has ability to survive and reproduce in a marginal environment and with minimal management. More important, consumers prefer the meat of the native chicken for its unique flavour and texture. To date, the native chicken remains an important source of high-quality protein food and additional income for many rural dwellers. It also performs other socio-economic and cultural roles as a form of savings against periodic shortages, and as a way of diversifying farm resources and allowing low-income farmers to meet their social and cultural obligations (Muchadeyi *et al.*, 2004).

Domestic chickens are reared in most parts of the world either in the backyard or commercial production system. Keeping poultry for commercial use has increased in recent decades. The most commonly kept poultry are chickens, ducks, geese and turkeys. Among these, domestic chickens are the most important. According to WATTAgNet.com (2009), worldwide poultry meat production has increased from 59.7 million tons in 1997 to 86.8 million tons in 2007. It acts as the source of meat, eggs, feathers and organic manure of high fertility. Of these, chicken meat productions impart 50.8 and 74.3 million tons in 1997 and 2007 respectively. Worldwide layer numbers have gone up slightly from 4,826 million in 1999 to approximately 6,200 million in 2010. World eggs output expanded by 34% between 1997 and 2007. Production in Asia and Africa has increased by 47% and 37% within this period. In contrast, over the same period, world market share for Europe showed a noticeable reduction from 20% to less than 16% due to parasite infection commonly *Ascaridia galli* (Mukaratirwa and Khumalo, 2010). Approximately 800 million chickens are found on the African continent. Approximately 80% of these are kept under traditional village production systems where mortality has been reported to be as high as 80-90% within the first year

after hatching (McAinsh *et al.*, 2004). The importance of rural poultry in the national economy of developing countries and its role in improving the nutritional status and income of many smallholder farmers and landless communities has been very significant (Permin *et al.*, 2002; Muchadeyi *et al.*, 2004). Rural poultry production is an important agricultural activity of almost all rural communities in Africa, providing scarce animal protein in the form of meat and eggs as well as being a reliable source of petty cash. Village chickens also fulfill a number of other functions for which it is difficult to assign any monetary value. These include the fact that rural chickens play an active role in pest control and are used for traditional ceremonies and festivals (Muchadeyi *et al.*, 2004). Strategic increases in the productivity of rural chicken flocks will, therefore, greatly assist in poverty alleviation, improve household food-security and protein intake of the rural communities and in the long term curb the massive urban migration of the youth. In the villages, the poultry is left scavenging around the house during daytime to obtain what feed they may be able to get from the environment often as offal, insects and seeds (Magwisha *et al.*, 2002). Owing to the free range and scavenging habits, traditional village poultry is in permanent contact with soil and insects. Soil, especially when humid and warm, may serve as an important reservoir and transmission site for external larval stages of helminthes (Horning *et al.*, 2003).

Many insects that may act as vectors for helminths are also favoured by high temperatures and to some extent humidity. These factors may explain the wide range and distribution of nematode and cestode species in poultry, especially during the tropical rainy season (Horning *et al.*, 2003). Traditional poultry production is often described as a low input/low output system. The low productivity is mainly caused by diseases, suboptimal management and lack of supplementary feed (Muchadeyi *et al.*,

2004; McAinsh *et al.*, 2004). Among diseases, parasitic infections are often neglected (Hotez *et al.*, 2008).

However, parasitism presents a main threat to the indigenous poultry production and cause heavy economic losses in the production of meat and eggs. Helminthosis is considered one of the most common diseases that affect free-range backyard chickens (Soulsby, 1982; Permin, *et al.*, 1997). Nematodes are the most important group of helminth parasites of poultry. *Ascaridia galli* is the most common worms especially in free-ranging birds and is the cause of economics losses such as retarded growth, reduced weight gain, decrease egg production, diarrhea and obstruction of intestine, morbidity and high mortality rate in modern poultry (Chadfield *et al.*, 2001). The increasing demand on poultry products from free-range and deep-litter production systems is restricted by the heavy burden of different poultry helminthes re-emerging in such production systems (Permin *et al.*, 1999; Fossum *et al.*, 2009).

2.8.2 Ascaridosis

Ascaridosis results in significant behavioural changes as the infected birds showed a higher food intake and lower locomotion activity during the prepatent and patent periods. Due to these factors the backyard (traditional) poultry production system is often characterized by low input, low output and periodic destruction of large proportion of the flock due to disease outbreaks. Thus, ascaridosis remains the major conundrum and cause of economic losses in poultry free-range and floor production systems (Ponnundurair and Chellappa 2001; Gauly, *et al.*, 2007).

2.8.3 Effects of parasitic infections on poultry husbandry

There has been an increased global demand and improved systems of poultry farming due to an ever increasing number of human populations. But with increased poultry management, a range of different parasitic infections are re-emerging, which pose

serious hindrance to successful economic output. Poultry production is relentlessly impeded by parasitic infections, of which the most persistent and most devastating is that of helminth parasites. Of the helminth parasites, *Ascaridia galli* (Schrank, 1788) Freeborn, 1923 (synonyms *A. lineata* Schneider, 1866; *A. perspicillus* Rudolphi, 1803) is inordinately the most pernicious and most prevalent among roundworms that cause debilitating health problems in poultry chickens and, resulting in poor performance, thereby, hamper poultry productivity (Ackert, 1931; Kates and Colglazier, 1970).

2.9. Helminths

2.9.1 *Ascaridia galli*

Ascaridia galli is the largest most ubiquitous and pathogenic species, and is the most significant nematode of poultry in terms of intensity and impact. It occurs worldwide in galliform birds of all ages (Peirce and Bevan, 1973; Yadav and Tandon, 1991). *Ascaridia galli* is ubiquitous and pathogenic species essentially parasitic in birds. The adult worms live in the lumen of the intestine, but are occasionally also found in the crop, gizzard and rarely in the oviduct or body cavity (Yamaguti, 1961) and most prevalent in fowl, principally in chicken, turkey, geese, guinea fowl and a number of wild birds (Permin and Hansen, 2003).

2.9.2 Scientific classification

Ascaridia galli is illustrated below, as described by Schrank, (1788)

Kingdom: Animalia
Subkingdom: Eumetazoa
Phylum: Nematoda
Class: Secernentea
Order: Ascaridida

Family: Ascaridiidae

Genus: *Ascaridia*

Species: *galli*

Binomial name: *Ascaridia galli*

2.9.3 Morphology

Ascaridia galli is a nematode parasite that causes ascariasis, or worm infection in poultry.

The body is semitransparent, cylindrical and has a creamy-white color. Like all other nematodes, *Ascaridia galli* is diecious with distinct sexual dimorphism (Ramadan and Znada, 1992). Females are longer than males with a length of 72-116 mm and a straight posterior terminal, whereas males are around 51-76 mm and possess a curved posterior terminal (Ashour, 1994). In the anterior end, both sexes have a prominent mouth with three distinct lips, bearing teeth-like denticles on their edges (Hassanain *et al.*, 2009). The entire body is covered with a thick cuticle, which is striated transversely throughout the length of the body. The eggs are oval and surrounded by three layers: the inner permeable layer called the vitelline membrane, a thick resistant shell and a thin albuminous layer (Ackert, 1931; Hansen *et al.*, 1956). These layers are a key factor for its resistance against desiccation and its long-term persistence in the environment their eggs measured 73-92 by 45-57 μm (Soulsby, 1982).



Plate 2.1: *Ascaridia galli* (photo shot 05-06-2013)

2.9.4 Occurrence

Ascaridia galli occurs worldwide in galliform birds of all ages. The adult worms live in the lumen of the intestine, but are occasionally also found in the crop, gizzard and rarely in the oviduct or body cavity and are essentially parasitic in birds (Yamaguti, 1961), and most prevalent in fowl, particularly in chicken and turkey, and also in geese, guinea fowl and a number of wild birds; the principal host manifestly being the chicken (Ackert, 1931; Permin and Hansen, 2003).

2.9.5 Life Cycle

The life cycle of *Ascaridia galli* is direct and involves a single host. Sexually mature worms live in the lumen of the small intestine, whereas eggs containing infective stage larvae (L3) develop in the environment (Permin, 1997). The eggs are oval and surrounded by three layers: the inner permeable layer called the vitelline membrane, a thick resistant shell and a thin albuminous layer (Ackert, 1931; Hansen *et al.*, 1956). These layers are a key factor for its resistance against desiccation and its long-term persistence in the environment. Larvae do not hatch in the environment; instead, they

moult inside the eggs until they become infective (L3). At optimal conditions (temperature and humidity) most of the fertile eggs within 24 hours, start dividing into the two-cell stage (Ramadan and Znada, 1992). In the next 24 hours, the second division takes place and gives rise to the three-cell stage. The four-cell stage is normally seen within three days in most of the eggs. After 3 days, a morula with blastomeres is formed, which is completed by the end of the fifth day. After eight days, the so called “tad pole” stage develops and after two additional days a vermiform embryo is developed. Within the next three to four days, this transforms into the coiled and fully mature infective L3 larva (Ramadan and Znada, 1992). The whole process may take between seven to twenty (20) days or longer depending on the temperature and relative humidity (Reid, 1960; Permin *et al.*, 1998) the life cycle is completed when new hosts ingest the infective eggs. After ingestion, the infective eggs are mechanically transported to the proventriculus and gizzard and further down to the duodenum where they hatch within the first 24 hours. Triggering factors that signal the larvae to hatch are believed to be temperature, carbon dioxide level and pH levels (Dick *et al.*, 1973; Salih and Saleem, 1987). Following hatching, the larvae burrow into the mucosal layer of the small intestine to enter the histotrophic phase (Ackert, 1931). The duration of the histotrophic phase is 3 to 54 days before the larvae return to the intestinal lumen where they reach final maturity (Permin *et al.*, 1998). However, this period is dose-dependent and probably very much related to the phenomenon of arrested development (Ikeme, 1971; Herd and McNaught, 1975). After the histotrophic phase, the mature worms settle down in the lumen of duodenum where they live and feed on ingesta and produce huge number of eggs that are passed with the faeces into the external environment where the life cycle continues (Ramadan and Znada, 1992). The prepatent period varies from 5-8 weeks (Pankavich *et al.*, 1974; Permin *et al.*, 1998).

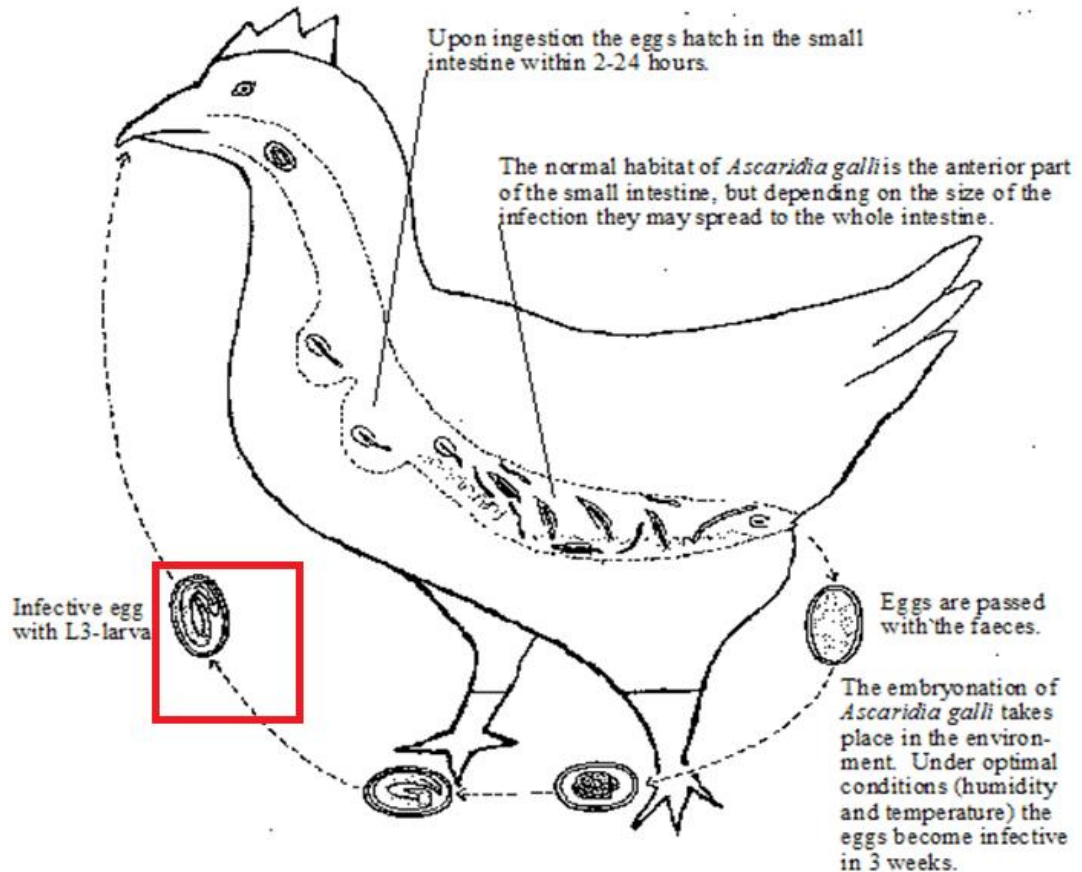


Plate 2.2: Life cycle of *Ascaridia galli*(www.WATTAgNet.com2009)

2.9.6 Pathogenesis and clinical symptoms of ascaridiosis

Ascaridia galli is the most prevalent helminth species in different poultry production systems (Permin *et al.*, 1999; Abdelqader *et al.*, 2008; Kaufmann *et al.*, 2011). Heavily infected birds may show droopiness of wings, bleaching of the head, emaciation and diarrhea. Diarrhea may be accompanied by anemia and intestinal obstruction in very heavy infections (Griffiths, 1978). The primary damage reduced efficiency of feed utilization, but death has been observed in severe infections. Reduced egg production and weight loss are common symptoms in broiler chickens. Young birds seem to be more susceptible to *Ascaridia galli* infection than adults and manifest greater degree of damage. Penetration of the parasite into the duodenal or jejunal mucosa may cause haemorrhagic enteritis, anaemia often associated with severe diarrhea as well as loss of

appetite, weakness, decrease activity, ruffled feathers and dirty cloacal region (Ikeme, 1971; Adang *et al.*, 2010). Established larvae in some cases cause destruction of the glandular epithelium (Permin, 1997). Moreover, adhesion of the mucosal villi may occur due to proliferation of secretory cells. Not only the larvae can cause pathological lesions, also adult worm can cause damage to the epithelium in the form of pressure atrophy upon villi (Ikeme, 1971) compromising the Integrity of intestinal mucosa and nutrient utilization can be affected which decreased weight gain (Ruff, 1999; Permin *et al.*, 1998; Kilpinen, *et al.*, 2005; Das, *et al.*, 2010). This worm is the major cause behind unremitting economic losses in poultry sector by causing reductions in growth rate and weight loss of fowl and induces damage to the intestinal mucosa, leading to blood loss, Partial or complete obstruction of the intestine, and increased mortality due to secondary infections (Ramadan and Znada, 1991; Permin *et al.*, 1999; Ackert and Permin, 2006).

Infection can spread very fast because of the nematode's direct life cycle and the environmental resistances of its eggs favour infections under poultry management systems (Permin and Ranvig, 2001).

2.9.7 Economic value

Economic losses are usually associated with *Ascaridia galli* infections because of treatment cost, decreased feed efficiency and impaired performance (Ruff, 1999; Martín-Pacho *et al.*, 2005). The chief economic importance lies in the ability of *Ascaridia galli* to act as a vector for transmission of other infectious organisms like *Salmonella enterica* and *Escherichia coli* (Chadfield *et al.*, 2001; Permin *et al.*, 2006). The development of drug resistance by helminths against chemotherapeutical products and the associated risks of chemical residues in poultry products have drawn the attention to alternative approaches. Different approaches were employed to sustainable

control of *Ascaridia galli* (Gauly *et al.*, 2002; Abdelqader *et al.*, 2007; Kaufmann *et al.*, 2011), nutrition of host animal (Daset *et al.*, 2010), biological control (Braga *et al.*, 2011), and the use of plants with anti-parasitic properties as well as the use of traditional herbal remedies (Siamba *et al.*, 2007; Kholhring *et al.*, 2009). Citrus peels extracts showed effective control against root-knot nematodes (Tsai, 2008).

2.9.8 Prevalence of *Ascaridia galli* infections

Research have proven that parasitic infection may have a negative effect on both welfare of the animal and productivity as well as on mortality in some cases (Kijlstra and Eijck 2006; Gauly *et al.*, 2007; Phiri *et al.*, 2007). The magnitude of the problems is illustrated by estimates from the World Health Organization (WHO, 1999) that, even in the midst of successful anthelmintic programs, 30% of the world's population harbours at least one species of helminth parasite; a staggering 2 billion people are affected.

Detailed analysis of the cross-sectional prevalence study of gastrointestinal helminthes conducted on 268 adult chickens randomly selected from 16 farms in Denmark revealed that *Heterakis gallinarum* (72.5%), *Ascaridia galli* (63.8%) and *Capillaria obsignata* (53.6%) were the most commonly detected helminths in organic layer production, while prevalence figures in hens in conventional cages were lower (Permin *et al.*, 1999). Moreover, Pennycott and Steel (2001) stated that a high proportion of free-range flocks in England carry parasitic worms, especially *Ascaridia galli* and *H. gallinarum*. Investigation on prevalence of ascarid infections in Swedish commercial laying hens in 2004 and 2008 showed that the overall prevalence was significantly higher in 2008 compared with 2004 and that parasite infection in non-cage systems in both years was considerably higher than in caged flocks (Jansson *et al.*, 2010). A later study in Germany on 144 laying hens from 11 free range farms (13 hens per farm, randomly selected) revealed that prevalence of infection with *Ascaridia galli*, *H. gallinarum* and

Capillaria sp. was 66.6%, 84% and 75.1%, respectively (Kaufmann and Gauly, 2009). Likewise, data from a study in Germany between 2007 and 2010 on 740 laying hens from 18 organic free range farms showed that almost all hens harbored at least one helminth species and the most prevalent species were *H. gallinarum* (98.0%) followed by *Ascaridia galli* (88.0%) and *Capillaria spp.* (75.3%) (Kaufmann *et al* 2011). A study of infection dynamics of *Ascaridia galli* in laying hens in both organic and conventional farms in Sweden discovered that all flocks became infected following the arrival of the birds with infective eggs presented in the environment (Höglund and Jansson, 2011). Data obtained from four rural localities of Africa showed that *Ascaridia galli* has a prevalence range of 22.2 - 43.8% was one of the most common parasites after *Tetrameres americanus* and *Gongylonema ingluvicola* (Mukaratirwa and Khumalo, 2010).

2.9.9: Reviews of some indigeneous anthelmintics

Indigenous system of herbal therapy is becoming an increasingly attractive approach to control parasitic infection, particularly in developing countries. Among parasites, helminth and helminthic infections (helminthiasis) remain one of the major neglected tropical health problems affecting billions of people and their animals all over the world (WHO, 2004; WHO 2010). They are the most common infectious agent of humans and animals that contribute in the wide spread occurrence of under nourishment, anaemia, eosinophilia and pneumonia (Bundy, 1994). They are also responsible for considerable economic losses to the livestock industry of marginal farmers, particularly of developing countries (Holden-Dye and Walker, 2007; Singh *et al* 2008; Ortega *et al.*,2010). People living in tropical and sub-tropical countries with low per capita income, poor hygienic conditions suffers most because of the presence of favourable conditions for the proliferation of the parasite (Hotez *et al.*, 2007), also for the

propagation of intermediate hosts that are an essential link in the life cycle of the parasite (Roy and Tandon, 1992). Over the last few decades the most common method of controlling helminthiasis all over the world include the use of commercial anthelmintic drugs of various types. WHO has listed several essential anthelmintic drugs which are safe to use, available in single dose and of low cost, e.g., mebendazole, albendazole, pyrantel pamoate and levamisole, which are found to be effective against soil transmitted helminths like *Ancylostoma duodenale*, *Ascarislumbricoides*, praziquantel for schistosomes and foodborne trematodes and both ivermectin and diethylcarbamazine (DEC) for curing filariasis (Albonico *et al.*, 1999; Kohler, 2009). However, despite having developed health care facilities, sophisticated instrumentations and advancement in chemotherapy, there is still lacking of proper and effective tools to deal with helminthic infections. Review of literature revealed that out of 1,556 new chemical entities marketed between 1975 and 2004, only few drugs were developed to treat helminthiasis and found effective (Chirac and Torrele, 2006; Hotez, 2008). Therefore, there has always been a need to find an alternative to anthelmintic drugs since current drugs do not control all parasitic infections well. Moreover, high treatment frequency, single-drug regiment or frequent use of the same anthelmintic has led to the development of resistance among helminth population (Geerts and Gryseels, 2000; Geerts and Gryseels, 2001). Similarly, its negative, undesired effects as well as limited availability to the rural areas further restricted the effective control of helminthiasis (Martin *et al.*, 1997; Waller, 1997; Satrija *et al.*, 2001; Suleiman, 2005), causing new threat to human society. Because of this limited availability and ineffectiveness of commercial drugs towards controlling helminthiasis, scientists are now looking for new drugs based on traditional knowledge and traditionally used medicinal plants as an alternative remedies (Roy, and Tandon, 1996; Mali and Mehta, 2008; Dasgupta *et al.*,

2010; Tandon *et al.*, 2011). Contribution of plants to fight against various diseases dates back several centuries, and has been documented by the ancient Chinese, Indian and North African Civilisations (Taylor *et al* 2001; Roy *et al.*, 2007; Swargiary and Roy, 2011). Though the advancement of synthetic medicines, to certain extent, has lifted the health care and livelihood of people, yet the use and importance of plants and its botanicals for the same has never been neglected: *Alpinia Nigra* (Family Zingiberaceae) is an Anthelmintic Medicinal Plant of North-East India used in various helminthic infections (Athanasiadou *et al.*, 2007; Diehlet *al.*, 2004; Roy *et al.*, 2008; Adama *et al.*, 2009; Roy *et al.*, 2010). Several such studies based on traditional medicinal knowledge were done in Indian subcontinent to test the putative anthelmintic activity of different plants (Ogawa *et al.*, 2000).

2.9.10. Some examples of indigineous anthelminths

Chenopodium ambrosioides (Family Chenopodiaceas) originated in Central America, though it has been distributed to more country of the world, it has been used as an anthelmintic (medicine for controlling internal parasites) for many years. In the early 1900s it was one of the major anthelmintics used to treat ascarids and hookworms in humans, cats, dogs, horses, and pigs. Usually, Essential oil of chenopodium was used. Chenopodium is still used to treat worm infections in humans and livestocks in many countries like Honduras and a few areas in Latin American countries (www.abc.cornell.edu).

Aloe Vera demonstrates powerful detoxifying effects by flushing out toxins and promoting healthy tissue formation. Traditionally, it has been used in Indian medicine for asthma, jaundice, as a carminative, for various musculoskeletal disorders, for suppression of menstrual disorders, as a tonic, purgative, aphrodisiac, antihelmintic, in various ophthalmological disorders, enlargement of the spleen, various forms of hepatitis,

vomiting, fever due to bronchitis, and for erysipelas(www.luresext.edu). *Allium sativum* (garlic) reportedly has nematocidal effects against *Ascaris lumbricoides*. The active component in garlic is allicin, which appears to interfere with sulfhydryl-dependent enzymes in microorganisms. However, because allicin can be degraded by heat, only large quantities of fresh raw garlic cloves may be useful for the treatment of worm infestations (Heron and Yarnell, 1999). Ginger was used historically in different regions of the world for the same basic therapeutic applications. These include: analgesic, anti arthritic, wound healing, anti ulcer, anthelmintic, stimulant and aphrodisiac properties and treatment of a variety of respiratory, reproductive and digestive complaints (www.herbs2000.com). Stem bark of *Mangifera indica* L. has been found to possess anthelmintic and anti allergic properties (Garcia et al., 2003). *Cassia alata* has been used for soothing inflammation and shingles, as a skin disinfectant, for constipation, oedema, herpes infections, hepatitis, liver discomfort, impetigo, worm infestation, as a laxative and an analgesic. Also it has been used for leprosy, wound healing, as an anthelmintic, anti-bacterial, diuretic, for snake bites, bronchitis, asthma, hasten child birth, eczema, stomachaches and uterine disorders.

The application of papaya latex that is probably of most interest to livestock producers is as an anthelmintic (dewormer) (Satrija *et al.* 1994). The study conducted by Satrija *et al* (1994). The result indicates that papaya latex is effective against *Ascaridia galli* in chickens. In another study, water extracts of papaya seeds decreased *Ascaridia galli* infections in chicks by 41.7% (compared to piperazine hexahydrate which decreased infections by 99%) (Satrija *et al.*, 1994).

In traditional veterinary medicine, papaya seeds also are used as dewormers. It also has been reported that papaya leaf extract is used as a profilaxis against malaria, though no studies on this use could be found in the literature (www.ansci.cornell.edu).

The Sensitive plant (*Mimosa pudica* L) is a creeping annual or perennial herb in Philippines. Makahiya is known as a diuretic, and is considered alterant and anti asthmatic. It is used for urinary complaints, and is useful in diseases arising from corrupt blood and bile and of late its usage as anthelmintic in Philippines (Cinco, 2006).

2.9.11 Prospective benefit from *Ascaridia Galli* infection

However, numerous studies have shown that modulation of the immuneresponse of the host by parasitic helminths can have a concomitant health benefit (reviewed by McKay, 2009). In addition, helminth-derived molecules are potent immunomodulators and can serve as templates for the design of novel anti-inflammatory drugs, and may also be candidates for vaccine development (Harnett and Harnett, 2008; Perrigoue *et al.* 2008). Parasitic helminths have been humans' long-time companion and have evolved as 'master regulators' of host immune responses (Maizels *et al.*, 2012). Consequently, knowledge of how the parasite modifies host immunity can be used to develop therapies for other diseases. In considering 'helminth therapy', one can either use viable infections or immunomodulatory molecules from the parasite; both strategies have been used to treat diseases in animal models. Infection with a variety of species of parasitic helminths has been shown to reduce the severity of airway, cerebral and intestinal inflammation in murine models of human disease (McKay, 2006), and a number of investigators are pursuing the mechanism(s) underlying this health benefit (Weinstock *et al.*, 2005). The use of helminth-derived molecules as drugs overcomes the concerns with the use of live parasites. However, the issues here are often technical and revolve around a single basic question: how can pure molecules be isolated from miniscule amounts of parasite material to allow testing in biological systems and to serve as blue prints for drug development (Weinstock *et al.*, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

All chemicals used in this study were of analytical grade and were products of May and Baker, England and Sigma Chemical Company: - anhydrous sodium sulphate salt, N-hexane, ethanol and acetone.

3.1.2 Equipments

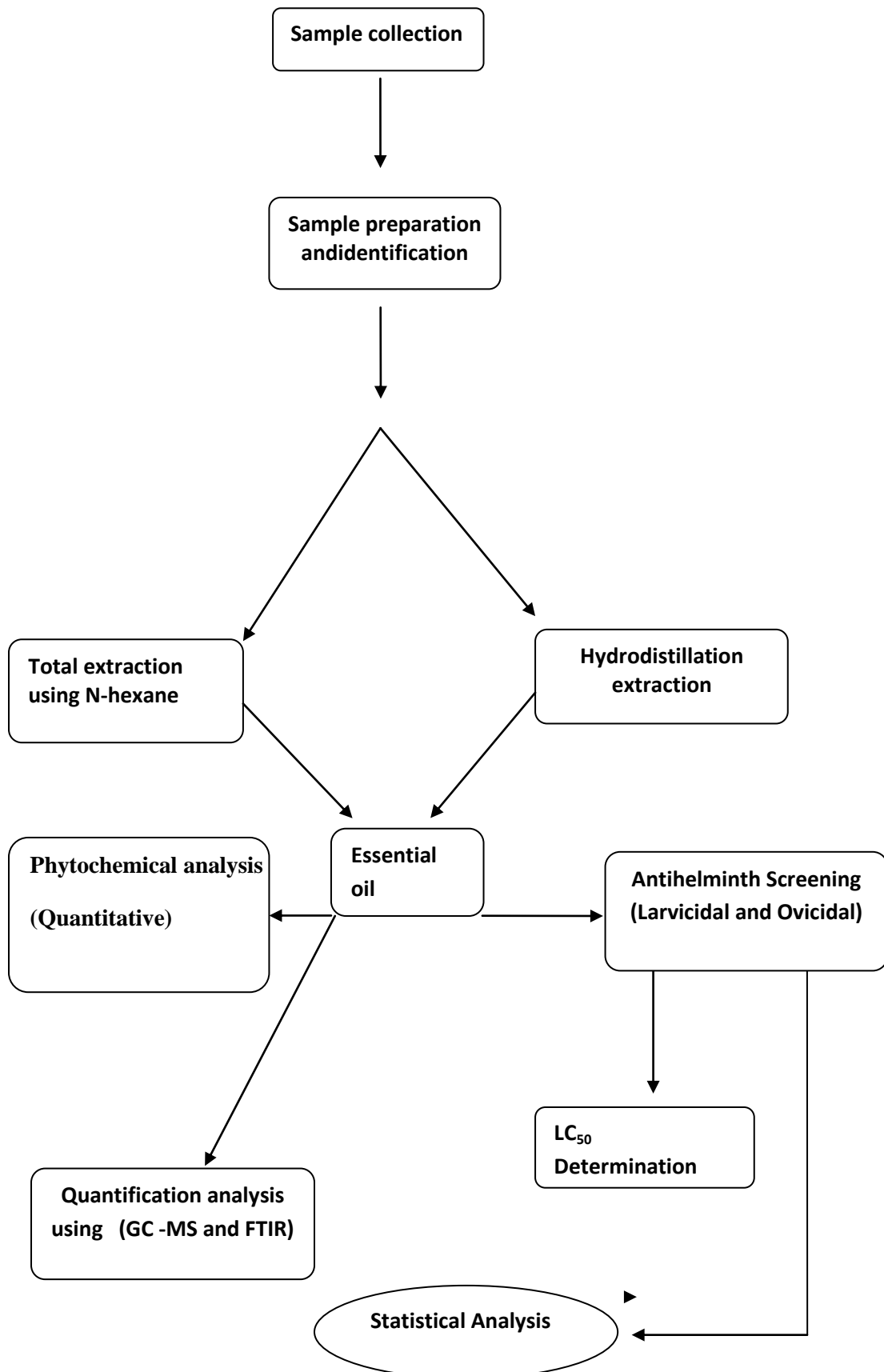
GC- MS machine (GCMS-QP2010PLUS Shimadzu, Japan), FTIR machine (8400s - Shimadzu Japan), Rotary evaporator, Distillating apparatus.

3.1.3 Collection, identification and preparation of *Ascaridia galli* eggs and larvae

Ascaridia galli were obtained from entrails of domestic chicken (*Gallus gallus domesticus*) obtained from Avian Physiology Section Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria. The nematodes were collected in phosphate buffer saline pH 7.2. The adult worms were identified and characterized using the identification key of Eslami (Eslami, 2006). The worms were washed in Goodwin physiologic saline (0.9% NaCl solution), worms were crushed the eggs were extracted by sedimentation method (Permin, 1997). Crushed worm was centrifuged and cell debris was washed off with tap water.

3.2. Methods

3.2.1 Experimental design



+3.2.2 Collection and identification of citrus peels

Citrus species were obtained from Institute of Agricultural Research A.B.U Zaria. The plants were identified at the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University Zaria and the voucher number documented as *Citrus sinensis* V/No:1432, *Citrus aurantifolia* V/No:4014, *Citrus limon* V/No:990.

3.2.3 Extraction of essential oil from orange peels

Orange peel oils extractions were carried out at Postgraduate Laboratory in the Department of Biochemistry, using steam distillation method. The oranges were washed with water, cleaned and peeled thinly. The peels were weighed (500g) with a digital weighing balance; then cut into small bits and blended into paste after being frozen in the refrigerator with electric blender, the paste was transferred into a 1000 ml flat bottom flask, with a large volume of water added to cover the peels paste, anti bumps were also added. The flask was then connected to Liebig condenser. This set up used heating mantle as source of heat. The steam generated from the boiling water extracted the volatile oils, which was condensed as part of steam into the distillation flask as it passed through a cooling system for 180 minutes in full glass apparatus. The distillate (a mixture of oil and water) was poured into a separating funnel where the mixture separate into two layers: oil at the upper layer and water at the lower layer. Water as allowed to run off while any remaining water droplets in the oil were dry over anhydrous sodium sulphate, the oil were collected in a brown bottle and refrigerated at 4.0⁰C until use.

3.2.4 Citrus peels N-hexane extraction

The peels cut into small bits, frozen and blended into paste with electric blender, and were mixed with N-hexane (1:3, w: v) ratio. The mixtures were placed in sealed glass bottles at room temperature for 78 hours. Each mixture was filtered separately through number one Whatman filter paper. N-hexane was removed under reduced pressure at 40 °C by a Rotary Evaporator. Percentage total oil obtained was calculated using this formular

$$\% \text{ Yield} = \frac{A}{B} \times 100.$$

Where A=amount of oils obtain in grams,

B=500grams of fresh citrus peels

$$\% \text{ Yield} = \frac{\text{mass of products}}{\text{mass of starting material}} \times 100 \quad \frac{\text{mass of products}}{\text{mass of starting material}} \times 100$$

$$\% \text{ Yield} = \frac{\text{mass of essential oils}}{\text{mass of citrus peels}} \times 100$$

An experimental composition (EC) was compounded by mixing equal citrus peels extracts with DMSO. The EC was essayed for active components as described by Hosni *et al.* (2010).

3.2.5 Anthelmintic assay

In vitro anthelmintic activities of different essential oils were conducted on ova and larvae of *Ascaridia galli*. The eggs were cultured for fourteen days in 1N Sulphuric acid solution to enabled larvae emanation; Larvalcidal assay' was employed using *larvae of Ascaridia galli*. Essential Oils were applied at three concentrations 0.1, 0.3 and 0.5µL/ml in phosphate buffer solution (PBS) plus 80% of DMSO (as carrier/emulsifier).

Peperazine citrate was used as a positive control at 0.05 µL/ml concentration; the effects were checked after thirty minutes under the microscope. Ovicidal assay the eggs were cultured with essential oil, DMSO, in 1N Sulphuric acid solution, similar to the concentrations above. Eggs and larvae were distributed to 20 treatment groups with about 25-30 larvae per well given a total of 1400-1500 larvae after correcting for infertile eggs.

3.2.6 Grouping

The isolated eggs and larvae of the worm were divided into 20 treatment groups; Groups tagged 2-4 were exposed to essential oils obtained through hydrodistillation while Groups tagged 5-7 were exposed to essential oils obtained through N-hexane extraction

Group 1: worms were exposed to 5ml DMSO+PBS (Normal.Control)

Group 2A: worms were exposed to 0.3 ml of *C.limon* oil extract/5ml DMSO+PBS

Group 2B: worms were exposed to 0.5ml of *C.limon* oil extract/5ml DMSO+PBS

Group 2C: worms were exposed to 1.0 ml of *C.limon* oil extract /5ml DMSO+PBS

Group 3A: worms were exposed to 0.3 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 3B: worms were exposed to 0.5 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 3C: worms were exposed to 1.0 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 4A: worms were exposed to 0.3ml of *C.aurantifolia oil extract*/5ml DMSO+PBS

Group 4B: worms were exposed to 0.5 ml of *C. aurantifolia* oil extract /5ml DMSO+PBS

Group 4C: worms were exposed to 1.0 ml of *C. aurantifolia* oil extract /5ml DMSO+PBS

Group 5A: worms were exposed to 0.3 ml of *C.limon* oil extract/5ml DMSO+PBS

Group 5B: worms were exposed to 0.5ml of *C.limon* oil extract/5ml DMSO+PBS

Group 5C: worms were exposed to 1.0 ml of *C.limon* oil extract /5ml DMSO+PBS

Group 6A: worms were exposed to 0.3 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 6B: worms were exposed to 0.5 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 6C: worms were exposed to 1.0 ml of *C. sinensis* oil extract /5ml DMSO+PBS

Group 7A: worms were exposed to 0.3ml of *C. aurantifolia oil extract*/5ml DMSO+PBS

Group 7B: worms were exposed to 0.5 ml of *C. aurantifolia* oil extract /5ml DMSO+PBS

Group 7C: worms were exposed to 1.0 ml of *C. aurantifolia* oil extract /5ml DMSO+PBS

Group 8: worms were exposed to 0.1µg/ml of perperizine citrate (Standard drug)

These twenty groups were exposed to the following treatments at controlled temperature ($37 \pm 1^\circ\text{C}$) up to the maximum period of 6 ½ hours:

Twenty five -thirty larvae or ova approximately were suspended in each of the above concentrations in separate Petri dishes, each containing Fifty milliliter of the formulation. The inhibition of motility of the worms kept in the above treatments followed by the fading away of their body colour was used as criteria for anthelmintic

activity (Yazwinski, *et al* 2003T). Larvae motility was observed after 30 minutes to one hour. The assays were carried out in triplicates for each treatment, to avoid any bias and to minimize other sources of errors. Percentage worm motility inhibitions (WMI) were determined according to Rabel *et al.* (1994).

Using the following Formula=

$$\frac{(\text{Number of mobile worms in negative control Petri dish} \times \text{number of mobile worms in treatment Petri dish}) \times 100}{[\text{Number of mobile worms in negative control Petri dish}]}$$

3.2.7 Quantitative phytochemical determination

Determination of saponins

Two grams of the sample was weighed into a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250ml round bottom flask for 3hrs, after which the other weighed 250ml round bottom flask containing methanol was fitted to the same extractor continued for another 3hrs. At the end of second extraction, the methanol was recovered by distillation and the flask oven-dried to remove the remaining solvent in the flask. The flask was allowed to cool in a desiccator and weighed (AOAC, 1984).

Calculation: % Saponin = $(A - B) / W \times 100$

Where A= weight of flask and extract (saponin),

B = weight of empty flask

W = weight of sample.

Determination of flavonoids

Flavonoid determination was done using the method of Boham and Kocipal-Abyazan, (1974). Plant samples were extracted with 100ml of 80% aqueous methanol at room

temperature. The whole solution was filtered through Whitman filter paper no. 42 (125mm), the filtrate was later transferred into a crucible and was evaporated to dryness in a water bath and weighed as flavonoid.

Calculation

% Flavonoid = weight of flavonoids/ weight of sample x 100/litre

Determination of tannins

Concentration of tannin was determined using the modified standard method described by AOAC, (1984). Ten millilitres (10ml) of the sample was boiled with 300ml of distilled water. This was diluted in a standard volumetric flask. A volume of 25ml of the infusion was measured into a 2 litre porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against 0.1N Oxalic acid) until the blue solution changed green, Then few drops of 0.1N potassium permanganate was added at a time until the solution turns golden yellow.

The tannin content in the sample was calculated by multiplying the volume of 0.1N potassium permanganate used (titre value) by 0.0066235g. Using the equation below;

1ml of 0.1N potassium permanganate (titre value) = 0.0066235g Tannins

Therefore Tannins content in the sample = titre value x 0.0066235g

Determination of alkaloids

This was done using method of Harborne, (1998). Five gram (5g) of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was

allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

3.2.8 Determination of Total phenolic content (TPC)

The total phenolic content was determined on the four extracts using the Folin-Ciocalteu method adopted by Amin *et al.* (2006)

Principle: The reaction is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product with a maximum absorption at 765 nm which can be measured spectrophotometrically (Amin *et al.*, 2006).

Procedure: The Folin-Ciocalteu reagent was diluted 10 times (2.5 ml) and mixed with 2 ml of saturated sodium carbonate (75 g/l) and 6050 μ l of sample (supernatant) and homogenized for 10 seconds and heated for 30 minutes at 45⁰C. The absorbance was measured at 765 nm after cooling at room temperature. The data obtained was calculated by comparison between a standard curve (μ g Gallic acid/ml) and the absorbance of each sample. The data obtained were expressed as mg Gallic acid equivalents per gram of dry matter.

3.2.9 Gas chromatography/mass spectroscopy analysis

Samples were taken to NARICT Zaria for GC-MS where the essential oil were quantified and analyze using Mexican Norm NMX-F-062-1974 (1974) GC Claurus 500 gas chromatograph coupled to aClaurus 500 MS mass spectrophotometer (Perkin-ElmerInc., Wesley, Massachusetts, USA).

The GC-MS instrument is made up of two parts. The gas chromatography (GC) portion which separates the chemical mixture into pulses of pure chemicals and the mass spectrometer (MS) portion which identifies and quantifies the chemicals.

Functional principle

The Gas Chromatography Mass Spectrometry (GC/MS) is one of the most accurate tools for analyzing environmental samples. The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecules which are compared with a “library” of known mass spectra, covering several thousand compounds which is stored on a computer.

3.2.10 Fourier Transform Infra-Red Spectrophotometer (FTIR)

Fourier Transform infra-red Spectrophotometer (FTIR) is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved, all of which will have characteristic vibrational frequencies in the infra-red range. It is a material analysis technique that makes use of molecular absorption and transmission radiation corresponding to the frequencies of vibrations between the bonds of the atoms making up the material, creating a molecular fingerprint of the sample, of which no two unique molecular structures produce the same infrared spectrum. This characteristic results in a positive identification (qualitative analysis) of different material functional groups and their unique combination of bonds within the atoms and possible reactions. Fourier Transform Infrared (FTIR) Analysis can be achieved through the unique collection of absorption bands to confirm the identity of a pure (quality or consistency) compound, to detect the presence of specific impurities and the amount of components in a mixture, Spectra of organic compounds have two general areas:-The Functional Group Region

(1500-400 cm^{-1}) Peaks in this region are characteristic of specific kinds of bonds, and therefore can be used to identify whether a specific functional group is present. The Fingerprint Region (4000-1500 cm^{-1}) Peaks in this region arise from complex deformations of the molecule which may be characteristic of molecular symmetry, or combination bands arising from multiple bonds deforming

Functional principle

FTIR relies on the fact that most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency ranges are measured as wave numbers typically over the range 4000 – 600 cm^{-1} .

The background emission spectrum of the IR source is first recorded, followed by the emission spectrum of the IR source with the sample in place. The ratio of the sample spectrum to the background spectrum is directly related to the sample's absorption spectrum. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample.

3.2.11 Statistical analysis of result

Standard data analyses were done using probit analysis and Analysis of variance (ANOVA) of statistical Package for the social Sciences (SPSS) version 20.0. Results of different treatment groups were expressed as mean \pm Standard deviation except otherwise stated. The difference between various treatments groups were compared using the Duncan Multiple Range Test. P values less than 0.05 ($P < 0.05$) were taken as significant

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield of Essential Oils from Citrus Peels

In the present study the variation in the yield and chemical composition of the essential oils isolated from fresh, peels of three *Citrus* species namely *Citrus lemon*, *Citrus sinensis* and *Citrus aurantifolia* were investigated. The hydro-distilled essential oil content from fresh has maximum yield was seen in *C.aurantifolia* 2.1%, *C.limon* 1.8% *C.sinensis* had the lowest 1.5%. From N-hexane fraction, maximum yield came from *Citrus aurantifolia* 10 %, *C.limon* 7.1 % and *C.sinensis* had the lowest 6 %. The yield was measure in the corresponding weight for hydrodistilled fraction of citrus peels 10.5, 9.0 and 7.5 in grams while N-hexane fraction of citrus peels had 50, 35.5 and 30 in grams as shown in the Table 4.1

Table 4.1: Percentage Yield of Three Citrus Peels Essential Oils

FRACTIONS		HYDRO-DISTILLED		N-HEXANE	
SN	SAMPLES	Yield (g)	% Yield	Yield (g)	% Yield
1	<i>C.sinensis</i>	7.5	1.5	30	6
2	<i>C. aurantifolia</i>	10.5	2.1	50	10
3	<i>C. limon</i>	9.0	1.8	35.5	7.1

Citrus lemon=*C. limon*, *Citrus sinensis* =*C. Sinensis*.,*Citrus auriantifolia* =*C. aurantifolia*.

4.2. Phytochemical Analysis

4.2.1 Quantitative phytochemical analysis

The quantitative phytochemical content of peels of *Citrus aurantifolia*, *Citrus limon* and *Citrus sinensis* are summarised in Table 4.3. These results reveal a significant ($p \leq 0.05$) difference in Alkaloids, Flavonoids, Tannins and Saponins in the different species.

Alkaloids content of *Citrus sinensis* (0.32 ± 0.06 mg/100g) was significantly ($p \leq 0.05$) greater than in *Citrus limon* and *aurantifolia*. However, there was no significant ($p \leq 0.05$) difference in alkaloid content of *Citrus aurantifolia* (0.26 ± 0.04 mg/100g) and *Citrus limon* (0.21 ± 0.03 mg/100g). Flavonoids were significantly ($p \leq 0.05$) greater in *Citrus limon* (0.27 ± 0.03 mg/100g) and *Citrus aurantifolia* (0.24 ± 0.04 mg/100g) than in *Citrus sinensis* (0.15 ± 0.04 mg/100g) where as tannin content was significantly ($p \leq 0.05$) greater in *Citrus sinensis* (0.10 ± 0.01 mg/100g) than in *Citrus limon* (0.06 ± 0.03 mg/100g) and *Citrus sinensis* (0.06 ± 0.02 mg/100g). *Citrus limon* had a significantly ($p \leq 0.05$) greater level of saponins than *Citrus sinensis* (0.11 ± 0.03 mg/100g), with no significant ($p \leq 0.05$) difference in saponin content of *Citrus aurantifolia* (0.13 ± 0.03 mg/100g) and *Citrus limon* (0.21 ± 0.05 mg/100g). Steroid content was significantly ($p \leq 0.05$) greater in *Citrus limon* (0.25 ± 0.04 mg/100g) which had a significantly ($p \leq 0.05$) greater level than *Citrus aurantifolia* (0.23 ± 0.05 mg/100g) and *Citrus sinensis* (0.21 ± 0.01 mg/100g).

Table 4.2 Quantitative Phytochemical Constituents in *Citrus Aurantifolia*, *Citrus Limon* and *Citrus Sinensis* Fruit Peels.

Citrus species	Phytochemical content (mg/100g)				
	Alkaloids	Flavonoids	Tannins	Saponins	Steroids
<i>C.aurantifolia</i>	0.26±0.04 ^{ab}	0.24±0.04 ^b	0.06±0.02 ^a	0.13±0.03 ^{ab}	0.23±0.05 ^{ab}
<i>C. limon</i>	0.21±0.03 ^a	0.27±0.03 ^b	0.06±0.03 ^a	0.21±0.05 ^b	0.25±0.04 ^c
<i>C. sinensis</i>	0.32±0.06 ^b	0.15±0.04 ^a	0.10±0.01 ^a	0.11±0.03 ^a	0.21±0.01 ^a

Values are mean ± Standard deviation, (n=3). Values with different superscripts down the column are significantly ($p \leq 0.05$) different.

Citrus aurantifolia- *C.aurantifolia*, *Citrus limon* -*C.limon*, *Citrus sinensis*- *C.sinensis*

4.2.2 Total polyphenol content

The total polyphenol Content of essential oils extracts of (N-hexane and hydrodistilled fractions) is shown in Figure 4.1. The result indicates a significant ($p \leq 0.05$) difference in total polyphenol content. The amount of total polyphenol of all N-hexane fractions were significantly ($p \leq 0.05$) lower than the hydrodistilled fractions in all cases, with hydrodistilled fractions of *Citrus aurantifolia* recording the highest amount 264.15 μ g/ml.

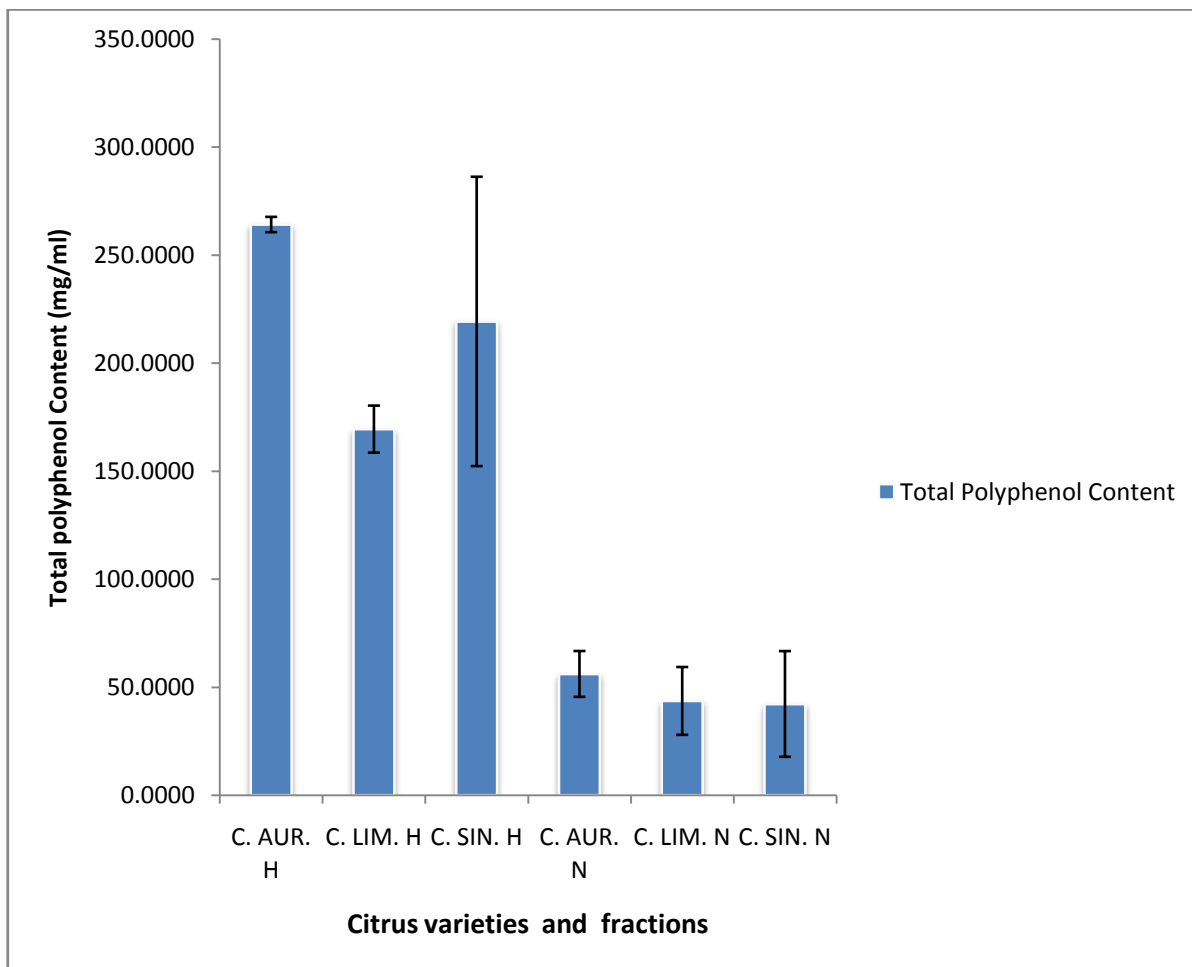


Figure 4.1 Total Polyphenol Content of Essential Oils (N-hexane and hydrodistilled fractions) of *Citrus aurantifolia*, *Citrus limon* and *Citrus sinensis*.

Values are mean \pm Standard deviation, (n=3) *Citrus aurantifolia*=*C.aur*, *Citrus limon*=*C.lim*, *Citrus sinensis*= *C.sin*, H=hydrodistilled fractions, N= n- hexane fraction.



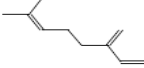








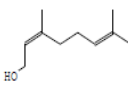

4.3.Citrus Peels Essential Oil Analysis

4.3.1 Gas chromatography-Microscopy analysis of chemical composition of essential oils from citrus peels

Essential oils obtained from the fresh Fruit Peels of *Citrus aurantifolia*, *Citrus limon* and *Citrus sinensis* by hydrodistilled, cold maceration using (N-hexane) and subjected to gas chromatography-mass spectroscopy (GC-MS). Forty-five components were identified.

GC-MS analysis of essential oils from peels *Citrus sinensis* Hydrodistilled fraction gave a total of thirteen (13) chemical constituents as identified with the standard library. they include:- alpha-Pinene, Nopinene, beta.-Myrcene,L- Limonene, Sylvestrene, Bicyclo [3.1.1]hept-2-ene, 2,6,6-trimethyl, beta.-Linalool, 1-Terpinen-4-ol, Decanal, p-menth-1-en-8-ol, beta.-Citronellol, cis-Geraniol,alpha-Citral with Sylvestrene having the highest percentage area of 57.42 followed by, L- Limonene 16.85 and cis-Geraniol 0.63 having the lowest percentage area as shown in Table 4.3.

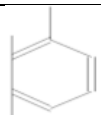
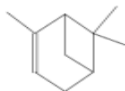

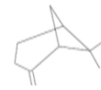
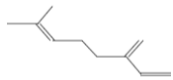
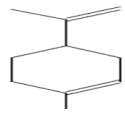
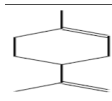

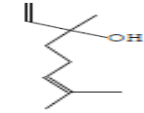
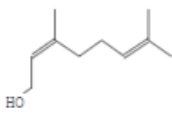
Table 4.3: Chemical Composition of Essential Oil from *Citrus sinensis* Hydrodistilled Fraction.

Peak No	R.time	Area ⁰ / ₀	Compound Name	M.weight	M.formular	Structure	S.I
1	6.032	1.83	α -Pinene,	136	C ₁₀ H ₁₆		97
2	6.763	5.18	β -Pinene,	136	C ₁₀ H ₁₆		94
3	6.849	7.25	β -Myrcene	136	C ₁₀ H ₁₆		90
4	7.591	16.85	Limonene	136	C ₁₀ H ₁₆		97
5	7.855	57.43	Sylvestrene	136	C ₁₀ H ₁₆		90
6	8.119	2.02	Bicyclo [3.1.1]hept-2-ene, 2,6,6-trimethyl	136	C ₁₀ H ₁₆		93
7	8.793	4.56	β Linalool	154	C ₁₀ H ₁₈ O		93
8	10.100	1.15	1-Terpinen-4-ol	154	C ₁₀ H ₁₈ O		92
9	10.238	0.84	Decanal, n-Decyl aldehyde	156	C ₁₀ H ₂₀ O		96
10	10.361	0.72	p-menth-1-en-8-ol	154	C ₁₀ H ₁₈ O		93
11	10.718	0.87	β -Citronellol	156	C ₁₀ H ₂₀ O		92
12	11.109	0.63	Cis-Geraniol	154	C ₁₀ H ₁₈ O		84
13	11.321	0.67	Citral,	152	C ₁₀ H ₁₆ O		92

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular, S.I: Similarity Index

GC-MS analysis of essential oils from peels *Citrus sinensis* N-hexane fraction gave a total of Ten (10) chemical constituents as identified with the standard library. They include:- Limonene 57.62, Toluene 16.51, beta.-Myrcene 7.44, beta.-Pinene 4.48, Linalool 4.15, Cajeputen 3.07, Sabinene 2.15, alpha.-Pinene 1.95, Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-, (1R) 1.74, cis-Geraniol 0.89 with limonene and Toluene having the highest percentage area of 57.62 and 16.51 while Cis-Geraniol have the lowest percentage area of 0.89 as shown in Table 4.4.

Table 4.4: Chemical composition of *Citrus sinensis*-N-hexane fraction







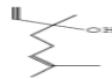



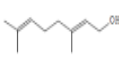
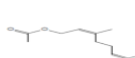
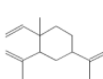

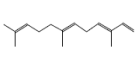

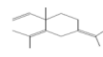
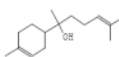
Peak No	R.time	Area ⁰ / ₀	Compound Name	M.weight	M.formular	Structure	S.I
1	3.913	16.51	Toluene	92	C ₇ H ₈		94
2	6.021	1.95	α -Pinene	136	C ₁₀ H ₁₆		97
3	6.651	2.15	Sabinene	136	C ₁₀ H ₁₆		92
4	6.742	4.48	β -Pinene	136	C ₁₀ H ₁₆		:93
5	6.839	7.44	β -Myrcene	136	C ₁₀ H ₁₆		91
6	7.574	57.62	Limonene	136	C ₁₀ H ₁₆		97
7	7.780	3.07	Cajeputen	136	C ₁₀ H ₁₆		87
8	8.024	1.74	α -Pinene	136	C ₁₀ H ₁₆		92
9	8.745	4.15	Linalool	154	C ₁₀ H ₁₈ O		93
10	10.743	0.89	Cis-Geraniol	154	C ₁₀ H ₁₈ O		90

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular,

S.I: Similarity Index

GC-MS analysis of essential oils from peels *Citrus auriantifolia* Hydrodistilled fraction gave a total of Eighteen (18) Chemical constituents as identified with the standard library. They include :- L-Limonene 25.36, beta.-Myrcene 11.13, 1-Terpinen-4-ol 8.45, beta.-Pinene 7.91, beta.-Citronellol 7.36, Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl 5.28, alpha.-Farnesene 5.25, beta.-Bisabolene 4.97, p-menth-1-en-8-ol 4.36, trans-Geraniol 4.31, Farnesene 3.79, alpha.-Pinene 3.40, beta.-Linalool 3.11, Cyclohexane 1.92, Nerol acetate 1.26, Artemesia triene 0.79, gamma.-Elemene 0.76, alpha.-Bisabolol 0.60, with L-Limonene, and beta.-Myrcene having the highest percentage area of 25.36 and 11.13 while alpha.-Bisabolol 0.60, have the lowest percentage area as shown in Table 4.5.








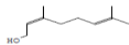
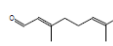

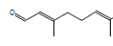



Table 4.5: Chemical Composition of *Citrus aurantifolia* Hydrodistilled Fraction.

Peak No	R time	Area (%)	Compound Name	M. weight	M. formular	Structure	S.I
1	6.023	3.40	α -Pinene	136	C ₁₀ H ₁₆		97
2	6.752	7.91	β -Pinene	136	C ₁₀ H ₁₆		93
3	6.844	11.13	β Myrcene	136	C ₁₀ H ₁₆		91
4	7.591	25.36	Limonene	136	C ₁₀ H ₁₆		97
5	8.099	5.28	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-,	136	C ₁₀ H ₁₆		94
6	8.517	0.79	Artemesia triene	136	C ₁₀ H ₁₆		93
7	8.761	3.11	β -Linalool	154	C ₁₀ H ₁₈ O		93
8	10.110	8.45	1-Terpinen-4-ol,	154	C ₁₀ H ₁₈ O		93
9	10.360	4.36	p-menth-1-en-8-ol	154	C ₁₀ H ₁₈ O		93
10	10.738	7.36	β -Citronellol	156	C ₁₀ H ₂₀ O		93
11	11.124	4.31	Trans-Geraniol	154	C ₁₀ H ₁₈ O		94
12	12.431	1.26	Nerol acetate	196	C ₁₂ H ₂₀ O ₂		94
13	12.973	1.92	2,4-Diisopropenyl-1-methyl-1-vinylcyclohexane	204	C ₁₅ H ₂₄		90
14	13.489	5.25	α -Farnesene	204	C ₁₅ H ₂₄		92
15	14.451	3.79	β -Farnesene	204	C ₁₅ H ₂₄		92
16	14.598	4.97	β -Bisabolene	204	C ₁₅ H ₂₄		90
17	15.777	0.76	γ -Elemene	204	C ₁₅ H ₂₄		90
18	18.019	0.60	α -Bisabolol)-,	222	C ₁₅ H ₂₆ O		92

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular, S.I: Similarity Index

GC-MS analysis of essential oils from peels *Citrus auriantifolia* H-hexane fraction gave a total of Fourteen (14) chemical constituents as identified with the standard library. They include:- beta-Farnesene 2.39, alpha.-Farnesene 3.48, 1,3,6,10-Dodecatetraene, 3,7,11-trimethyl 3.03, alpha.-Citral 2.5, trans-Geraniol 1.48, beta-Citral 1.52, cis-Geraniol 0.62, p-menth-1-en-8-ol 0.99, 6,6 Dimethyl-2 methylenebicyclo[3.1.1]heptane 5.42, L-Limonene 57.67, beta-Pinene 12.36, Sabinene 2.06, alpha.-Pinene 1.11, Toluene 5.29, with L-limonene and beta-pinene having the highest percentage area of 57.67 and 12.36 while cis-Geraniol 0.62 have the lowest percentage area as shown in Table 4.6.



















Table 4.6: Chemical Composition of *Citrus auriantifolia* N-Hexane Fraction

Pea k No	R. time	Area (%)	Compound Name	M. weight	M. formular	Structure	S.I
1	3.918	5.29	Toluene	92	C ₇ H ₈		95
2	6.019	1.11	α-Pinene	136	C ₁₀ H ₁₆		97
3	6.652	2.06	Sabinene	136	C ₁₀ H ₁₆		92
4	6.750	12.3 6	β-Pinene,	136	C ₁₀ H ₁₆		:93
5	7.590	57.6 7	L-Limonene	136	C ₁₀ H ₁₆		97
6	8.012	5.42	, 6,6 Dimethyl-2 methylenebicyclo[3.1.1]heptan e	136	C ₁₀ H ₁₆		93
7	10.344	0.99	p-menth-1-en-8-ol	154	C ₁₀ H ₁₈ O		92
8	10.747	0.62	Cis-Geraniol	154	C ₁₀ H ₁₈ O		91
9	10.906	1.52	α-Citra	152	C ₁₀ H ₁₆ O		93
10	11.108	1.48	trans-Geraniol	154	C ₁₀ H ₁₈ O		94
11	11.329	2.57	alpha.-Citral,	152	C ₁₀ H ₁₆ O		94
12	13.488	3.03	(ZE)alpha.-Farnesene,	204	C ₁₅ H ₂₄		90
13	14.443	3.48	alpha.-Farnesene,	204	C ₁₅ H ₂₄		93
14	14.586	2.39	β -Farnesene	204	C ₁₅ H ₂₄		90

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular, S.I: Similarity Index

GC-MS analysis of essential oils from peels *Citrus limon* Hydrodistilled fraction gave a total of Eighteen (18) chemical constituents as identified with the standard library. They include:- L-Limonene 24.66, Limonene 20.40, beta.-Pinene 6.97, 1,5-Heptadiene 6.11, p-menth-1-en-8-ol 5.73, cis-Geraniol 5.27, alpha.-Farnesene 4.75, 1-Terpinen-4-ol 4.64, 4-Isopropenyl-1-methyl-1,2-cyclohexanediol 3.39, beta.-Citronellol 3.18, Caryophyllene oxide 3.16, beta.-Linalool 2.82, Patchulane 2.72, alpha.-Citral 1.79, Santolina triene 1.74, alpha.-Pinene 1.15, trans-p-Mentha-2,8-dienol 0.86, 2-Cyclohexen-1-ol 0.65, with L-Limonene and Limonene having the highest percentage area of 24.66 and 20.40 while Cyclohexen-1-ol 0.65, have the lowest percentage area as shown in Table 4.7.













Table 4.7: Chemical composition of *Citrus limon* hydrodistilled Fraction.

Peak No	R. time	Area (%)	Compound Name	M. weight	M. formular	Structure	S.I
1	6.019	1.15	α -Pinene,	136	C ₁₀ H ₁₆		97
2	6.761	6.97	β -Pinene,	136	C ₁₀ H ₁₆		94
3	7.587	24.66	L-Limonene	136	C ₁₀ H ₁₆		96
4	7.804	20.40	Limonene,	136	C ₁₀ H ₁₆		91
5	8.769	2.82	β -Linalool	154	C ₁₀ H ₁₈ O		93
6	9.240	0.86	trans-p-Mentha-2,8-dienol	152	C ₁₀ H ₁₆ O		80
7	9.474	0.65	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)	152	C ₁₀ H ₁₆ O		89
8	10.117	4.64	1-Terpinen-4-ol	154	C ₁₀ H ₁₈ O		91
9	10.773	5.73	p-menth-1-en-8-ol	154	C ₁₀ H ₁₈ O		92
10	10.773	3.18	β -Citronellol	156	C ₁₀ H ₂₀ O		90
11	11.142	5.27	Cis-Geraniol	154	C ₁₀ H ₁₈ O		84
12	11.340	1.79	α -Citral	152	C ₁₀ H ₁₆ O		89
13	12.177	1.74	Santolina triene,	136	C ₁₀ H ₁₆		90
14	12.861	3.39	1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)	170	C ₁₀ H ₁₈ O ₂		86
15	12.985	2.72	Patchulane	206	C ₁₅ H ₂₆		89
16	13.522	4.75	alpha.-Farnesene	204	C ₁₅ H ₂₄		94
17	14.360	6.11	1,5-Heptadiene, 6-methyl-2-(4-methyl-3-cyclohexen-1-yl)-,	204	C ₁₅ H ₂₄		92
18	16.458	3.16	Caryophyllene oxide,	220	C ₁₅ H ₂₄ O		86

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular, S.I: Similarity Index

GC-MS analysis of essential oils from peels *Citrus limon* N-hexane Fraction gave a total of Twelve (12) chemical constituents as identified with the standard library. They include:- L-Limonene 44.28, n-Decane 10.27, Nonane 8.81, Cyclohexane 6.00, Ethylbenzene 5.81, 1-ethyl-2-methyl 5.43, Cyclogeraniolane 4.87, n-Undecane 4.33, 2-undecene 3.82, n-Octane 3.38, n-Tridecane 1.52, n-Dodecane 1.47, with L-Limonene and n-Decane having the highest percentage area of 44.28 and 10.27 while n-Dodecane 1.47, have the lowest percentage area as shown in Table 4.8.

Table 4.8: Chemical Composition of *Citrus limon* N-hexane Fraction

Peak No	R. time	Area ⁰ %	Compound Name	M. weight	M. formular	Structure	S.I
1	3.942	5.43	Cyclohexane, 1-ethyl-2-methyl	126	C ₉ H ₁₈		82
2	4.080	3.38	n-Octane	114	C ₈ H ₁₈		96
3	4.629	4.87	Cyclogeraniolane	126	C ₉ H ₁₈		92
4	5.190	5.81	Ethylbenzene	106	C ₈ H ₁₀		95
5	5.370	8.81	n-Nonane	128	C ₉ H ₂₀		96
6	5.952	6.00	Cyclohexane, (2-methylpropyl)	140	C ₁₀ H ₂₀		88
7	6.419	3.82	Erythro Dimethyl-2-undecene	182	C ₁₃ H ₂₆		85
8	6.881	10.27	n-Decane	142	C ₁₀ H ₂₂		96
9	7.598	44.28	L-Limonene	136	C ₁₀ H ₁₆		97
10	8.430	4.33	n-Undecane	156	C ₁₁ H ₂₄		96
11	11.377	1.52	n-Tridecane	184	C ₁₃ H ₂₈		97
12	12.744	1.47	n-Dodecane	170	C ₁₂ H ₂₆		95

R.time: Retention Time, M.weight: Molecular weight, M.formular: Molecular formular, S.I: Similarity Index

In summary, GC-MS Chromatograms for typical *Citrus* oil analyzed are shown in appendices 3-8. The chemical compositions of *C. sinensis* peel essential oil are shown on Table 4.3. The total numbers of compounds identified in the essential oils from hydrodistilled and N-hexane peels of *C. sinensis* were 13 and 10, respectively. Limonene (16.86 and 57.62 %), β -myrcene (7.25 and 7.44%) and β -pinene (5.18 and 4.48%) were the main constituents of essential oil of *C. sinensis*, respectively. In addition, sylvestrene (57.43%) and toluene (16.51%) exhibit considerable amount for hydrodistilled and N-hexane peel oil separately.

The chemical compositions of *C. auriantifolia* peel essential oil are shown on table 4.5. The total numbers of compounds identified in the essential oils from hydrodistilled and N-hexane peels of *C. auriantifolia* were 18 and 14, respectively. Limonene (25.36 and 57.67 %), β -myrcene (11.13%) only in hydrofraction, β -pinene (7.91 and 12.36%) were the main constituents of essential oil of *C. sinensis*, respectively. In addition, β -citronelol (7.36%) and 6,6 Dimethyl-2methylene bicyclo[3.1.1]heptane (5.42%) exhibit considerable amount for hydrodistilled and N-hexane peel oil separately.

The chemical compositions of *C. limon* peels essential oil are shown in table 4. 7. The total numbers of compounds identified in the essential oils from hydrodistilled and N-hexane peels of *C. limon* were 18 and 12, respectively. Limonene (24.66 and 44.28 %), while Cajeputen (20.40%), β -Pinene (6.97%) cis-Geraniol (5.27) and N-Decane (10.27%), Nonane (8.81%) and Cyclohexane (6.00%) all in N-hexane and 4.48%) were the main constituents of essential oil of *C. limon*, respectively.

Generally, comparative analysis of chemical composition of hydrodistilled and N-hexane fractions of essential oil from three Nigerian citrus peels revealed more chemical constituents in hydro distilled fractions are more in numbers compared to N-hexane fractions that is, the hydrodistilled fraction extracts more component compare to

N-hexane and only L-limonene was common to all the fractions in high quantities as shown in Table 4.9

Table 4.9 Comparative Analysis of Chemical Composition of Hydrodistilled and N-Hexane Fractions of Essential Oil from Three Citrus Peels.

S/ N	CONSTITUENTS	<i>C. sinensis</i>		<i>C. auriantifolia</i>		<i>C. limon</i>	
		H. distill	N-hexane	H. distill	N-hexane	H. distill	N-hexane
		%Area	%Area	%Area	%Area	%Area	%Area
1	Limonene	16.85	57.62	25.36	57.67	24.66	44.28
2	β -Myrcene,	7.25	7.44	11.13	-	-	-
3	1-Terpinen-4-ol	1.15	-	8.45	-	4.64	-
4	α -Pinene	1.83	1.95	3.40	1.11	1.15	-
5	β -Pinene,	5.18	4.48	7.91	12.36	6.97	-
6	p-menth-1-en-8-ol	0.72	-	4.36	0.99	5.73	-
7	β -Linalool,	4.56	4.15	3.11	-	2.82	-
8	β -Citronellol	0.87	-	7.36	-	3.18	-
9	cis-Geraniol	0.63	0.89	-	0.62	5.27	-
10	Trans-Geraniol	-	-	4.31	1.48	-	-
11	α -Farnesene	-	-	3.79	3.48	4.75	-
12	β -Farnesene	-	-	5.25	2.39	-	-
13	Bicyclo [3.1.1]hept-2-ene, 2,6,6-trimethyl	2.02	-	5.28	-	-	-
14	α -Citral	0.67	-	-	2.57	1.79	-
15	Toluene	-	16.51	-	5.29	-	-
16	Sabinene	-	2.15	-	2.06	-	-
17	Cajeputen	-	3.07	-	-	20.40	-
18	Sylvestrene	57.43	-	-	-	-	-
19	1R- α -Pinene	-	1.74	-	-	-	-
20	γ -Elemene	-	-	0.76	-	-	-
21	α -Bisabolol	-	-	0.60	-	-	-

22	Cyclohexane	-	-	1.92	-	-	-
23	β -Bisabolene	-	-	4.97	-	-	-
24	Decanal,	0.84	-	-	-	-	-
25	Artemesia triene	-	-	0.79	-	-	-
26	Nerol acetate	-	-	1.26	-	-	-
27	6,6 Dimethyl- 2methylenebicyclo [3.1.1]heptane	-	-	-	5.42	-	-
28	4-Isopropenyl-1- methyl-1,2- cyclohexanediol	-	-	-	-	3.39	-
29	Caryophyllene oxide	-	-	-	-	3.16	-
30	1,5-Heptadiene	-	-	-	-	6.11	-
31	Santolina triene	-	-	-	-	1.74	-
32	trans-p-Mentha- 2,8-dienol	-	-	-	-	0.86	-
33	Patchulane	-	-	-	-	2.72	-
34	2CyclohexeN-1-ol,	-	-	-	-	0.65	-
35	Cyclogeraniolane	-	-	-	-	-	4.87
36	n-Dodecane	-	-	-	-	-	-
37	N-Tridecane	-	-	-	-	-	1.52
38	N-Octane	-	-	-	-	-	3.38
39	2-undecene	-	-	-	-	-	3.82
40	N-Undecane	-	-	-	-	-	4.33
41	1-ethyl-2-methyl	-	-	-	-	-	5.43
42	Ethylbenzene	-	-	-	-	-	5.81

43	Cyclohexane	-	-	-	-	-	6.00
44	Nonane	-	-	-	-	-	8.81
45	N-Decane	-	-	-	-	-	10.27

H. distill = hydrodistilled fraction

4.3.2 Fourier Transform Infrared (FTIR) Analysis

The FTIR result for *Citrus sinensis* hydrodistilled fraction, gave Twenty two (22) peaks, only Twenty one (21) of the peaks could be identified as functional groups with the standard library. These Functional groups includes:- Alkyl halide(R-I), Alkenes($\text{RCH}=\text{CR}-\text{R}$), Ethers(AO-R), Alcohols [$\text{RR}-\text{R}-\text{OH}$] (3^0), Alkyls, Aromatic compounds, Amides, Acyl chlorides[$\text{R}-\text{C}(\text{O})-\text{Cl}$], Anhydrides [$\text{R}-\text{C}(\text{O})-\text{O}-\text{C}(\text{O})-\text{R}$], Nitriles($\text{R}-\text{C}\equiv\text{N}$), Carboxylic acids and Alkanes as shown on table 4.10.

Table 4.10: Functional groups in Essential oil of *Citrus Sinensis* Hydrodistilled Fraction.

PEAK NO	ABSORBANCE (nm)	CLASS OF COMPOUND	FUNCTIONAL GROUP	INTENSITY
1-6	380.95- 438.82	Alkyl halide (R-I)	C-I Stretch	Strong
7	798.56	Alkenes(RCH=CR(R)(R'))	=C-H bend	Strong
8	891.14	Alkenes (RR'C=CH ₂)	=C-H bend	Strong
9	1032.92	Ethers (Ar-O-R)	=C-O-C symetry	M.strong
10	1110.07	Ethers(R-O-R')	C-O-C Stretch	Strong
11	1152.51	Alcohols [RR'R''OH] (3 ⁰)	C-O Stretch	M.strong
12	1242.02			
13	1322.25	Alkyl halide (R-F)	C-F Stretch	V. strong
14	1377.22	Alkyl	C-H bend	Strong
15	1443.77	Aromatic compoud Alkyls	C=C Stretch C-H bend	M. strong Strong
16	1643.41	Alkene Amides	C=C Stretch C=O Stretch	V. weak.m Strong
17	1806.4	Acyl chlorides [R-C(O)-Cl] Aldehydes [R-C(O)-O-C(O)-R]	C=O Stretch C=O symetry	Strong S.strong
18	2258.72	Nitriles(R-C≡N)	C≡N Stretch	M.strong
19	1923.59	-	-	-
20	2616.53	Carboxylic acids	O-H Stretch	S. broad
21	2919.36	Alkanes	C-H Stretch	Strong
22	3075.06	Aromatic compounds	ArC-H Stretch	Medium

The FTIR result for *Citrus sinensis* N-hexane fraction, gave Twelve (12) peaks, only Ten (10) of the peaks could be identified as functional groups with the standard library. These Functional groups includes:-alkyl halide[R-I], Alkenes [RCH=CR R R], aromatic compounds (m-distribution), Alcohols [C=C-CH₂-OH], alkyls, amines, carboxylic acids and amines [R-NH₂] as shown on table 4.11.

Table 4.11: Functional groups in Essential oil of *Citrus sinensis* N-hexane Fraction

PEAK NO	ABSORBANCE (nm)	CLASS OF COMPOUND	FUNCTIONAL GROUP	INTENSITY
1	445.57	Alkyl halide [R-I]	C-I Stretch	Strong
2	803.38	Alkenes[RCH=CR R] Aromatic compounds m-distribution	=C-H bend -C-H bend	Strong Strong
3	893.07	Alkenes[RR C=CH ₂]	=C-H bend	Strong
4	1037.74	Alcohols [C=C-CH ₂ -OH]	C-O Stretch	M. strong
5	1367.58	Alkyl s	-CH(CH ₃) ₂ bend	Medium
6	1441.84	-	-	-
7	1587.47	Amines	N-H bend	W.medium
8	2357.09	-	-	-
9	2737.08	Carboxylic acids	O-H Stretch	S-broad
10	2869.21	Alkanes	C-H Stretch	Strong
11	2923.22	Alkanes Carboxylic acids	C-H Stretch O-H Stretch	Strong S-broad
12	3555.89	Amines [R-NH ₂]	N-H symmetry	Weak

The FTIR result for *Citrus aurantifolia* hydrodistilled fraction, gave Twenty one (21) peaks, only Nineteen (19) of the peaks were identified as functional groups with the standard library. These Functional groups includes:-Alkyl halides [R-I], Alkynes [$\equiv\text{C-H}$], Alkenes [$\text{RCH}=\text{CR}-\text{R}$], Alcohols [$\text{C}-\text{O}-\text{H}$], Alkyl halide [R-F], Alcohol [$\text{RR}-\text{O}-\text{H}$], Ester acetate [$\text{O}=\text{C}-\text{O}-\text{C}$], Alkyls, Amides [$\text{C}=\text{O}$], Acyl chloride [$\text{R}-\text{C}(\text{O})-\text{Cl}$], Anhydrides [$\text{R}-\text{C}(\text{O})-\text{O}-\text{C}(\text{O})-\text{R}$], Carboxylic acids [$\text{O}-\text{H}$], Alkanes and Aromatic compound [$\text{ArC}-\text{H}$] as shown on table 4.12.

Table 4.12: Functional groups in Essential oil of *Citrus aurantifolia* Hydrodistilled Fraction.

PEAK NO	ABSORBANCE (nm)	CLASS OF COMPOUND	FUNCTIONAL GROUP	INTENSITY
1	438.82	Alkyl halide [R-I]	C-I Stretch	Strong
2	664.5	Alkynes	\equiv C-H Stretch	S.broad
		Alkyl halide[R-Br]	C-Br Stretch	Strong
3	759.98	Alkyl halide[R-Cl]	C-Cl Stretch	Strong
4	797.59	Alkenes[RCH=CR ₁ R ₂]	C-H bend	Strong
5	887.28	Alkenes[RR ₁ C=CH ₂]	=C-H bend	Strong
6	914.21	Alkenes[RCH=CH ₂]	C-H bend	M-Strong
7	955.76	Alkenes[RCH=CH ₂]	C-H bend	M-Strong
8	1018.45	Alkyl halide[R-F]	C-F Stretch	Very strong
9	1077.28	Alcohols [C=C—CH ₂]	C=O Stretch	M-Strong
		Alkyl halide[R-F]	C-F Stretch	Very strong
10	1151.54	Alcohol [RR ₁ R ₂ COH(3 ⁰)]	C-O Stretch	M-Strong
11	1236.41	Ester acetate	O=C-O-C Stretch	Very strong
12	1375.25	Alkyl	CH ₃ CH bend	Medium
13	1441.84	-	-	-
14	1645.33	Amides	C=O Stretch	S.broad
15	1782.21	Acyl chloride [R-C(O)-Cl]	C=O Stretch	Strong
		Anhydrides [R-C(O)-O-C(O)-R]	C=O symmetry	S.Strong
16	2363.84	-	-	-
17-18	2726.47-2837.38	Carboxylic acids	O-H Stretch	S-broad

19	2921.29	Alkanes	C-H Stretch	Strong
20-21	3010.02- 3078.49	Aromatic compound	ArC-H Stretch	Medium

The FTIR result for *Citrus aurantifolia* N-hexane fraction, gave Eighteen (18) peaks, only Fifteen (15) of the peaks were identified as functional groups with the standard library. These Functional groups includes:- Alkyl halides [R-I], Alkynes[≡C-H], Alkenes [RCH=CR□ R□ □], Aromatic compounds [polisubstitution], Alkenes[RR□ C=CH₂], Alcohol[C=C-CH₂-OH], Ethers [R-O-R□], Alkyls ,Amides[N-H], Alkanes and Carboxylic acids as shown on table 4.13.

Table 4.13: Functional groups in essential oil of *Citrus aurantifolia* N-hexane Fraction.

PEAK NO	ABSORBANCE (nm)	CLASS OF COMPOUND	FUNCTIONAL GROUP	INTENSITY
1	367.45	Alkyl halide [R-I]	C-I Stretch	Strong
2	612.42	Alkynes	\equiv C-H Stretch	S.broad
3	814.95	Alkenes [RCH=CR R R]	=C-H bend	Strong
		Aromatic p-disubst	C-H bend	Strong
4	890.18	Alkenes[RR C=CH ₂]	=C-H bend	Strong
5	1046.42	Alcohol[C=C-CH ₂ -OH]	C-O Stretch	M-Strong
6	1108.14	Ethers [R-O-R]	C-O-C Stretch	Strong
7	1148.65	Ethers [R-O-R]	C-O-C Stretch	Strong
8	1218.09	Ethers[Ar-O-R]	=C-O-C symmetry	M-Strong
9	1377.22	Alkyl S	CH ₃ C-H bend	Medium
10	1450.52	Alkyl S	C-H bend	Strong
11	1625.08	Amides	N-H bend	M-Strong
12	1731.17	Aldehydes [R-CH=O]	C=O Stretch	Strong
13	2087.05	-	-	-
14	2349.38	-	-	-
15	2864.39	Alkanes	C-H Stretch	Strong
		Carboxylic acids	O-H Stretch	S-broad
16	2925.15	Alkanes	C-H Stretch	Strong
		Carboxylic acids	O-H Stretch	S-broad
17	3457.52	Carboxylic acids	O-H Stretch	S-broad

18	3933.95	-	-	-
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The FTIR result for *Citrus limon* hydrodistilled fraction, gave Sixteen (16) peaks, only eleven (11) of the peaks were identified as functional groups with the standard library. These Functional groups includes:- Alkyl halides{[R-I] [R-Br] [R-Cl]}, Alkenes[R-R C=CH], Alcohols [RR-R-OH(³)], Amides[C=O], Carboxylic acids[O-H], alkanes[C-H] and Aromatic compound[ArC-H] as shown on table 4.14.

Table 4.14: Functional groups in essential oil of *Citrus limon* Hydrodistilled Fraction.

PEAK NO	ABSORBANCE (nm)	CLASS OF COMPOUND	FUNCTIONAL GROUP	INTENSITY
1	381.92	Alkyl halides[R-I]	C-I Stretch	Strong
2	544.91	Alkyl halide[R-Br]	C-Br Stretch	Strong
3	793.73	Alkyl halide[R-Cl]	C-Cl Stretch	Strong
4	892.11	Alkenes[R ¹ R ² C=CH ₂]	=C-H bend	Strong
5	1034.84	-	-	-
6	1135.15	Alcohols [RR ¹ R ² COH(3 ^o)]	C-O Stretch	M-Strong
7	1380.11	Alkyls	CH ₃ C-H bend	Medium
8	1442.8	-	-	-
9	1643.41	Amides	C=O Stretch	S-broad
10	1883.55	-	-	-
11	2062.94	-	-	-
12	2347.45	-	-	-
13	2738.05	Carboxylic acids	O-H Stretch	S-broad
14	2929.97	Alkanes	C-H Stretch	Strong
15	3098.75	Aromatic compound	ArC-H Stretch	Medium
16	3454.62	Carboxylic acids	O-H Stretch	S-broad

The FTIR result for *Citrus limon* N-hexane fraction, gave Eleven (11) peaks, only Ten (10) of the peaks were identified as Functional groups with the standard library. These Functional groups includes:-Alkyls, Alkyl halides {[R-I] [R-F] [R-Cl]}, Alkenes [R-R C=CH], Alcohols [RR-R-OH(3⁰)], Amides [C=O], Carboxylic acids [O-H], alkanes [C-H] and Aromatic compound [ArC-H], Amide as shown in chromatogram Table 4.15.

Table 4.15: Functional groups in Essential Oil of *Citrus Limon* N-hexane Fraction.

PEAK NO	ABSORBANCE (nm)	CLASS OF FUNCTIONAL COMPOUND	OF FUNCTIONAL GROUP	INTENSITY
1	380.95	Alkyl halides[R-I]	C-I Stretch	Strong
2	440.75	Alkyl halides[R-I]	C-I Stretch	Strong
3	807.24	Alkyl halide[R-Cl]	C-Cl Stretch	Strong
		Alkene trans [RCH=CHR]	=C-H bend	Strong
4	895.0	Alkenes[R R C=CH]	=C-H bend	Strong
5	1044.49	Alkyl halides[R-F]	C-F Stretch	V. Strong
		Alcohols [C=C-CH ₂ -OH]	C-O Stretch	M-Strong
6	1374.33	Alkyl	CH ₃ C-H bend	Medium
7	1453.41	Alkyl	C-H bend	Strong
		Aromatic compounds	Ring C=C	M-Strong
8	1599.04	Amines	N-H bend	W-medium
		Amides	N-H bend	M-Strong
9	2358.06	-	-	-
10	2863.42	Alkanes	C-H Stretch	Strong
11	2924.18	Alkanes	C-H Stretch	Strong

4.4. Anthelmintics Effects of Essential Oils from *Citrus aurantifolia*, *Citrus limon* and *Citrus sinensis* Fruit Peels.

4.4.1 Lethal concentrations (LC₅₀ and LC₉₀)

The lethal concentration (LC) at 50% and 90% for larvacidal and ovicidal activities of essential oil from the citrus fruit peels are shown in Tables 4.16 and 4.17 respectively. Larvacidal and ovicidal potential is directly proportional to lethal concentration value. LC₅₀ and LC₉₀ mortality for larvacidal and ovicidal activity of hydrodistilled fractions is less than that of N-hexane fractions for all citrus species.

The lethal concentration at 50% inhibition for ovicidal activity is higher for hydrodistilled fractions than those of N-hexane fractions while lethal concentration is greater for N-hexane fractions than the hydrodistilled fractions at 90% inhibition, for all citrus species with the exception of *Citrus sinensis*. The least LC₅₀ and LC₉₀ (in µg/ml) were recorded for hydrodistillate fractions of *C. sinensis* (0.089 µg/ml) and *C. limon* (6.249 µg/ml) respectively. On the other hand, greatest LC₅₀ and LC₉₀ (in µg/ml) were recorded for N-hexane fractions of *C. limon* (0.147 µg/ml) and *C. limon* (34.399 µg/ml) respectively. The ovicidal and larvicidal activities show significance activities in all for both hydro distilled and N-hexane fractions after Thirty (30) minutes of administration.

Table 4.16: Lethal Concentrations at 50 and 90 percent of essential oils from three species of *Citrus* peels and peperazine citrate against *Ascaridia galli* larvae

Group	LC ₅₀ (ug/ml)	LC ₉₀ (µg/ml)	Regression equation
SD	0.400	0.863	y=0.8259x-8.941
NC	0.00	0.00	
<i>C. sinensis</i> H	0.801	1.314	y = 0.4086x - 3.9338
<i>C. sinensis</i> N	0.403	0.985	y = 0.9508x - 9.6941
<i>C. aurantifolia</i> H	0.515	1.395	y = 0.3428x - 3.3995
<i>C. aurantifolia</i> N	0.414	0.644	y = 0.7976x - 8.4796
<i>C. limon</i> H	0.066	0.215	y = 17.68x - 205.74
<i>C. limon</i> N	0.236	0.732	y = 94.431x - 1101.9

NC=Normal control, *C. sinensis* H=*Citrus sinensis*-hydrodistilled fraction, *C. sinensis* N= *Citrus sinensis*-N-hexane fraction, *C. aurantifolia*.H =*Citrus aurantifolia*-hydrodistilled fraction, *C. aurantifolia*.N= *Citrus aurantifolia* -N-hexane fraction,*C. limon* H= *Citrus limon*-hydrodistilled fraction, *C. limon* N=*Citrus limon* N-hexane fraction,SD=standard drug-(peperazine citrate)

Table 4.17: Lethal concentration at 50 and 90 percent of essential oils from three species of peels and peperazine citrate against eggs of *Ascaridia galli*

Group	LC ₅₀ (ug/ml)	LC ₉₀ (ug/ml)	Regression equation
SD	0.286	0.703	y=2374.0x-531.03
NC	0.000	0.000	
<i>C. sinensis</i> H	0.890	1.740	y = 2467.9x - 551.03
<i>C. sinensis</i> N	0.217	0.529	y = 821.77x - 181.56
<i>C. aurantifolia</i> H	0.330	0.624	y = 21.093x - 4.3307
<i>C. aurantifolia</i> N	0.194	0.344	y = 18.678x - 215.49
<i>C. limon</i> H	0.172	0.712	y = 13.602x - 156.62
<i>C. limon</i> N	0.147	0.750	y = 8509.1x - 101112

NC=Normal control, *C.sinensis*H=*Citrus sinensis*-hydrodistilled fraction, *C.sinensis* N= *Citrus sinensis*-N-hexane fraction, *C. aurantifolia*. H= *Citrus aurantifolia*-hydrodistilled fraction, *C. aurantifolia* N= *Citrus aurantifolia* -N-hexane fraction,*C. limon* H= *Citrus limon*-hydrodistilled fraction, *C. limon* N=*Citrus limon* N-hexane fraction, SD=standard drug-(peperazine citrate)

4.4.2 Ovicidal effects of individual essential oils from *Citrus sinensis* peels on hatchability of *Ascaridia galli* eggs

Comparative ovicidal effects of essential oils of citrus peels specifically from hydrodistilled and N-hexane fraction of *Citrus sinensis* at concentrations of 0.1, 0.3 and 0.5 µg/ml are displayed in Figure 4.2. The result indicates a significant ($p \leq 0.05$) increase in percentage (%) ovicidal effects in all treated groups compared to the normal control at all concentrations. The ovicidal activity of all treated groups was significantly ($p \leq 0.05$) greater than that of Normal control

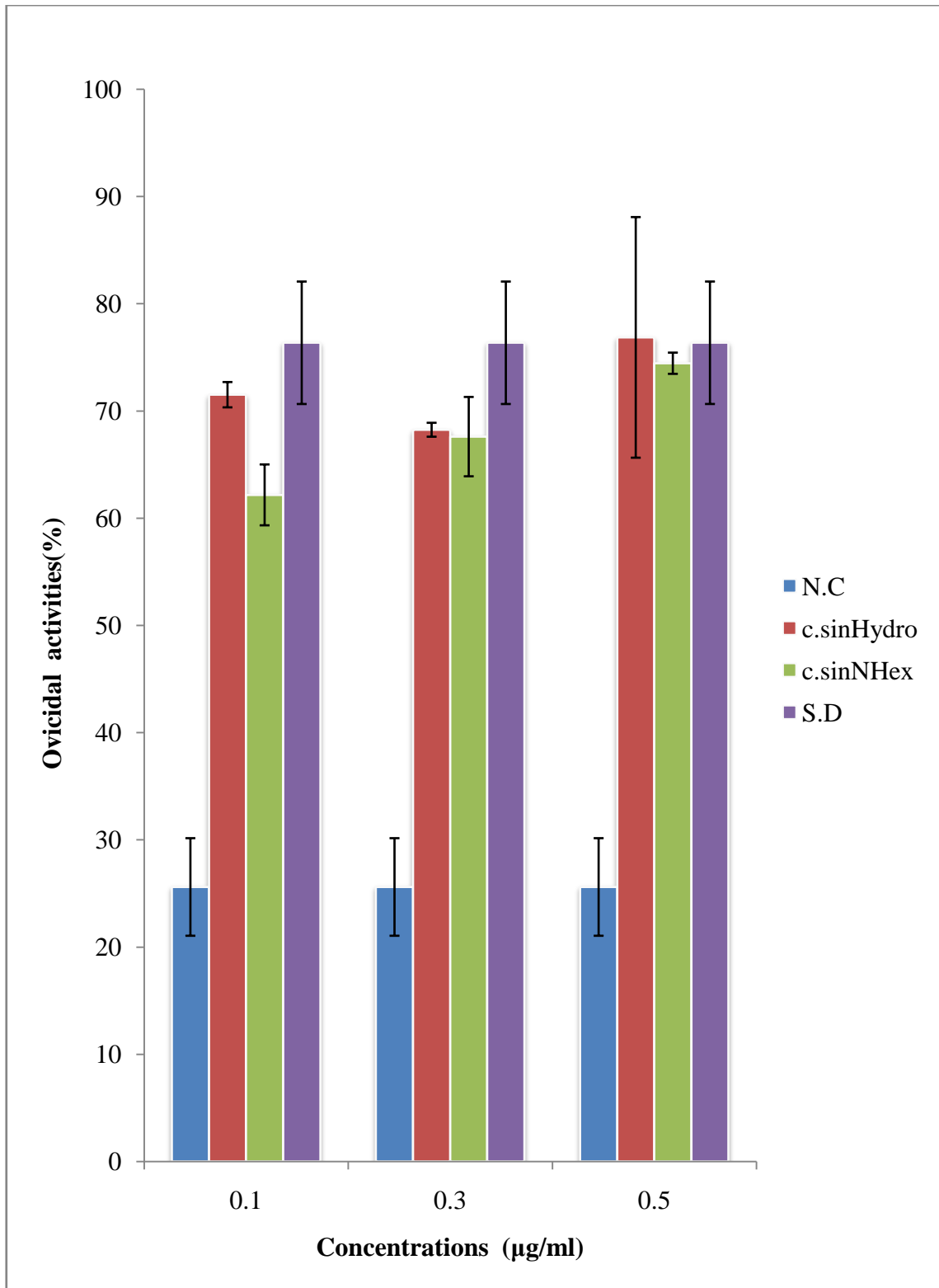


Figure 4.2. Ovicidal activities of essential oil of *Citrus sinensis* Peels on eggs of *Ascaridia galli*.

NC=Normal control, SD=standard drug, *C. sinensis* H =*Citrus sinensis* hydrodistilled fractions, *C. sinensis* N =*Citrus sinensis* hexane fraction

The comparative ovicidal effects of essential oils of citrus *aurantifolia* peels specifically from hydrodistilled and N-hexane fractions at concentrations of 0.1, 0.3 and 0.5 $\mu\text{g/ml}$ are shown in Figure 4.3. The result indicates a significant ($p \leq 0.05$) increase in % ovicidal effects in all treated groups compared to the normal control at all concentrations. The ovicidal activity of all treated groups was significantly ($p \leq 0.05$) greater than that of Normal control. In all, there was an increasing level of activities across the concentrations showing that an activity is directly proportional to the concentrations. The ovicidal effect was higher in the hydrodistilled fractions than in the N-hexane fraction, although there were higher activities in concentration 0.3 and of 0.5 compared to that of standard drug Figure 4.3.

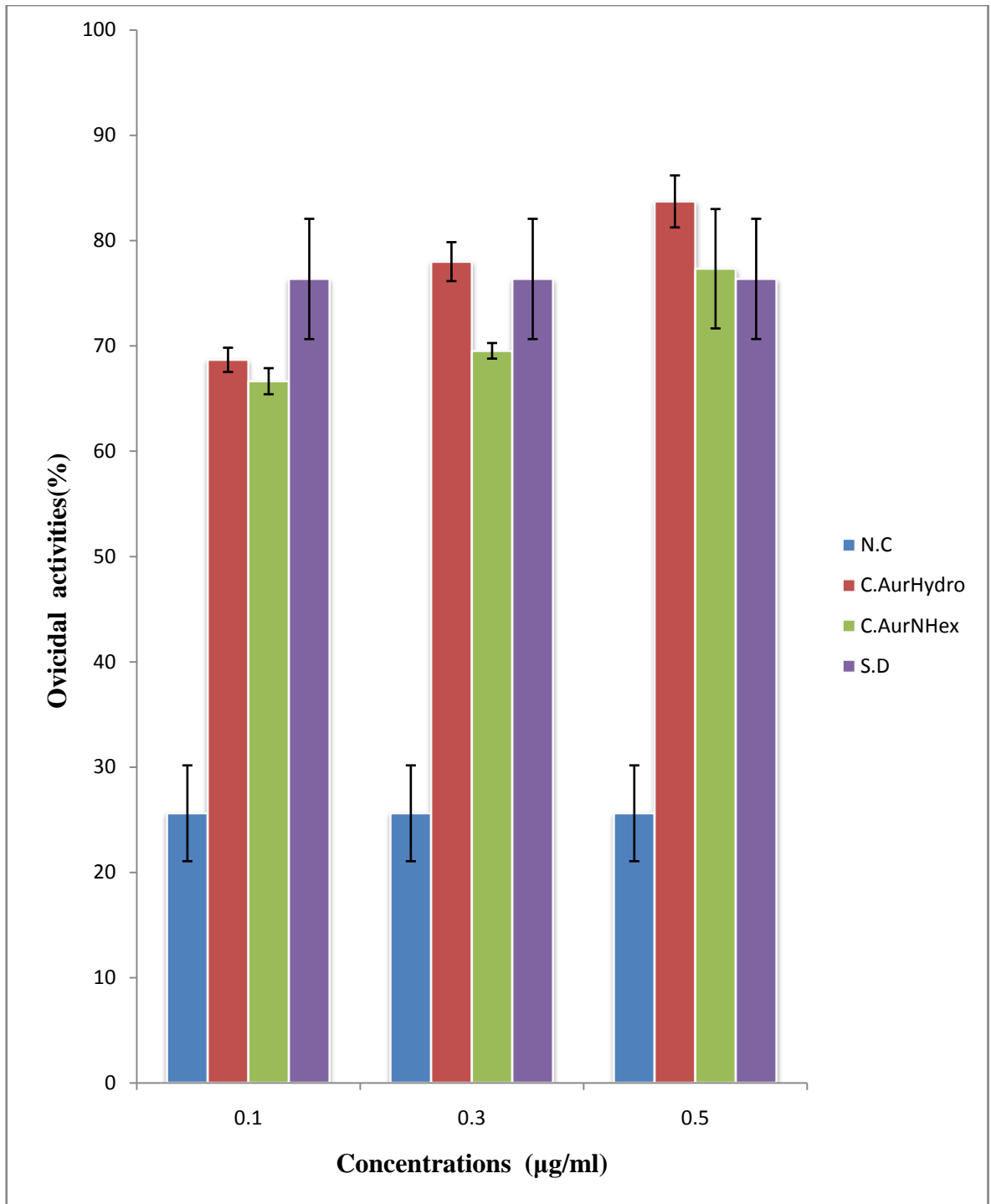


Figure 4.3. Ovicidal activities of essential oil of *Citrus aurantifolia* peels on egg of *Ascaridia galli*.

NC=Normal control, SD=standard drug, *C. aurantifolia* H =*citrus aurantifolia* hydrodistilled fractions, *C. aurantifolia* N = *Citrus aurantifolia* hexane fraction

The comparative ovicidal effects of essential oils of *Citruslimon* peels specifically from hydrodistilled and N-hexane fractions at concentrations of 0.1, 0.3 and 0.5 $\mu\text{g/ml}$ is shown in Figure 4.4. The result indicates a significant ($p \leq 0.05$) increase in % ovicidal effects in all treated groups compared to the normal control at all concentrations. The ovicidal activity of all treated groups was significantly ($p \leq 0.05$) greater than Normal control. In all, there was an increasing level of activities across the concentrations showing that an activity is directly proportional to the concentrations. The ovicidal effect was higher in the hydrodistilled fractions than in the N-hexane fraction Figure 4.4.

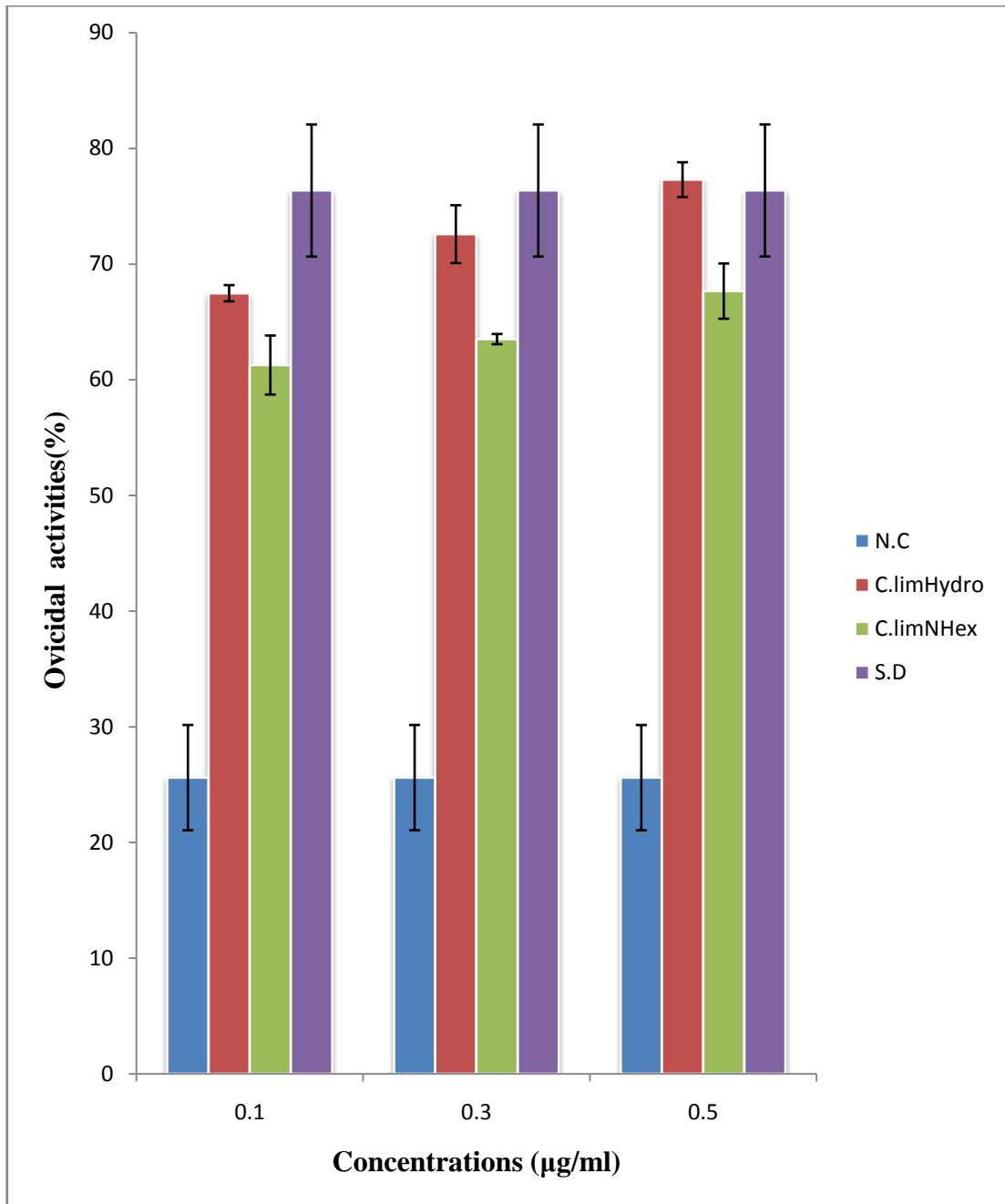


Figure 4.4: Ovicidal activities of essential oil of citrus limon peels on egg of *Ascaridia galli*.

NC=Normal control, SD=standard drug, *C. limon H* =*Citrus limon* hydrodistilled fractions, *C. limon N* =*Citrus limon* hexane fraction

4.4.3 Combine ovicidal effect of individual samples on hatchability of *Ascaridia galli* eggs.

The table of ovicidal effects of essential oil from citrus peels on *Ascaridia galli* is shown in Appendix 1. The result indicates a significant ($p \leq 0.05$) increase in percentage (%) ovicidal effects in all treated groups compared to the normal control and standard drugs at all concentrations, there was a significant ($p \leq 0.05$) increase in ovicidal activity of all treated groups compared to the normal control. At concentration of 0.1 $\mu\text{g/ml}$, there was no significant ($P > 0.05$) difference in ovicidal activity of groups treated with hydrodistillate fraction of *Citrus sinensis* compared to the standard drug. At concentration of 0.3 $\mu\text{g/ml}$, there was no significant ($p \leq 0.05$) difference in groups treated with hydrodistillate fractions of *Citrus aurantifolia* and *Citrus limon* compared to normal control.

In all concentrations, *Citrus sinensis*-hydrodistilled fraction, *Citrus limon*-hydrodistilled and *Citrus aurantifolia*-hydrodistilled and N-hexane fraction *Citrus limon*-hydrodistilled exhibits the highest ovicidal activities respectively followed by *Citrus aurantifolia*-hydrodistilled fraction in concentration 0.1 $\mu\text{g/ml}$, *Citrus aurantifolia*-N-hexane fraction in concentration 0.3 $\mu\text{g/ml}$ and *Citrus aurantifolia*-hydrodistilled fraction in concentration 0.5 $\mu\text{g/ml}$ respectively while *Citrus limon* N-hexane fraction exhibits the lowest ovicidal activities in all the concentrations respectively. There was no significant difference ($p \leq 0.05$) in ovicidal activity of all groups treated with fractions compared to the group treated with standard drug at when 0.5 $\mu\text{g/ml}$ of essence was administered.

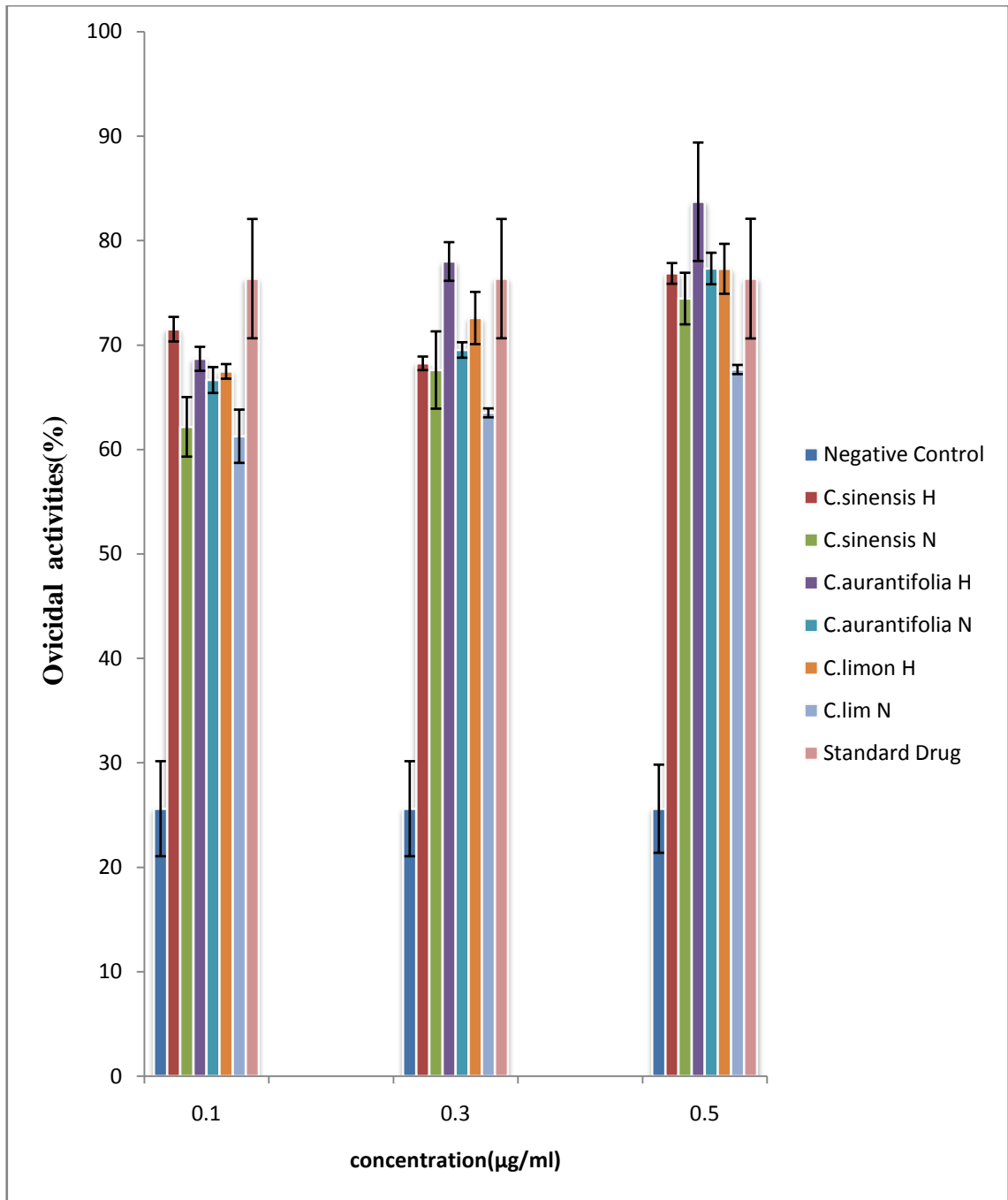


Figure 4.5 Ovicidal activities of essential oil of *Citrus sinensis*, *Citrus aurantifolia*, *Citrus limon* peels and peperazine citrate against the eggs of *Ascaridia gali*.

NC= Normal control, *C. sinensis*H = *Citrus sinensis*-hydrodistilled fraction, *C. sinensis* N = *Citrus sinensis*-N-hexane fraction, *C. aurantifolia*.H = *Citrus aurantifolia*-hydrodistilled fraction, *C. aurantifolia*.N = *Citrus aurantifolia* -N-hexane fraction,, *C. limon* H= *Citrus limon*-hydrodistilled fraction, *C. limon* N=*Citrus limon* N-hexane fraction,SD=standard drug-(peperazine citrate)

4.4.4 Larvicidal effects of individual citrus essential oil on the larvae of *Ascaridia galli*

The comparative larvicidal effects of Citrus peels essential oil from hydrodistilled and N-hexane fractions of *Citrus sinensis* at concentrations of 0.1, 0.3 and 0.5 ($\mu\text{g/ml}$) Figure 4.6. The result indicates a significant ($p \leq 0.05$) increase in percentage larvicidal effects in all treated groups compared to the normal control at all concentrations.

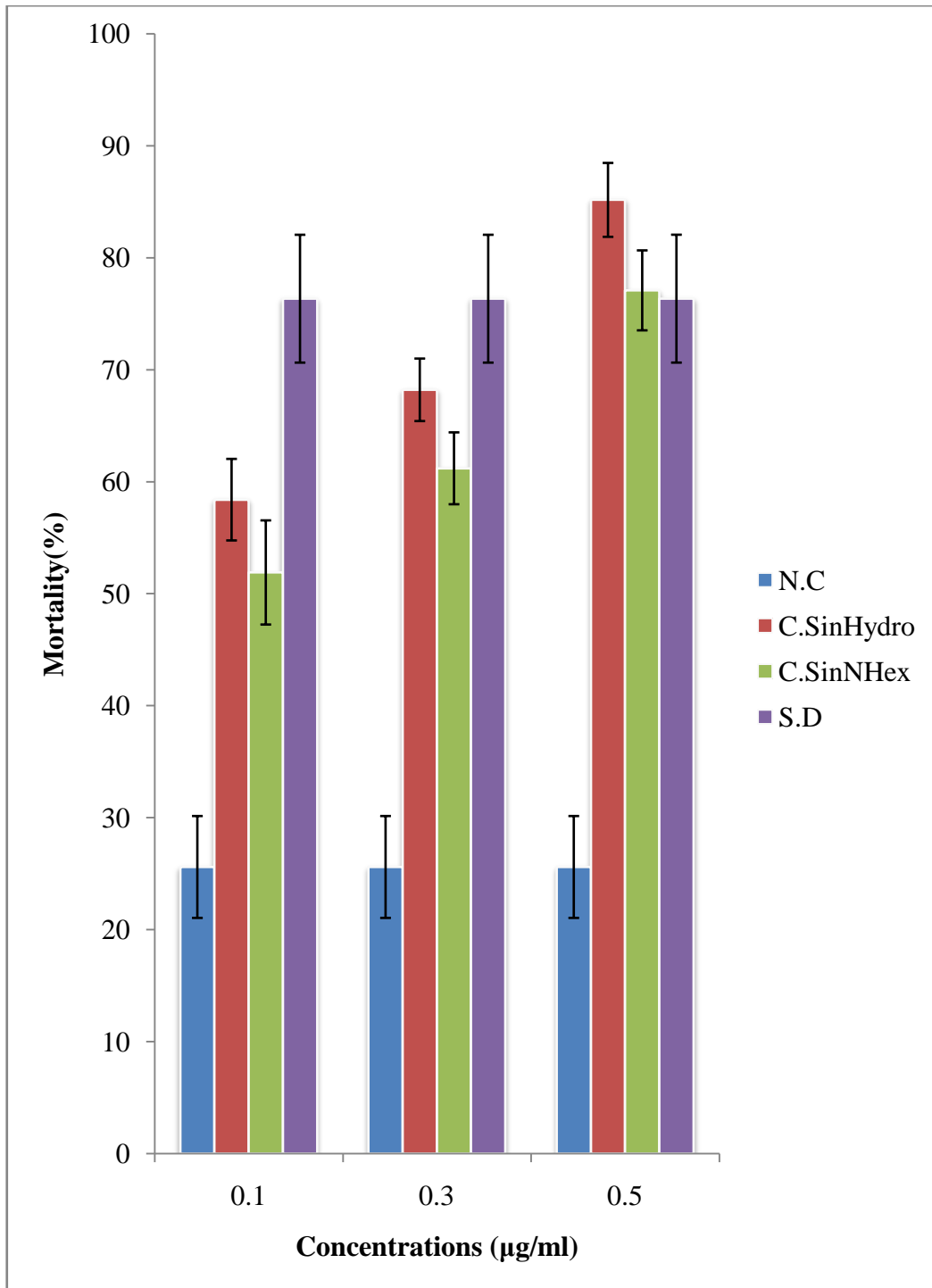


Figure 4. 6 Larvicidal activities of essential oil of some *Citrus sinensis* peels on the larvae of *Ascaridia galli*

C. sinensis H =*Citrus sinensis* hydrodistilled fractions, *C. sinensis* N =*Citrus sinensis* N-hexane fraction NC=Normal control, SD=standard drug.

The comparative larvicidal effects of essential oils of citrus peels specifically from hydrodistilled and N-hexane fraction of *Citrus aurantifolia* at concentrations 0.1, 0.3 and 0.5($\mu\text{g/ml}$) shown on Figure 4.7. The result indicates a significant ($p\leq 0.05$) increase in percentage larvicidal effects in all treated groups compared to the normal control at all concentrations.

The larvicidal activity of all treated groups was significantly ($p\leq 0.05$) greater than that of Normal control while the effects in concentrations 0.3 and 0.5 $\mu\text{g/ml}$ were higher than the standard drug with 0.5 $\mu\text{g/ml}$ having the highest activity.

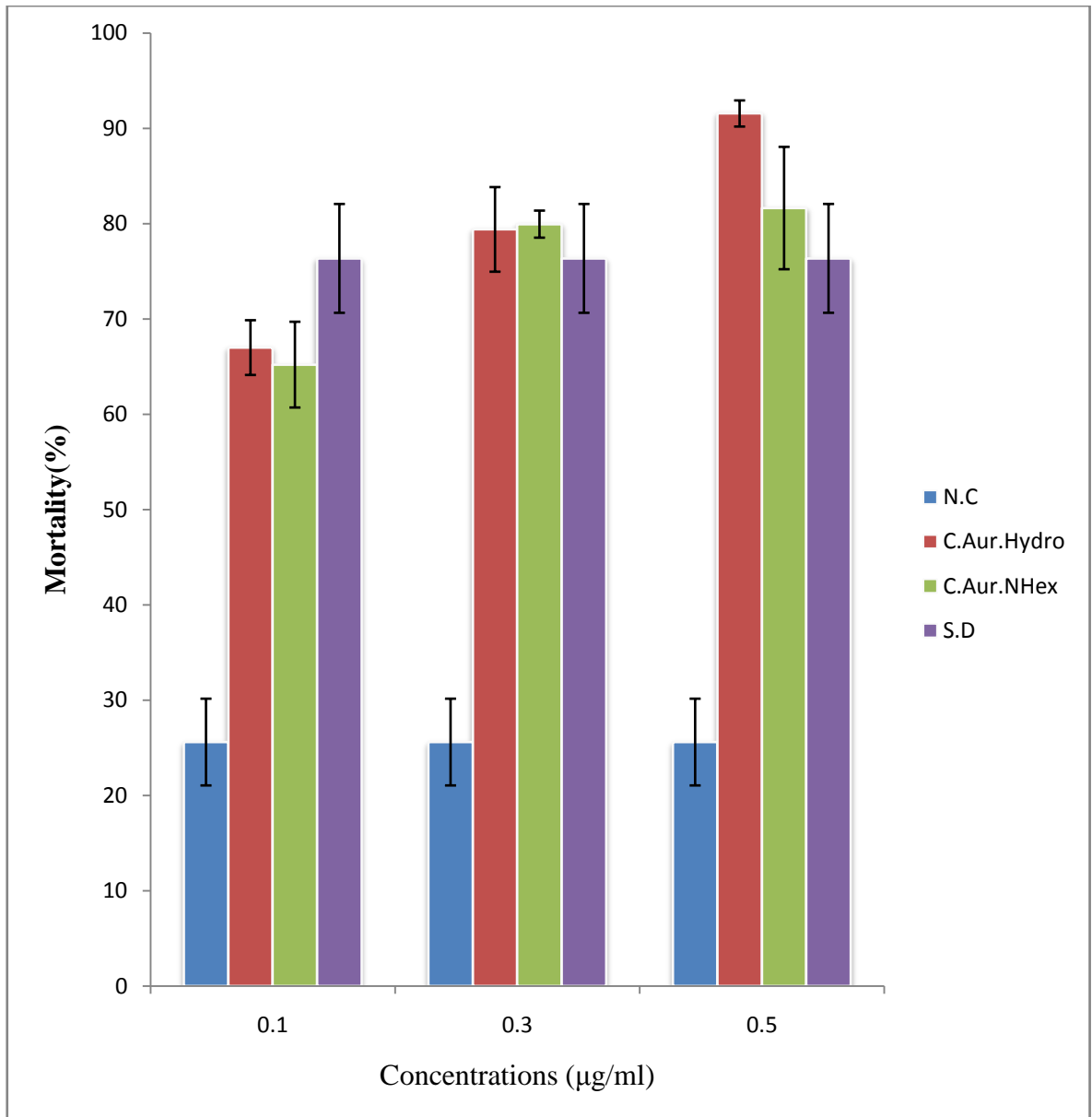


Figure 4.7. Larvicidal activities of essential oil of *Citrus aurantifolia* peels on the larvae of *Ascaridia galli*

C. aurantifolia H =*Citrus aurantifolia* hydrodistilled fractions, *C. aurantifolia* N =*Citrus aurantifolia*N-hexane fraction NC=Normal control, SD=standard drug,

The comparative larvicidal effects of essential oils of citrus peels specifically from hydrodistilled and N-hexane fraction of *Citrus limon* at concentrations of 0.1, 0.3 and 0.5 ($\mu\text{g/ml}$) are shown on Figure 4.8. The result indicates a significant ($p \leq 0.05$) increase in % larvicidal effects in all treated groups compared to the normal control at all concentrations.

The larvicidal activity of all treated groups was significantly ($p \leq 0.05$) greater than that of Normal control while there was a steady increase in level of activities across the concentrations showing that an activity is directly proportional to the concentrations. In *Citrus limon* the larvicidal effects was higher in the hydrodistilled fractions than in the N-hexane fraction and it was seen that concentration 0.5 $\mu\text{g/ml}$ was even higher compared to that of the standard drug.

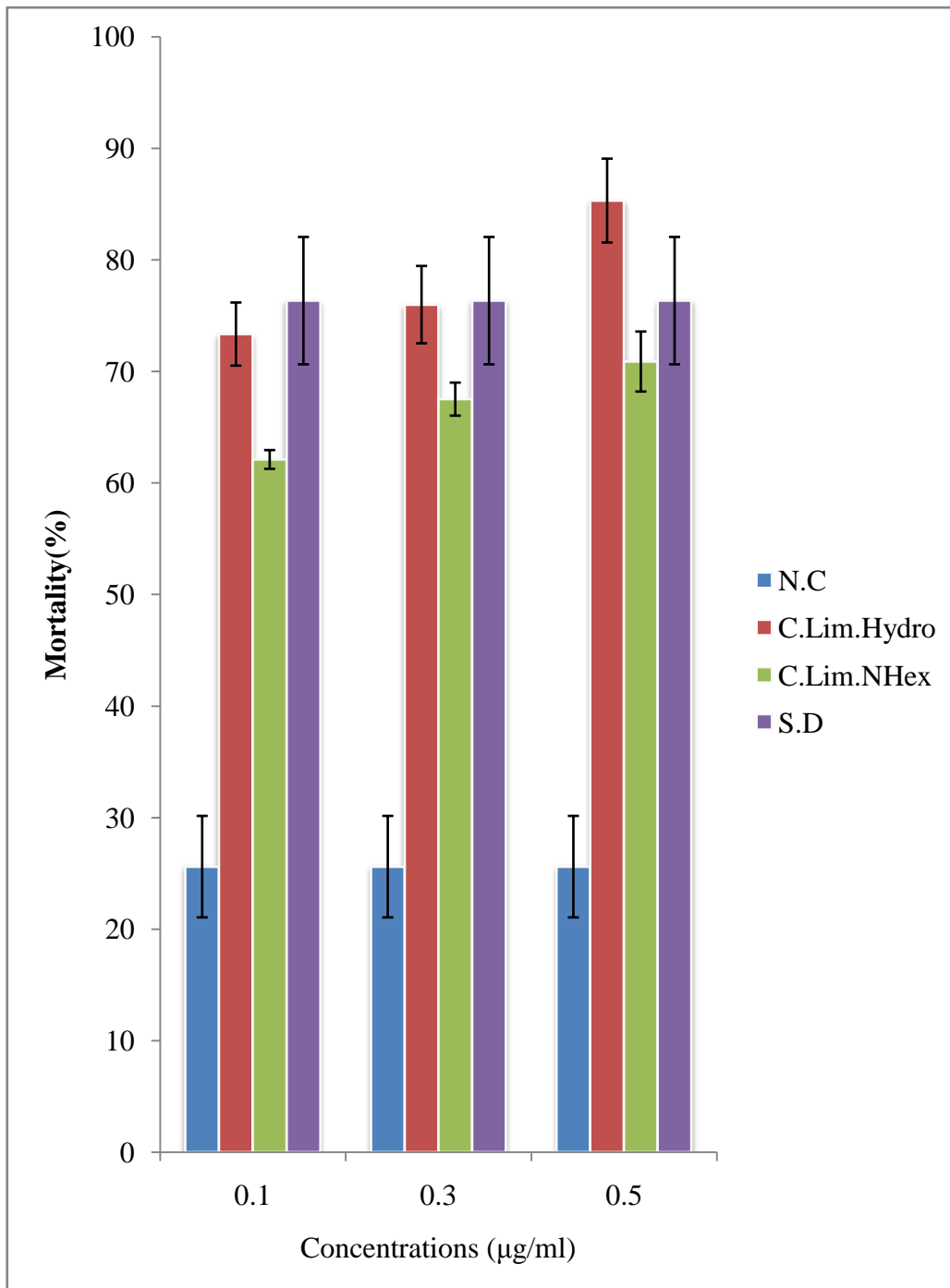


Figure 4. 8. Larvicidal activities of essential oil of *Citrus limon* peels on the larvae of *Ascaridia galli*

C. limon H = *Citrus limon* hydrodistilled fractions, *C. limon* N = *Citrus limon*, N-hexane fraction NC=Normal control, SD=standard drug,

4.4.5 Combine larvicidal effects of individual *Citrus* essential oils against the larvae of *Ascaridia galli*

The larvacidal effects of essential oils of citrus peels at concentrations of 0.1, 0.3 and 0.5 µg/ml are shown on Appendix 2. The result indicates a significant ($p \leq 0.05$) increase in % percentage larvicidal effects in all treated groups compared to the Normal control at all concentrations. The larvicidal activity of all treated groups was significantly ($p \leq 0.05$) greater than that of Normal control

At concentrations 0.1, 0.3 and 0.5 µg/ml, *Citrus limon* hydrodistilled in concentration 0.1 µg/ml, *Citrus aurantifolia* hydrodistilled, *Citrus aurantifolia* N-hexane and *Citrus limon*-hydrodistilled in concentration 0.3 µg/ml exhibits the high larvicidal activities respectively while *Citrus sinensis* hydrodistilled fraction, *Citrus sinensis* N-hexane fraction, *Citrus aurantifolia* hydrodistilled fraction, *Citrus aurantifolia* N-hexane fraction *Citrus limon* hydrodistilled fraction, exhibit larvicidal activities greater than the standard drug, followed by *Citrus aurantifolia*, *Citrus limon* and *Citrus aurantifolia*-hydrodistilled fractions respectively while *Citrus limon* N-hexane fraction exhibits the lowest larvicidal activities in all the concentrations. as also shown in Figure 4.9.

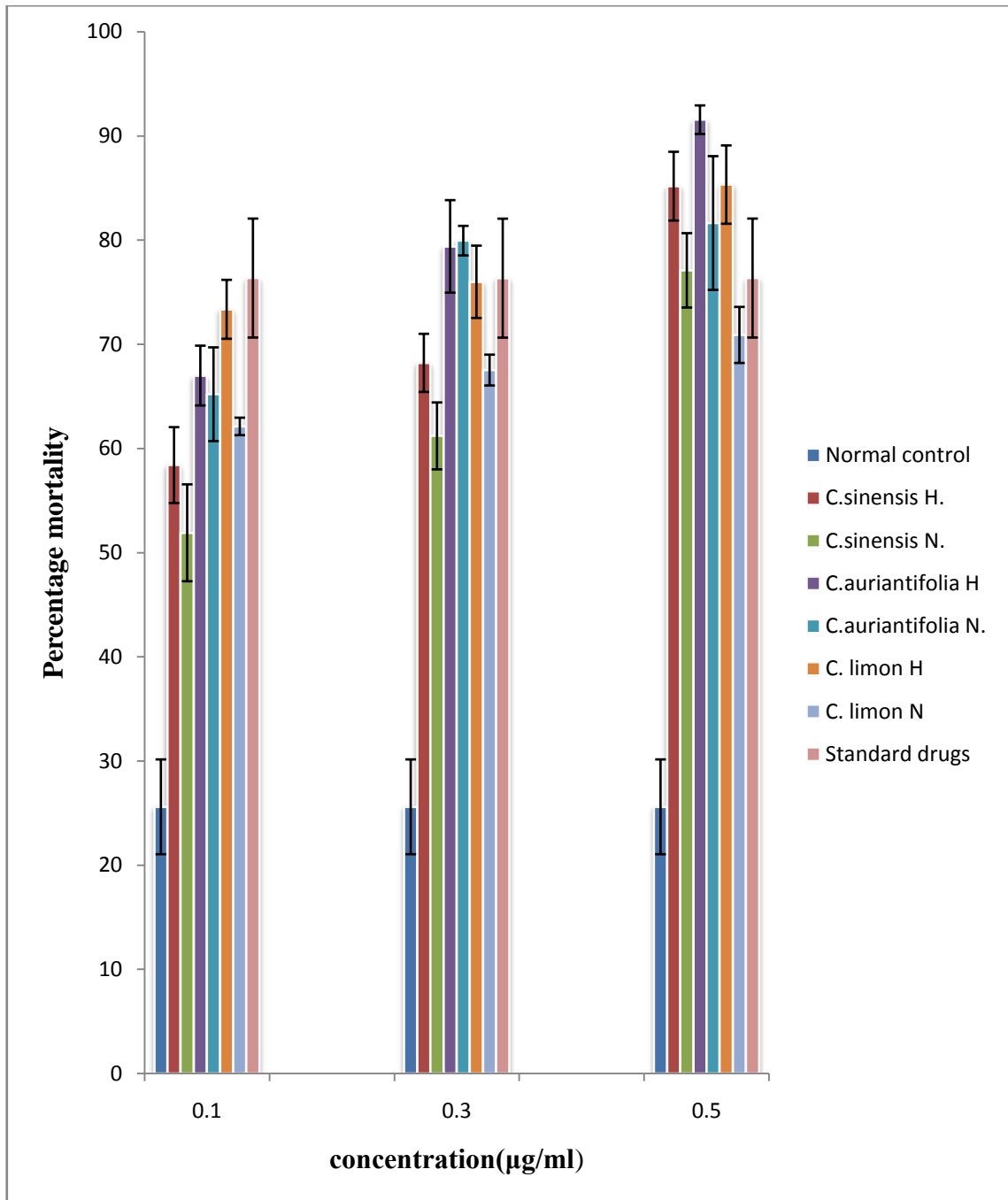


Figure 4.9 Larvicidal activities of essential oils from *Citrus sinensis*, *Citrus aurantifolia*, *Citrus limon* peels and peperazine citrate against the L3 larvae of *Ascaridia gali*.

NC=Normal control, C. *sinensis* H=*Citrus sinensis*-hydrodistilled fraction, C. *sinensis* N=*Citrus sinensis*-N-hexane fraction, C. *aurantifolia*.H= *Citrus aurantifolia*-hydrodistilled fraction, C. *aurantifolia*.N= *Citrus aurantifolia* -N-hexane fraction, C. *limon* H= *Citrus limon*-hydrodistilled fraction, C. *limon* N=*Citrus limon* N-hexane fraction, SD=standard drug-(peperazine citrate)

4.4.6 Microscopic View of Anthelmintic Activities of Citrus Essential oil on Eggs and larvae of *Ascaridia galli*

Anthelmintic activity of Essential Oils, from *Citrus sinensis*, *aurantifolia* and *limon* L., fruit peels of hydrodistilled and N-hexane fractions were evaluated (plate 4.10). Each concentration of essential oils obtained from different plant species exhibited varied anthelmintic activities. Change in the colour of the dead, disruption of developmental processes in larvae, eggs, and eggs hatching inhibition were indicative parameters of the Essential Oils (*Citrus sinensis*, *aurantifolia* and *limon* L) harmful potentials on the membrane of larvae and eggs of the helminth. Some of the physical parameters are shown in plate 4.10

Before Administration of essential oil. After Administration of essential oil.

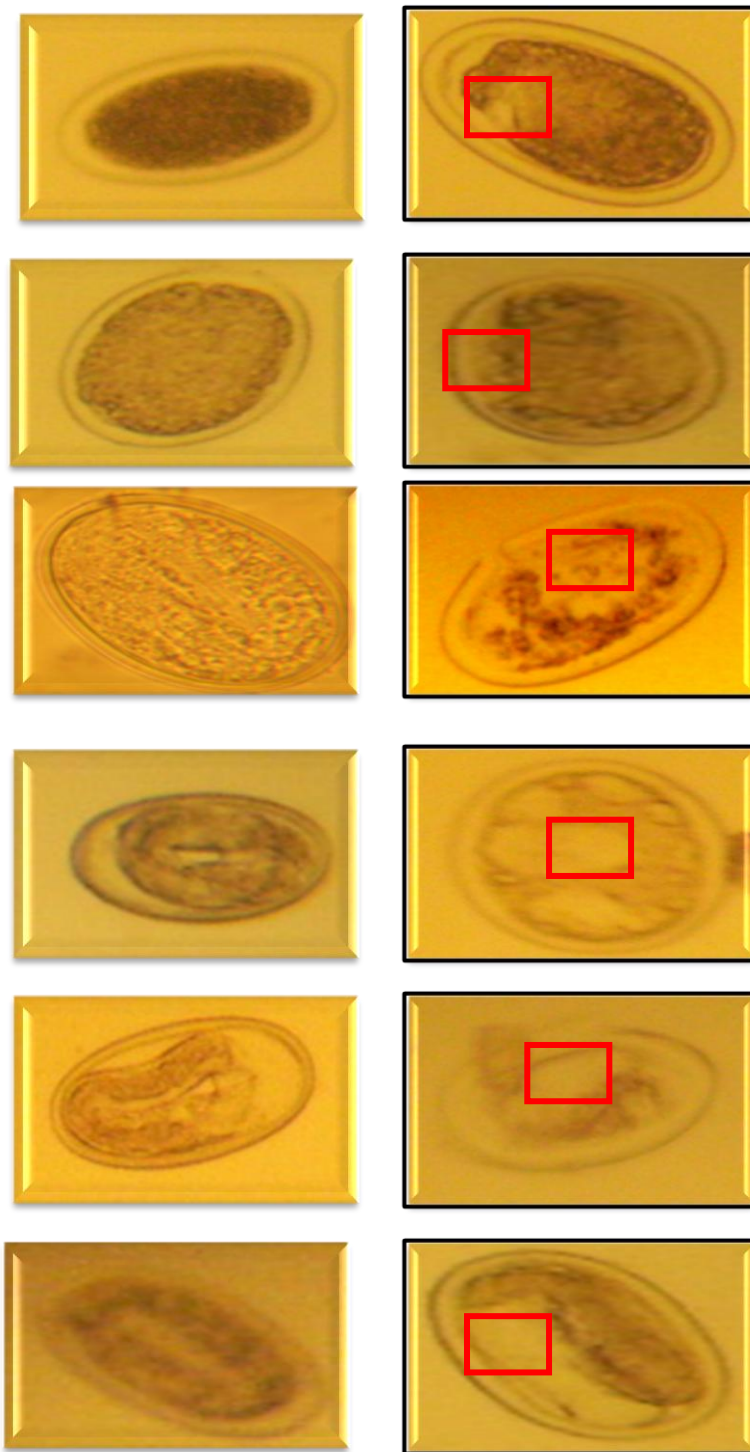


Plate 4.1: Microscopic views of effect of Essential Oils of *C.sinensis*, *C.aurantifolia*, *C.limon* on Eggs and larvae of *Ascaridia Galli* before and after administration

CHAPTER FIVE

5.0 DISCUSSION

Essential oil from hydrodistilled fraction gave the maximum yield as seen in *C.aurantifolia* 2.1% and *C.sinensis* had the lowest with 1.5%. These yields agreed with the findings of Tue *et al.* (2002), they reported that yield of *Citrus* essential oils differed with individual plant species ranging in most of the cases from 0.2-2.0%. Although it differs for n- hexane fraction which has maximum and minimum percentage yields from *Citrus auriantifolia* 10%, and *C.sinensis*6%,the yield agreed with the findings of Tue *et al.* (2002), it reported that yield of *Citrus* essential oils differences also depends on the method employed in extraction of essential oil, a findings which was supported in work done by Ncube *et al.* (2008),which posited that variations in different extraction methods affect quantity and quality of secondary metabolite composition of an extract, these depend upon: type of extraction,time of extraction, temperature, nature of solvent, solvent concentration and polarity (Ncube *et al.*, 2008).

In general, N-hexane Fractions of *Citrus* peels had higher yield than hydrodistilled fractions it follows the review work of Prashant *et al.*(2011) that cited Cowan (1999) who reported that terpenoids are more concentrated in hexane, chloroform and methanol phase while occasionally tannins and terpenoids were found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Prashant *et al.*,2011).The yields, content and composition of essential oils of *Citrus* species were significantly affected by treatments and several other parameters including seasonal variation (Guedes *et al.*, 2004), phenological cycle (Schwob *et al.*, 2004), geographic distribution and methods of oil extraction.

Essential oils from citrus flavedo composed mainly of volatile oils and these phytochemicals possessed anthelmintic properties, Substantial presence of these phytochemicals support the display of anthelmintics properties of the three citrus essential oils from both fractions as confirmed by the works of Camurça-Vasconcelosa *et al.* (2007), it revealed the presence of volatile oil as one of the chemical constituent of essential oil and shown that these phytochemicals possess anthelmintic activities against worms (Sollmann, 1918;Pessoaa *et al.*, 2002; Camurça- Vasconcelosa *et al.*, 2007). Singh and Malhotra (2007) posited that the lipophilic compounds have the ability of disrupting the membrane and transcutaneous penetration into the body, there by may uncouple oxidative phosphorylation, inhibition of enzymes, they can inhibit the glucose uptake and deplete the glycogen content in *worms* or interaction of volatile oils with glycolytic enzymes and succinic acid production in worm and activated nicotinic cholinergic receptor in the worms resulting in either persistent depolarization or hyperpolarization could be the possible mechanism of action as agreed by Martinez *et al.* (2008), who reported that sesquiterpene isolated from *Butea monosperma* has anthelmintic activity with possible mode of action as inhibition of glucose uptake and depletes the glycogen content in *Ascaridia galli*. It further added that terpenoids,steroids, sterols, and cardiac glycosides., are major components of many essential oils,they can also act as irritants when applied externally or consumed internally and that a number of them broadly have antimicrobial (particularly antiprotozoal) and neurotoxic action (Martinez *et al.*, 2008).

Many other works confirmed the anthelmintics properties of these phytochemicals in essential oils thus: Alkaloids are these nitrogenous compounds which function as Anthelmintic by diminishing the support of glucose to the helminthes and acts on central nervous system (CNS) causing paralysis. Saponins Possesses membrane

permeabilizing properties, Leads to vacuolization and disintegration of teguments while they also pointed out that Saponins are extremely poisonous and cause irritation to mucous membranes, as they cause hemolysis of cell wall. Terpenoids act through Membrane disruption, Polyphenols and Tannins Bind to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption and metal ion complexation (Kar, 2007; Mali *et al.*, 2007; Cruz, 2008; Bachaya *et al.*, 2009; Mute, 2009; Shaibani *et al.*, 2009; Maniyar *et al.*, 2010; Patel *et al.*, 2010; Roy, 2010; Sharma *et al.*, 2010; Sutar *et al.*, 2010; Vidyadhar *et al.*, 2010; Wang *et al.*, 2010).

The FTIR result from the six fractions: *Citrus sinensis*, *Citrus aurantifolia* and *Citrus limon* hydrodistilled and N-hexane fractions showed, similar functional groups Alkyl halide (R-I), Alkenes (RCH=CR¹R²), Ethers (A-O-R), Alcohols [RR¹R²OH] (3⁰), Alkyls, Aromatic compounds, Amides [N-H], Acyl chlorides [R-C(O)-Cl], Aldehydes [R-C(O)-O-C(O)-R], Nitriles (R-C≡N), Carboxylic acids and Alkanes, Amines [R-NH₂] although they have different stretch and distribution patterns as shown in Table 4.3-4.8. The Functional groups pattern in the sample serve as a pointer to significant anthelmintic potentials of citrus essential oils, confirmed by earlier work done by Griffin *et al.*, (1999), and Kar, (2007), who reported that Tannins are acidic in reaction property that is attributed to the presence of phenolics or carboxylic group which form complexes with proteins, carbohydrates, gelatin and alkaloids (Kar, 2007).

Besides being active, ketones, aldehydes and alcohols have different specificity and activity levels. It points to the multifunctional nature of the essential oils. It confirmed the existing relationship between the compounds' chemical structure and the anthelmintic activities they exert (Griffin *et al.*, 1999). Several data suggest that great antimicrobial potential could be ascribed to the oxygenated terpenes because of the

functional groups and bonding pattern (Panizi *et al.*, 1993; Adam *et al.*, 1998; Saroglou *et al.*, 2007).

The anthelmintic property of plants is dependent on numerous substances that are found in them. These could be alkaloids, sugars, saponins, aromatic oils, resins and other medicinally useful chemicals (Lejoly *et al.*, 1996). Oryema (1997) reported that substances like steroids, coumarins, tannins, and triterpoids and other chemical constituents of plants like alkaloids, glycosides, enzymes, anthraquinones, gums, fixed oils, fats, waxes, volatile oils, proteins and carbohydrates all have medicinal or pharmaceutical values.

GC-MS analysis indicated that, the essential oils mainly contained monoterpene hydrocarbons, with significantly varied chemical composition in relation to *Citrus* species these are linked to the differences in their genetic makeup as well as the oil extraction regimes employed. In general, the compositions chemical components, of *Citrus* essential oils analyzed from Nigeria citrus peels were quite comparable with *Citrus peels* oil reported from other regions of the world; however in some cases a notable variation in the composition of oils was also observed, this is in agreement with the earlier studies which revealed considerable variation in the chemical compositions of peels essential oil with respect to varieties and extraction conditions (Laranja *et al.*, 2003; Asekun *et al.*, 2006; Asekun *et al.*, 2007a; Asekun *et al.*, 2007b).

In the analysis, limonene and β -myrcene were determined to be the two major components in the *C. sinensis* peel essential oil. Similar to the findings in the work of Lota *et al.*, (2000) which examined limonene and γ -terpinene as the two major monoterpenes in *Citrus sinensis* peels essential oil. Feger *et al.*, (2003) reported that limonene was the major component in peel oils from commercial Brazilian Murcot

Tangerines. Choi and Sawamura (2000) investigated limonene (80.35-82.39%), α -terpinene (7.71-9.03%), myrcene (2.11-2.28%), linalool (1.37-2.01%), and α -pinene (1.17-1.43%) as the most prevalent components in Hyuganatsu oils. Anon, (2004) also investigated that limonene and citral were the main chemical components in sweet orange peel essential oil. Our results are also in agreement with the findings of Gancel *et al.*, (2003) who worked on the chemical composition of *Citrus paradisi* oil. Vekiari *et al.*, (2002) reported that the main components of *Citrus* essential oils were limonene, β -pinene, myrcene, neral, geranial, neryl acetate and β -caryophyllene. Lota *et al.*, (2001) found limonene and α -pinene as the main compounds in the peel oils of sour orange. Ahmad *et al.*, (2006) extracted essential oils from the peels of Malta (*C. sinensis*), Mousami (*C. sinensis*), Grapefruit (*C. paradisi*) and Eureka lemon (*C. limon*) through cold pressing method. According to them the main constituents detected in Malta peel oil were limonene (61.08%), citronellol (4.18%), citral (7.74%), borneol (7.63%), α -terpinolene (2.06%) and linalool (1.28%). The principal compounds in Mousami essential oils were limonene (76.28%), α -pinene (1.26%), β -pinene(5.45%), citral (1.74%), and linalool (2.32%) while limonene (86.27%), myrcene (6.28%), γ -terpinene(2.11%) and α -pinene (1.26%) in Grapefruit essential oils (Ahmad *et al.*, 2006). According to the investigation carried out by Choi and Sawamura (2000a), the essential oil of *Citrus tamurana* contained hydrocarbons (95.95-96.95%), aldehydes (0.33- 0.62%), alcohols (1.91%-2.64%), ketones (0.40 0.62%), esters (0.28-0.39%), oxides (0.04-0.06%), acids (0.01%) and trace amounts of eugenol methyl ether (Choi and Sawamura., 2000a).

Result of larvicidal analysis, there is an increasing level of activities across the concentrations showing that activity is directly proportional to the concentrations. In citrus sinensis, *citrus aurantifolia* the larvicidal effects was higher in the hydrodistilled

fractions than in the N-hexane fractions this is in line with the earlier work on indigenous system of medicine which reports a number of *in vitro* anthelmintic activity from medicinal plants that have been investigated from different regions of the world (Akhtar *et al.*, 2000; Tagboto and Townson, 2001; Athanasiadouet *al.*, 2007). Dixit and Varma in their study reported that the oils of the rhizomes of *Hedychium coronarium* (Zingiberaceae) and *H. spicatum* (Zingiberaceae) possess better anthelmintic activity than piperazine phosphate against earthworms and tapeworms. *Caraca papaya*, *Sapindus trifoliatum* (Sapindaceae), *Butea frondosa* and *Momordica charantia* has been found to possess good *in vitro* anthelmintic activity against *Ascaridia galli* worms (Lalet *al.*, 1976).

Kalesaraj and Kurup(1975) reported that rhizomes of *Zingiber zerumbet* (Zingiberaceae) bear significant anthelmintic activity against human *A. lumbricoides*, whereas the alcoholic extract of the bark of *Albizzialebbek* (Leguminosae), the bulb of *Allium sativum* (Litiaceae), rhizomes of *Alpinia calcarata* (Zingiberaceae), rind of *Citrus acida* (Rutaceae) rind of *Citrus aromatica* (Rutaceae), rind of *Citrus medica* (Rutaceae), rhizomes of *Cucuruma aromatica* (Zingiberaceae), rind of *Punica granatum* show moderate level of anthelmintic activity against round worm (Kalesaraj and Kurup, 1962; Kalesaraj, 1975).

The anthelmintic activity of *Zanthoxylum alatum* (Rutaceae) has been found to be comparable to that drug against roundworms, while the essential oil from the fruits of *Z. limonella* has been reported to bear better anthelmintic efficacy than that of piperazine phosphate.

Hydrodistilled and N-hexane fractions show significance inhibition of transformation of eggs to filariform larvae of *Ascaridia galli*, this shows high lethal concentration (LC) at

50% and 90% for ovicidal activities of essential oil from citrus fruits peels as shown in Table 4.3. In all, there was an increasing level of *Ovicidal* activities across the concentrations showing that the activity is directly proportional to the concentrations. In citrus sinensis the ovicidal effects was higher in the hydrodistilled fractions than in N-hexane fraction, this has been confirmed by several works done earlier by the following authors, Koné *et al.*,(2005), made a pilot study on 79 plant species for their anthelmintic efficacy using *H. contortus* as the test parasite and found they possess either significant larvicidal or ovicidal activity, while *Cardiospermum halicacabum* extract when tested *in vitro* for its efficacy against L3 of *Strongyloides stercoralis* showed reduction in the viability of larvae (Kone *et al.*, 2005).

In vitro anthelmintic activities of crude aqueous and hydro-alcoholic extracts of the seeds of *Croton macrostachyus* and *Ekebergia capensis* showed significant activity on the egg and adult of *H. Contortus* (Egualé *et al.*, 2006). *Trachyspermum ammi* seeds used locally in Pakistan as anthelmintic for worm control in sheep were evaluated for their ovicidal activity against *H. contortus* eggs and were reported to possess some anthelmintic properties (Jabbar *et al.*, 2006). The anthelmintic activity of *Croton zehntneri* and *Lippia sidoides* essential oils and their major constituents, anethole and thymol were determined by *in vitro* assays with the eggs and larvae of *H. Contortus* (Camurça-Vasconcelos *et al.*, 2007).

Kataki (2010) reported the anthelmintic activity of ethanolic extract of *Anana comosus* L. tender leaves were used in Indian adult earth worms. Similarly, the anthelmintic activity of *Eucalyptus staigeriana* essential oil has been well established using egg hatching test and the inhibition of larval development of *H. contortus*. Its *in vivo* anthelmintic effects were also noticed through fecal egg count reduction test in goats (Macedo *et al.*, 2010).

Marie-Magdeleine *et al.* (2010), investigated the anthelmintic effects of aqueous, methanolic and dichloromethane extracts on four developmental stages of *H. contortus* using egg hatching assay, larval development, L3 migration inhibition assay and observed that the methanolic extract of plant leaves possesses significant efficacy against larval development (Kataki, 2010; Macedo, *et al.*, 2010; Marie-Magdeleine *et al.*, 2010). High presence of tannin may be implicated for that as shown by the effects of condensed tannins extracted from five species of plants (*Lotus pedunculatus*, *Lotus corniculatus*, *Dorycnium pentaphyllum*, *Dorycnium rectum* and *Rumex obtusifolius*) were investigated using egg hatching and larval development bioassays against *Ostertagia circumcincta* and it was concluded that condensed tannins from these plants are able to disrupt the life cycles of nematodes (Molan and Faraj, 2010).

In another study, the chloroform methanol and crude tannin extracts of *Leucas indica* (L) showed very good activity. Paralysis and death time of crude tannins, isolated from methanol extract, were very close to standard drug Albendazole (Ramalingam *et al.*, 2010). In many studies, the helminth parasites' tegument/cuticle has been ascertained as one of the principal target site for mode of action of synthetic or natural anthelmintic products (Mehlhorn *et al.*, 1983; Alvarez *et al.*, 2006). It is against this background that several workers while investigating the putative anthelmintic efficacy of plants have also extended their studies to investigate the mode of action of plants with the help of scanning electron microscopy (SEM). The fresh tuber extract of *Flemingia vestita* which was reported to bring about paralysis of *A. suum* under *in vitro* conditions, it showed wrinkles and cracks on lips and body cuticle following treatment with plant extract (Yadav *et al.*, 1992). Vacuolization and pit formation was also recorded in *Artyfechinostomum sufrartyfex* (Roy and Tandon, 1996).

In another study, Tandon, *et al.* (1997), reported exposure of *R. echinobothrida* to genistein, an active principle of *F. vestita*, caused spontaneous loss of movement of cestode parasite followed by structural alteration in its tegumental architecture. The isoflavones of *F. vestita* has been shown to alter carbohydrate metabolism and the activity of nitric oxide synthase leading to change in the concentration of cGMP in *R. echinobothrida* at paralytic time (Tandon and Das, 2007; Das *et al.*, 2009).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

From the present study, phytochemical qualitative analysis of citrus peels essential oils from the three citrus varieties shows presence of alkaloids, flavonoids, tannins, saponins and steroids. Quantitative analysis revealed that in *Citrus aurantifolia* and *Citrus limon*, flavonoids (0.24 ± 0.04 and 0.27 ± 0.03 respectively) and Saponins (0.13 ± 0.03 and 0.21 ± 0.05) were higher than their content in *Citrus sinensis*, alkaloids were higher in *Citrus aurantifolia* (0.26 ± 0.04) and *Citrus sinensis* (0.32 ± 0.06), steroids were significantly greater in *Citrus limon* (0.25 ± 0.04). No significant difference in tannin content was recorded.

Gas Chromatography Mass Spectroscopy results revealed myraids of chemical components mostly terpenes thus: *Citrus sinensis* peels essential oils Hydrodistilled fraction had a total of Thirteen (13), chemical constituents with Sylvestrene had the highest percentage area (57.42) followed by L- Limonene (16.85) and cis-Geraniol (0.63) had the lowest percentage area while N-hexane fraction had a total of Ten (10), chemical constituents with limonene having the highest percentage areas (57.62), followed by Toluene (16.51) and Cis-Geraniol had the lowest percentage area (0.89) respectively.

Citrus aurantifolia peel essential oils Hydrodistilled fraction had a total of Eighteen (18), chemical constituents, L-Limonene had the highest percentage area (25.36) followed by β -Myrcene (11.13) and α -Bisabolol (0.60), had the lowest percentage area while N-hexane fraction had a total of Fourteen (14), chemical constituents, L-limonene

had the highest percentage area (57.67) followed by β -pinene (12.36) and cis-Geraniol (0.62) had the lowest percentage area respectively.

Citrus limon peels essential oils of Hydrodistilled fraction had a total of Eighteen (18,) chemical constituents, L-Limonene had the highest percentage area (24.66) followed by Limonene (20.40) and Cyclohexen-1-ol (0.65) had the lowest percentage area while N-hexane fraction had a total of Twelve (12), chemical constituents L-Limonene (44.28) had the highest percentage area followed by Decane (10.27) and n-Dodecane (1.47), had the lowest percentage area respectively

Fourier transform infrared spectroscopy of hydrodistilled and N-Hexane fractions for the three varieties gave numbers of peaks and their corresponding functional groups, *Citrus sinensis* show Twenty two (22), Twelve (12) peaks, corresponding functional groups were identified with the standard library.

Citrus aurantifolia, Twenty one (21), Eighteen (18) peaks, corresponding functional groups were identified with the standard library respectively. *Citrus limon* hydrodistilled fraction, Sixteen (16), Eleven (11) peaks corresponding functional groups were identified with the standard library respectively..

The lethal Concentrations for percentage larval mortality for both fractions at 50% and 90% of essential oil from citrus varieties peels were calculated using linear regression equations as LC₅₀ and LC₉₀ ($\mu\text{g/ml}$), least LC₅₀ and LC₉₀ were recorded for hydrodistilled fractions of *C. limon* (0.066 $\mu\text{g/ml}$) and *C. aurantifolia* (0.396 $\mu\text{g/ml}$), greatest were *C. sinensis* (0.801 $\mu\text{g/ml}$) and (1.314 $\mu\text{g/ml}$), similarly, least were recorded for n-hexane fractions are *C. sinensis* (0.236 $\mu\text{g/ml}$) and *C.aurantifolia* (0.644 $\mu\text{g/ml}$), greatest *C. aurantifolia* (0.414 $\mu\text{g/ml}$) and *C. sinensis* (0.985 $\mu\text{g/ml}$) respectively.

On the other hand, percentage reduction in eggs hatchability; least LC₅₀ and LC₉₀ were recorded for hydrodistilled fractions of *C. limon* (0.175 µg/ml) and *C. aurantifolia* (0.625 µg/ml), greatest LC₅₀ and LC₉₀ were *C. sinensis* (0.890 µg/ml) and *C. sinensis* (1.741 µg/ml) LC₅₀ and LC₉₀ (in µg/ml) similarly, least were recorded for n-hexane fractions of *C. limon* (0.147 µg/ml) and *C.aurantifolia* (0.344 µg/ml), greatest *C. sinensis* (0.217 µg/ml) and *C. limon* (0.751 µg/ml) respectively.

The ovicidal effects of essential oils of citrus peels on egg of *Ascaridia galli* indicated a significant ($p \leq 0.05$) increase in % ovicidal effects in all treated groups compared to the normal control and standard drugs at all concentrations, concentrations of 0.1(µg/ml), had the highest and lowest effects as recorded in *C. sinensis* – Hydrodistilled fraction (71.51±1.18) and *C. limon* – N-hexane fraction (61.26 ±2.55) concentrations 0.3(µg/ml) had the highest and lowest effects as recorded in *C. aurantifolia*- Hydrodistilled fraction (77.99±1.85) and *C. limon* N-hexane fraction (63.50±0.44); concentrations 0.5(µg/ml) had the highest and lowest effects as recorded in *C. aurantifolia*- Hydrodistilled fraction (83.71± 2.47) and *C. limon* – N-hexane fraction (67.65 ± 2.39).

The larvacidal effects of essential oils of citrus peels indicating a significant ($p \leq 0.05$) increase in % larvacidal effects in all treated groups compared to the normal control in all concentrations (µg/ml). For 0.1, had the highest and lowest effects as recorded in *C.limon* –Hydrodistilled fraction (73.35 ± 2.83) while *C. sinensis* N-hexane fraction (51.90 ± 4.65). For concentrations 0.3(µg/ml) had the highest and lowest effects as recorded in *Citrus aurantifolia*- N-hexane fraction (79.94 ±1.42) and *C. sinensis*- N-hexane (61.20 ± 3.21); concentrations 0.5(µg/ml) had the highest and lowest effects as recorded in *C. aurantifolia*-H (91.55 ± 1.37) and *C. limon*- N-hexane (70.89 ± 2.69).

6.2 Conclusion

Essential oil from peels *C. sinensis*, *C. aurantifolia*, *C. limon* hydrodistilled and N-hexane fractions are reservoir of phytochemicals and active principles, which were mostly monoterpenes and monoterpene alcohols. Essential oils considered in this study, have been found to demonstrate significant dose dependent *in vitro* anthelmintic (larvacidal and ovicidal) potentials on poultry worm *Ascaridia galli* Which may be due to single or a synergistic effect of mixtures of chemical groups found in them and could be considered as the scientific basis for its traditional use and as an alternative therapeutics for helminths.

6.3 Recommendations

The results from this research are quite promising for the use of *C. sinensis*, *C. aurantifolia*, *C. limon* Linn, essential oil from hydrodistilled and N-hexane fraction as anthelmintics there is a need for further studies to confirm the *in-vivo* anthelmintic activities

1. Using solvent extracts of citrus peels of these Citrus species should also be tested for in-vitro anthelmintic activity.
2. The anthelmintic activities attributed to *C. sinensis*, *C. aurantifolia*, *C. limon*, require further clinical studies and trials should be conducted to support their therapeutic use in multiple animal-based models using a variety of suitable biochemical markers to understand their full mechanism of action and thus check the toxicity, standardize doses and develop a drug since they have been used successfully in Ayurvedic and traditional medicine for centuries.

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APPENDICES

Appendix1

Ovicidal effects of essential oils of *Citrus sinensis*, *Citrus aurantifolia*, *Citrus limon* peels and peperazine citrate against the eggs of *Ascaridia gali*

SN	SAMPLE	0.1µg/ml	0.3µg/ml	0.5µg/ml
1	NC	25.60 ± 4.55 ^a	25.60 ± 4.55 ^a	25.60 ± 4.55 ^a
2	C.sin -H	71.5067±1.18 ^{de}	68.2367±0.65 ^{bc}	76.85 ± 11.22 ^{bc}
3	C.sin -N	62.1633±2.84 ^{bc}	67.5967±3.70 ^{bc}	74.44 ± 0.99 ^{bc}
4	C.aur.-H	68.6667±1.15 ^d	77.9933±1.85 ^d	83.71 ± 2.47 ^{bc}
5	C.aur -N	66.6367±1.24 ^{bcd}	69.5233±0.74 ^c	77.32 ± 5.67 ^c
6	C.lim-H	67.4700±0.70 ^{cd}	72.5767±2.50 ^{cd}	77.29 ± 1.50 ^{bc}
7	C.lim -N	61.2633±2.55 ^b	63.5033±0.44 ^b	67.65 ± 2.39 ^b
8	SD	76.3467±5.71 ^e	76.3467±5.71 ^d	76.35±5.71 ^{bc}

NC=Normal control, C. sinH=*Citrus sinensis*-hydrodistilled fraction, C. sinN= *Citrus sinensis*-N-hexane fraction, C .aur. H =*Citrus aurantifolia*-hydrodistilled fraction, C. aur. N= *Citrus aurantifolia* -N-hexane fraction, C. lim. H= *Citrus limon*-hydrodistilled fraction, C. lim N=*Citrus limon* N-hexane fraction, SD=standard drug-(peperazine citrate)

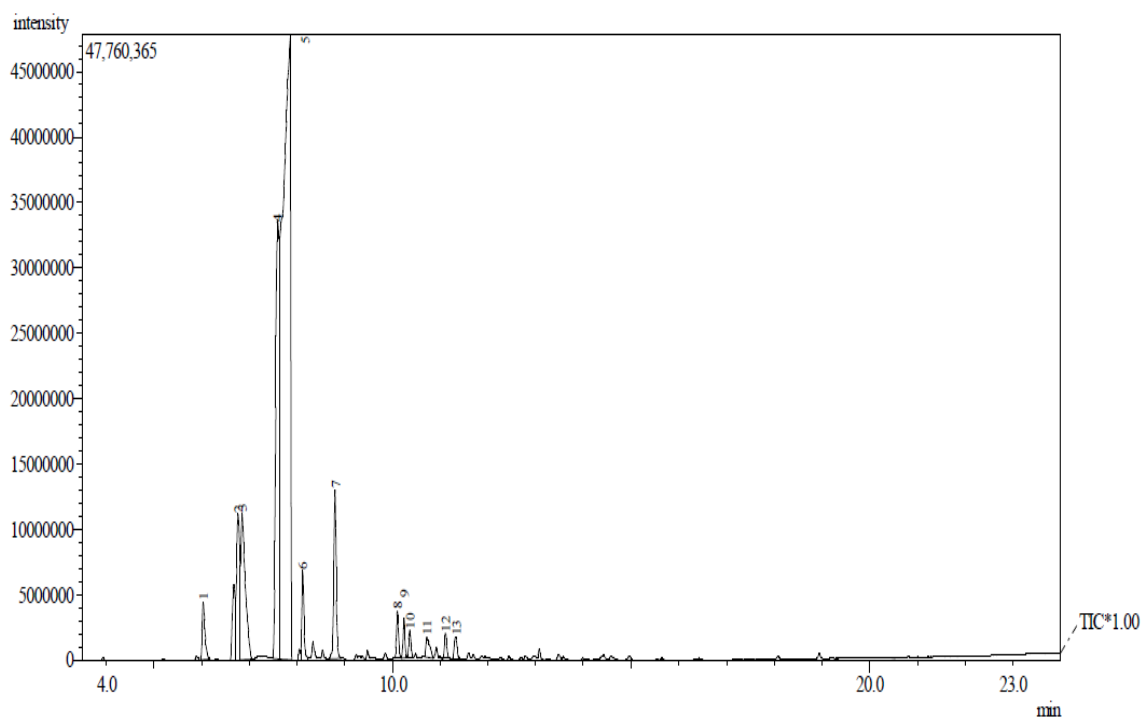
Appendix 2

Larvicidal effects of essential oils of *Citrus sinensis*, *Citrus aurantifolia*, *Citrus limon* peels and peperazine citrate against the L3larvae of *Ascaridia galli*

SN	SAMPLES	CONC.0.1µg/ml	CONC.0.3µg/ml	CONC.0.5µg/ml
1	NC	25.60 ± 4.55 ^a	25.60 ± 4.55 ^a	25.60 ± 4.55 ^a
2	C. sin-H	58.40 ± 3.64 ^c	68.21 ± 2.79 ^c	85.17 ± 3.30 ^{de}
3	C. sin-N	51.90 ± 4.65 ^b	61.20 ± 3.21 ^b	77.09 ± 3.57 ^{bc}
4	C. aur-H.	66.99 ± 2.87 ^{de}	79.39 ± 4.44 ^d	91.55 ± 1.37 ^e
5	C. aur.-N	65.20 ± 4.50 ^{cd}	79.94 ± 1.42 ^d	81.63 ± 6.42 ^{cd}
6	C. lim-H	73.35 ± 2.83 ^{ef}	75.99 ± 3.47 ^d	85.32 ± 3.76 ^{de}
7	C. lim-N	62.11 ± 0.84 ^{cd}	67.52 ± 1.48 ^{bc}	70.89 ± 2.69 ^b
8	S D	76.35 ± 5.71 ^f	76.34 ± 5.71 ^d	76.35 ± 5.71 ^{bc}

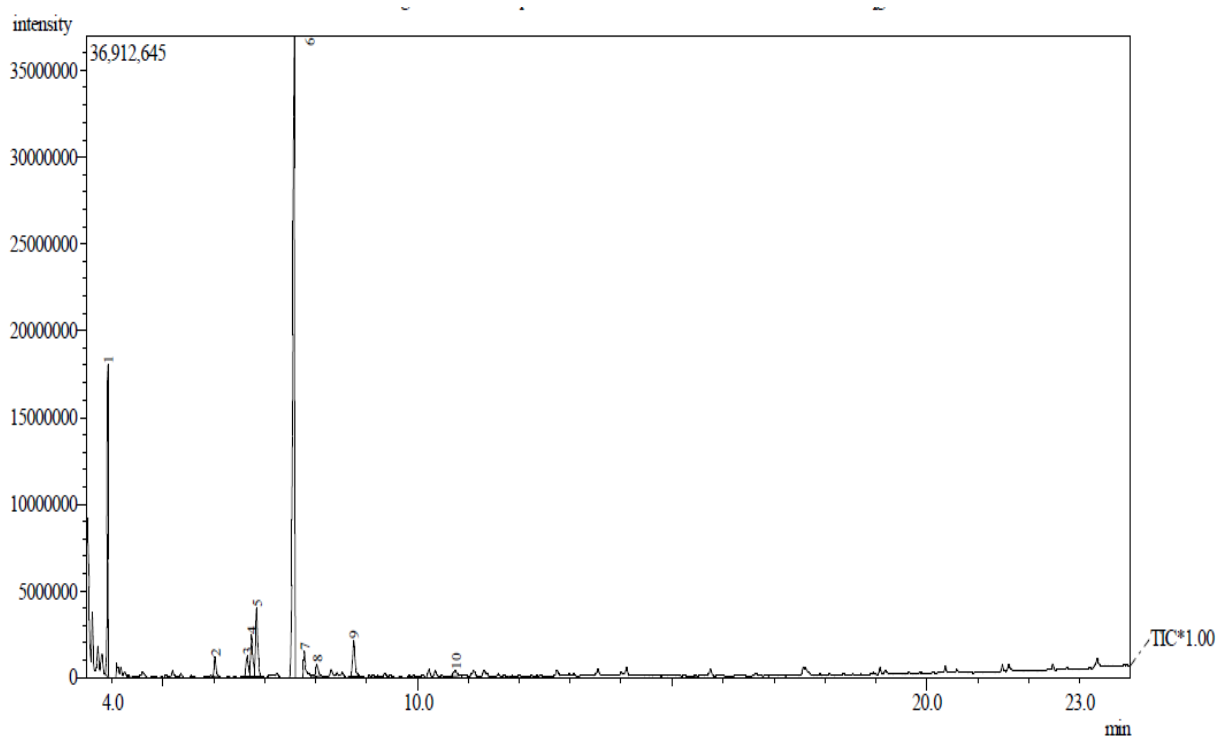
NC=Normal Control, C. *sinensis* H=*Citrus sinensis* hydrodistilled fraction, C. *sinensis* N= *Citrus sinensis* N-hexane fraction, C. *aurantifolia*.H=*Citrus aurantifolia* hydrodistilled fraction, C. *aurantifolia*.N= *Citrus aurantifolia* N-hexane fraction,, C. *limon* H= *Citrus limon* hydrodistilled fraction, C. *limon* N=*Citrus limon* N-hexane fraction,SD=standard drug-(peperazine citrate)

Appendix 3



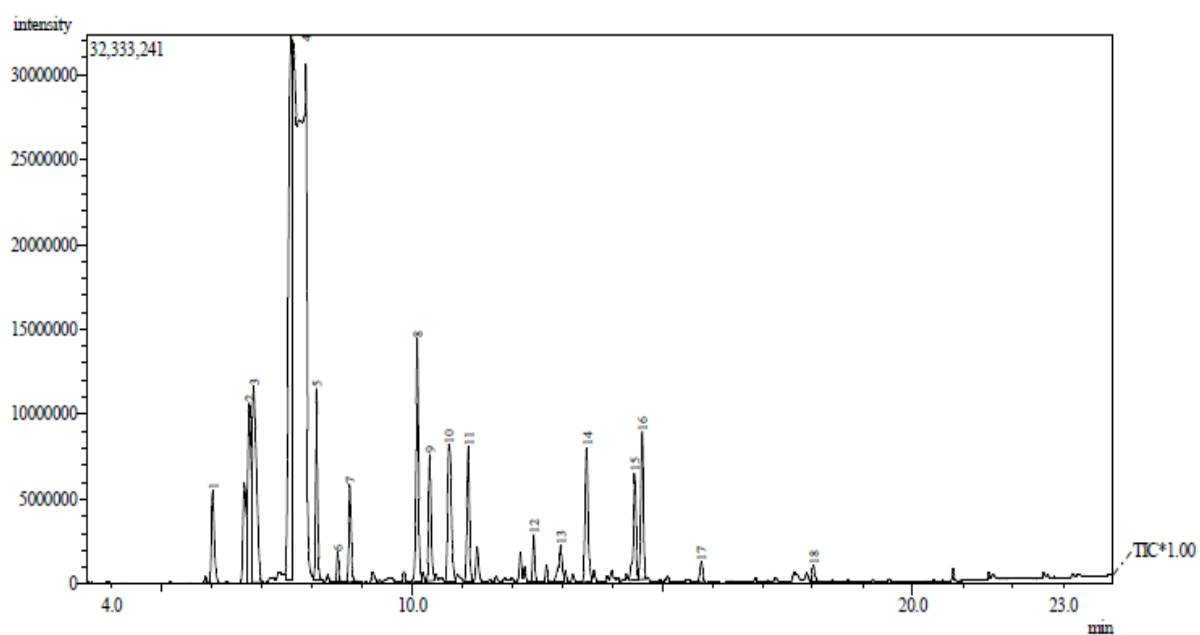
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus Sinesis* Hydrodistilled Fraction Showing Various Components

Appendix 4



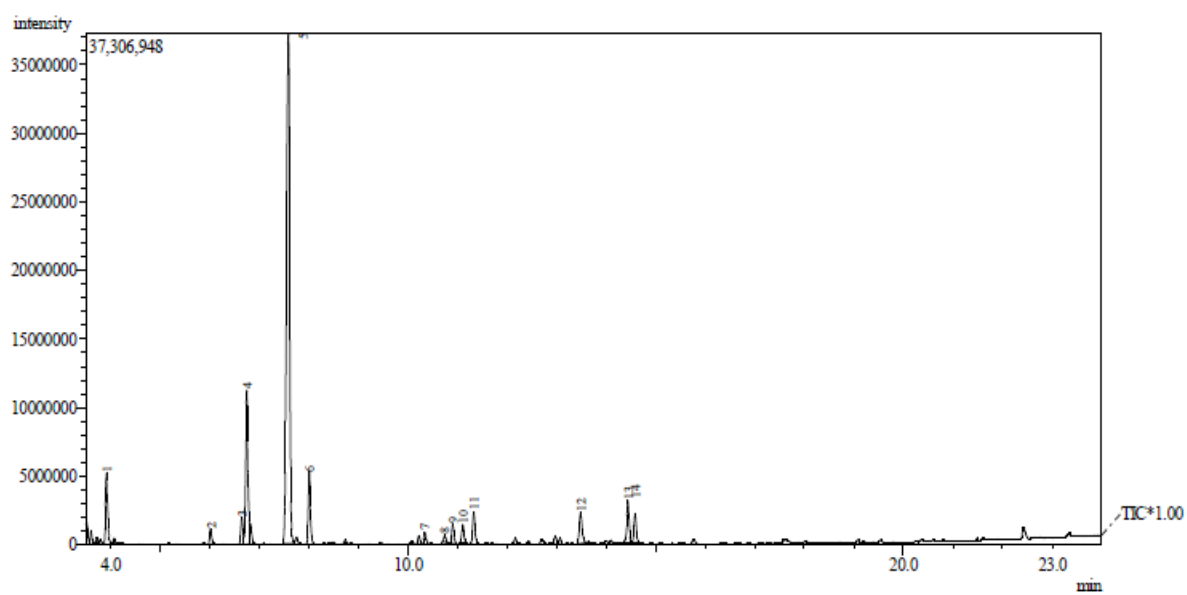
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus sinensis* N-hexane Fraction Showing Various Components

Appendix 5



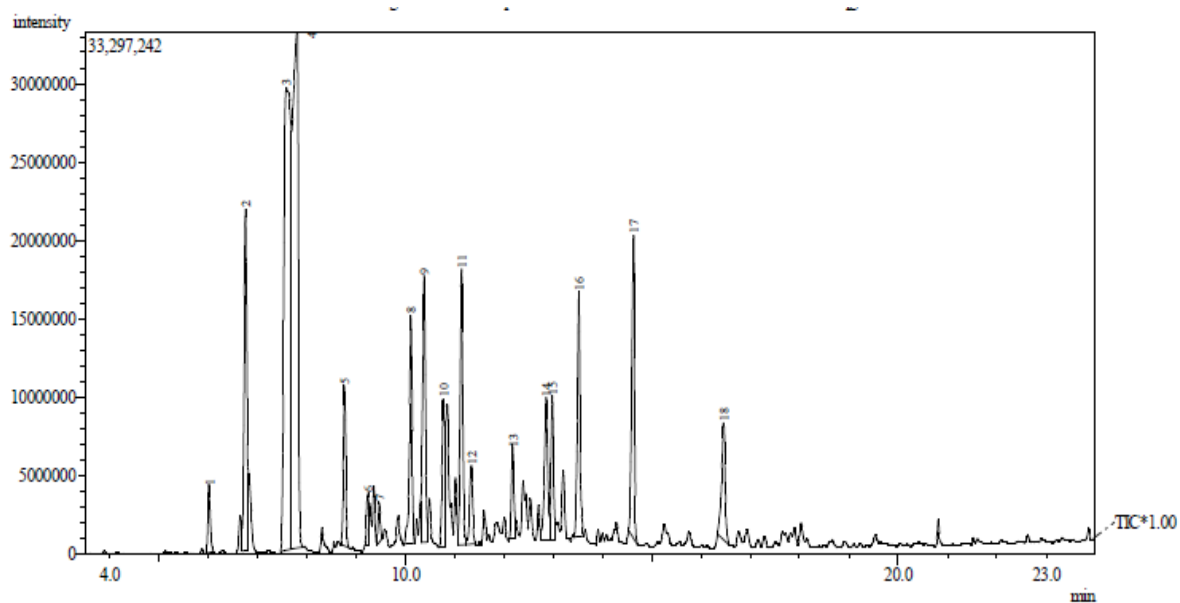
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus aurantifolia* Hydrodistilled Fraction Showing Various Components

Appendix 6



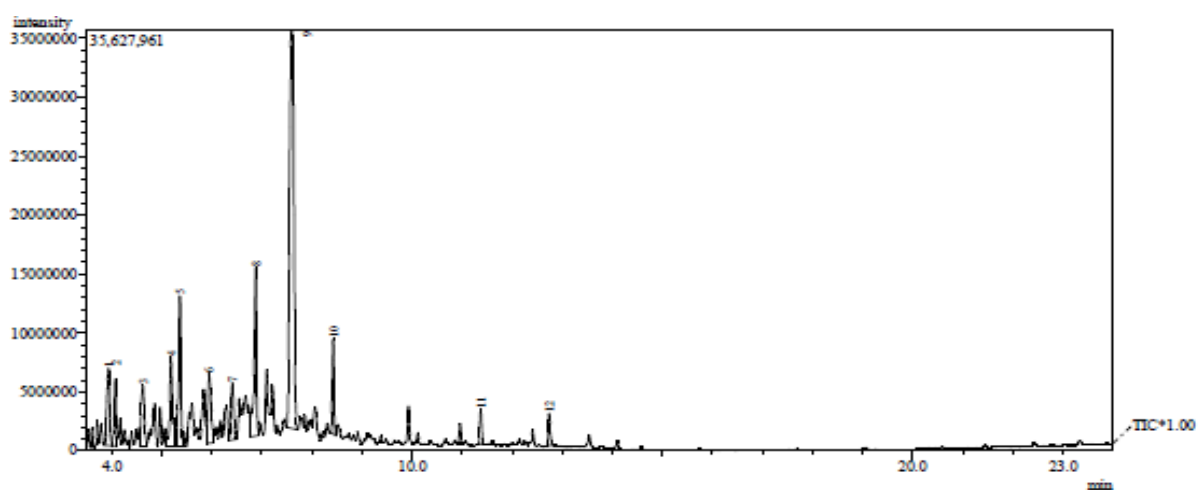
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus aurantifolia* N-hexane Fraction Showing Various Components

Appendix 7



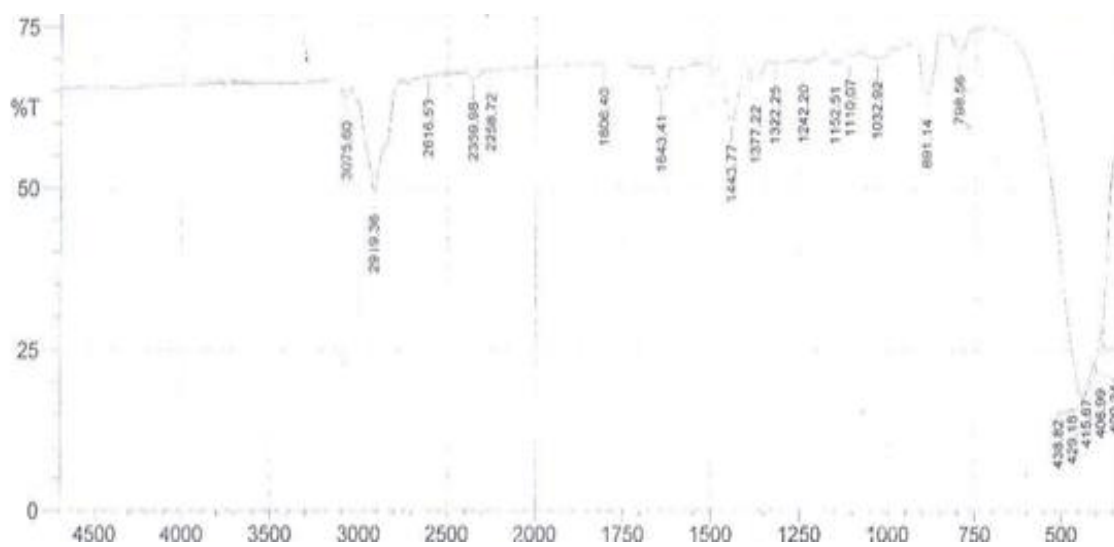
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus limon* Hydrodistilled Fraction Showing Various Components.

Appendix 8



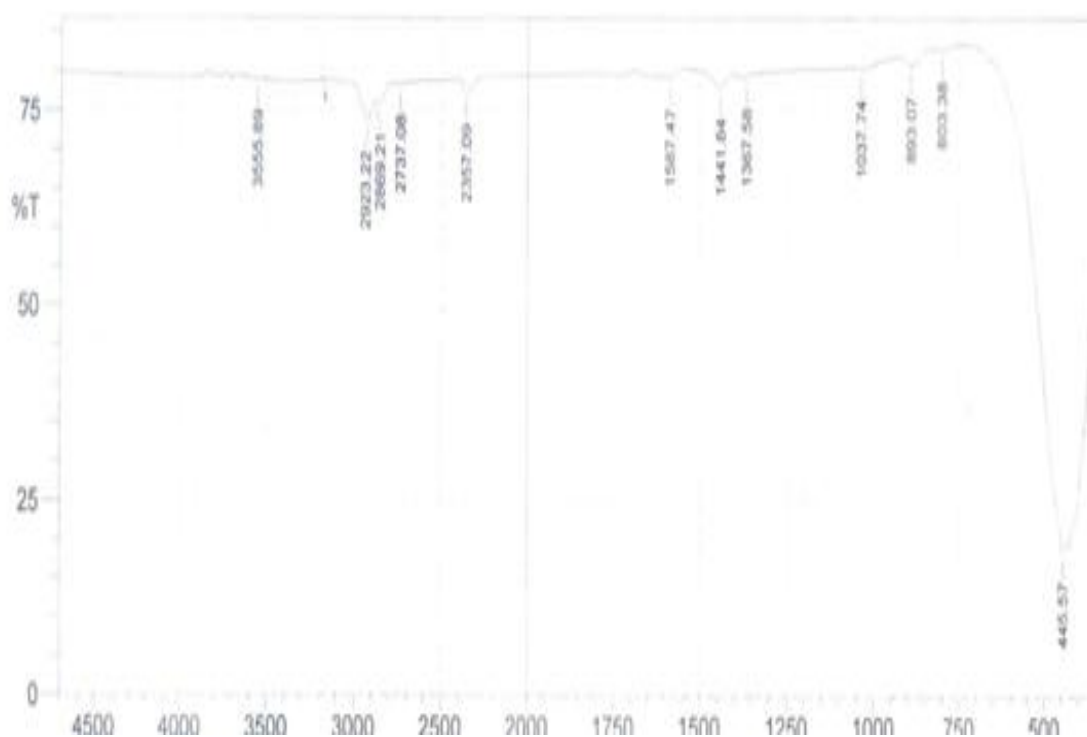
Gas Chromatography-Mass Spectroscopy Chromatogram of Essential Oil from *Citrus limon* N-Hexane Fraction Showing Various Components

Appendix 9



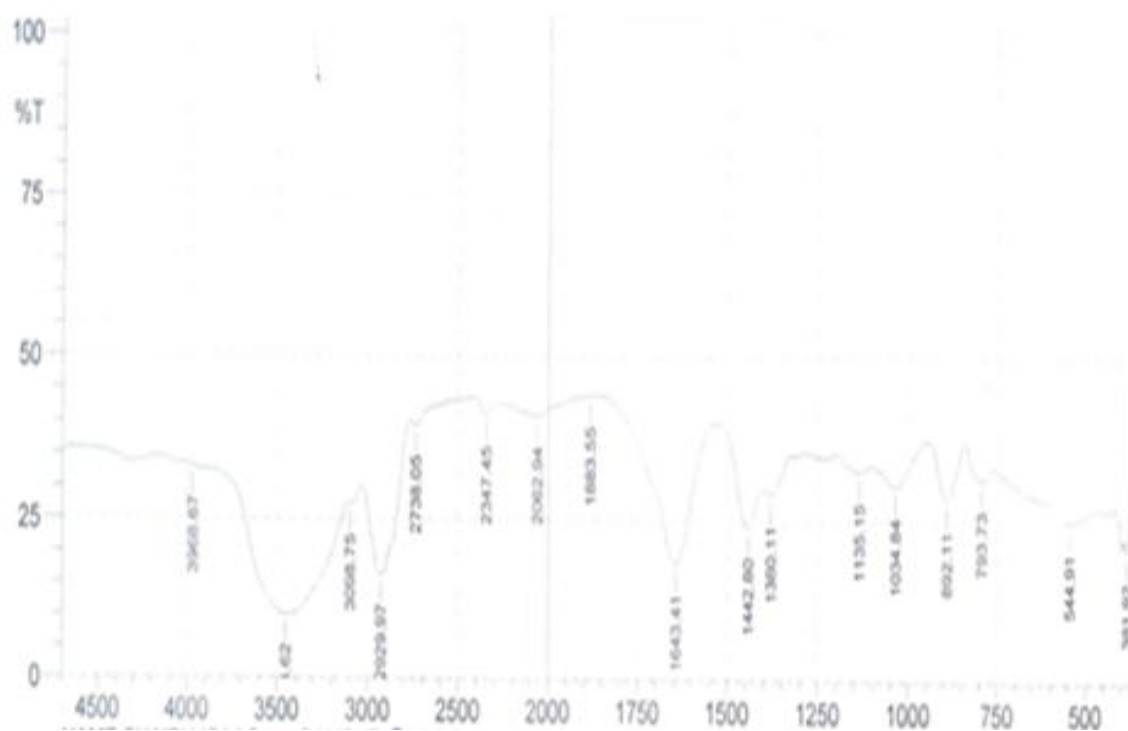
Fourier Transformed Infrared Spectroscopy Chromatogram of Functional Groups in Essential Oil of *Citrus sinensis* Hydrodistilled Fraction

Appendix 10



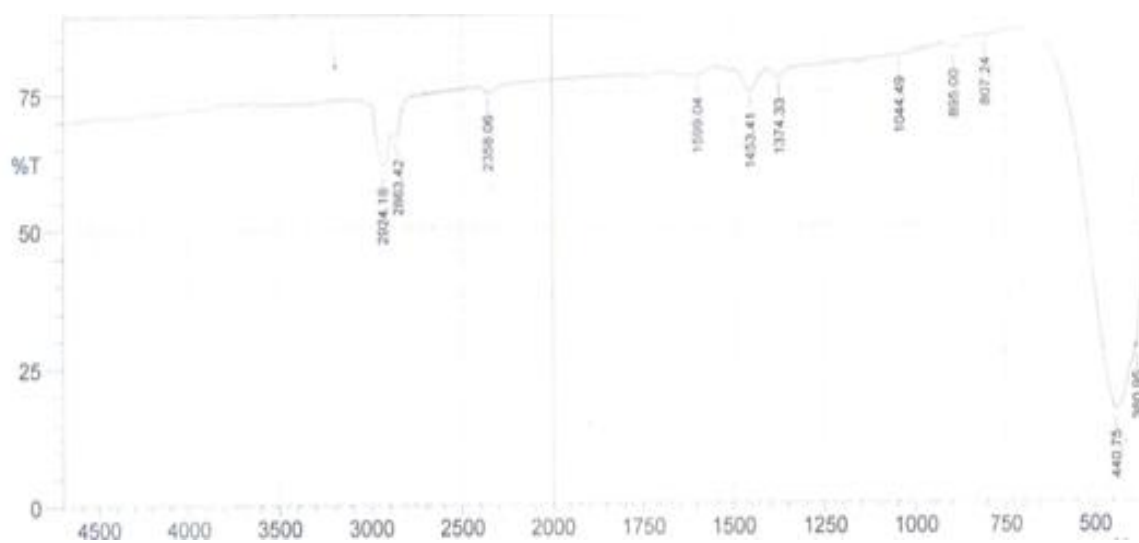
Fourier Transformed Infrared Spectroscopy Chromatogram of Functional Groups in Essential Oil of *Citrus sinensis* N-Hexane Fraction

Appendix 11



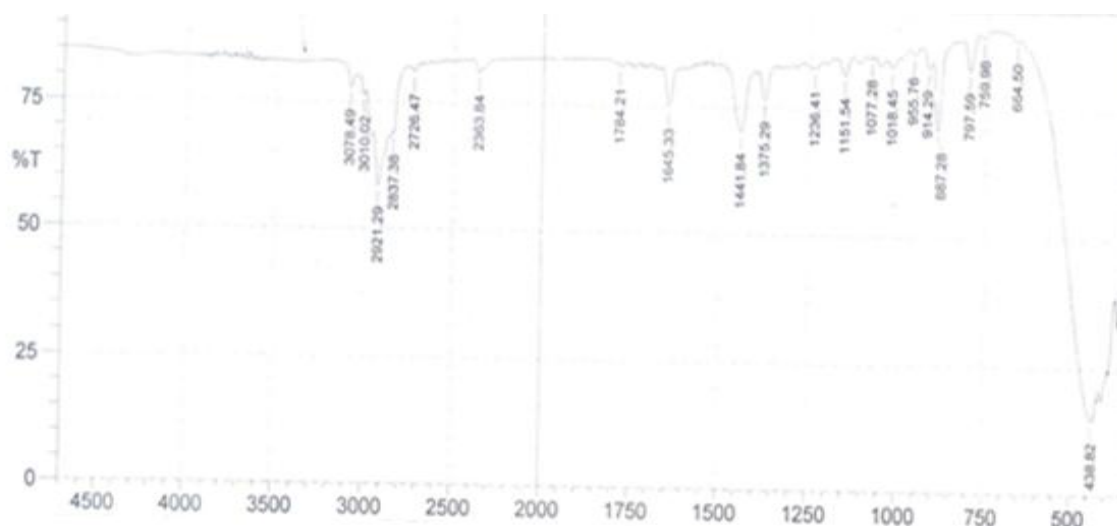
Fourier Transformed Infrared Spectroscopy Chromatogram of Functional Groups in Essential Oil of *Citrus aurantifolia* Hydrodistilled Fraction.

Appendix 12



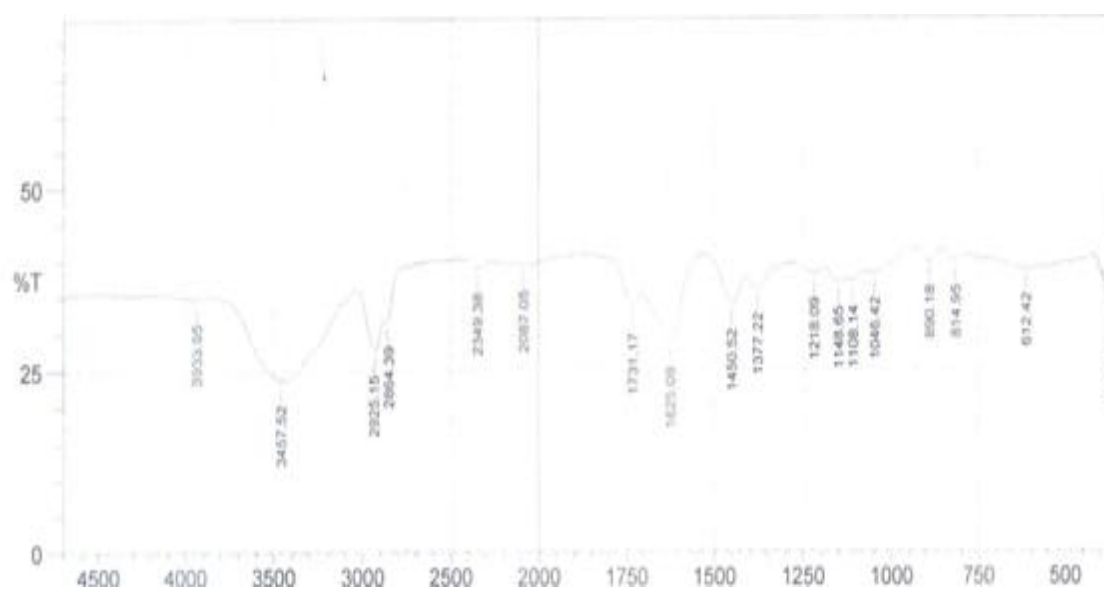
Fourier Transformed Infrared Spectroscopy chromatogram of functional groups in Essential Oil of *Citrus aurantifolia* N-Hexane Fraction.

Appendix 13



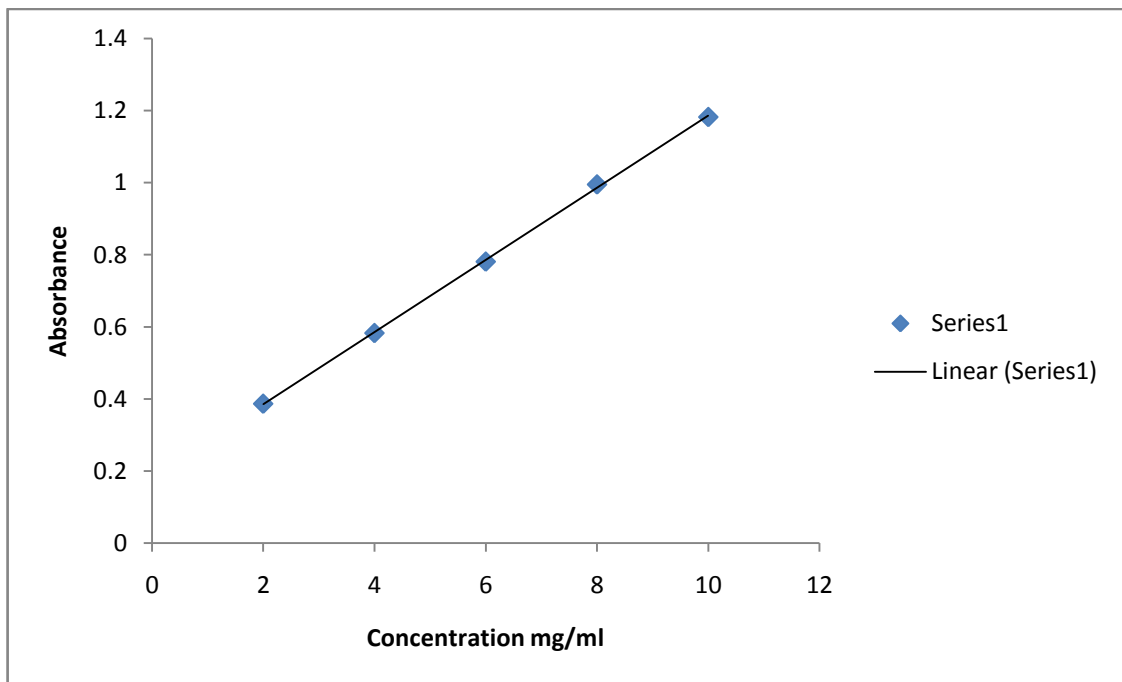
Fourier Transformed Infrared Spectroscopy chromatogram of functional groups in essential oil of *Citrus limon* Hydrodistilled fraction.

Appendix 14



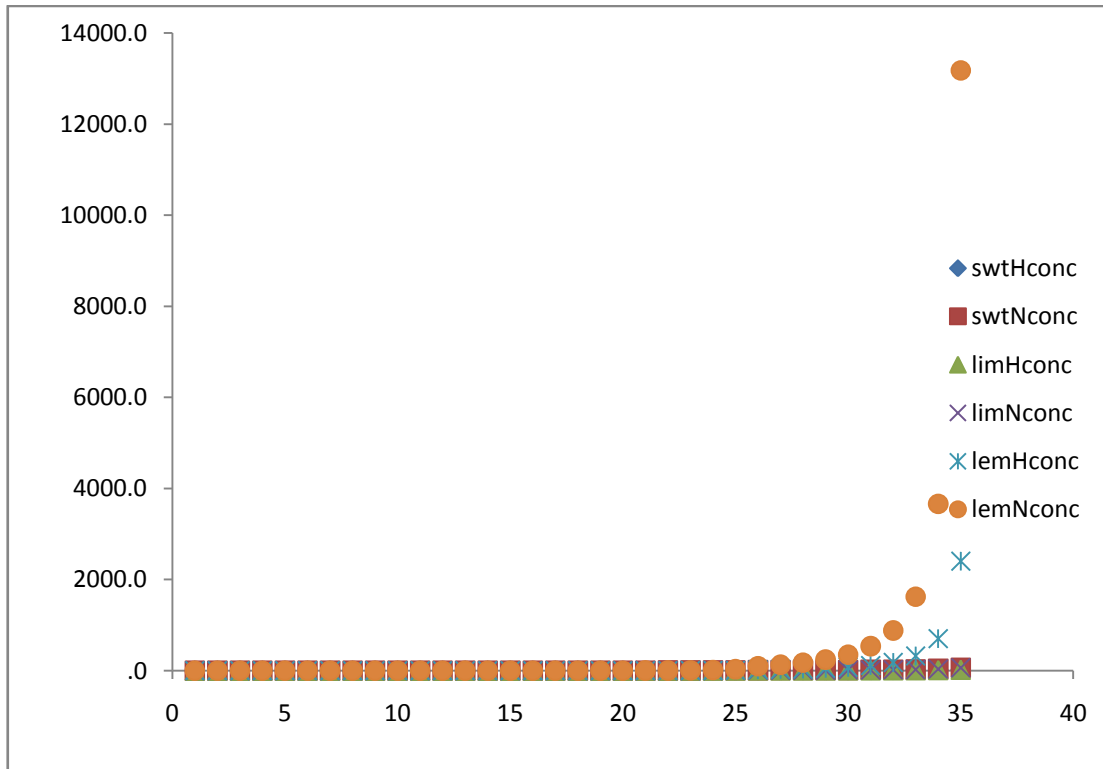
Fourier Transformed Infrared Spectroscopy chromatogram of functional groups in essential oil of *Citrus limon* N- Hexane fraction.

Appendix 15



Standard Curve of Gallic Acid

Appendix 16



Standard Curve for Probit Analysis

Appendix 17

General Preparation Protocol of Phosphate Buffer Saline

A total of 8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, 0.25g of KH₂PO₄, were measured and dissolved in 800 ml of distilled water, the pH of the solution was adjusted to 7.4 slowly adding in dropwise 1 M HCl acid using pH meter. the volume of the solution was finally made up to one litre.