KAMPALA INTERNATIONAL UNIVERSITY- WESTERN CAMPUS

SCHOOL OF PHARMACY:

INVESTIGATION OF ANTIBACTERIAL ACTIVITY, PHYTOCHEMICAL SCREENING AND THE EXTRACTIVE VALUE OF DIFFERENT SOLVENTS ON *LAWSONIA INERMIS* (HENNA) PLANT LEAF EXTRACT.

THE RESEARCH REPORT SUBMITTED TO THE SCHOOL OF PHARMACY KAMPALA INTERNATIONAL UNIVERSITY, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF PHARMACY DEGREE.

BY: PAUL MAYENGO MARANYA

REGISTRATION NUMBER: BPH/0017/61/DF

SUPERVISOR: DR. SSEMIREMBE RICHARD (BVM)

APRIL, 2010

DECLARATION:

I, **PAUL MAYENGO MARANYA** registration No; BPH/0017/61 do hereby declare that this research report entitled **"investigation of antibacterial effect, Phytochemical screening and the extractive value of different solvents on *Lawsonia inermis* (henna)plant extracts"** is my original work and has not been presented for the award of a Bachelor of Pharmacy degree in any University.

PAUL M. MAYENGO 20/05/**2010**

BPH/0017/61/DF Signed. Date.

DEDICATION:

I dedicate this book to my beloved wife, **Asia Hamis Issa** for being a powerful role model throughout my study.

ACKNOWLEDGEMENT:

With honor and heart of gratitude, I wish to thank my supervisor Dr. Ssemirembe RICHARD for his advice and writing collections.

My sincere appreciations goes to the Associate Dean School of Pharmacy-Mr. Joseph EZEONWUMELU who had been with me for advice and all necessary assistance to ensure the success of this work throughout the study.

My sincere gratitude also goes to Dr. Agwu EZRA for his guidance in microbiology area in this study.

I wish to thank all my lecturers in and outside of school of Pharmacy-KIU for building me in a scientific model.

Also I would like to thank my beloved wife Asia H.I. and all my family friends in particular Mr. and Mrs. Kalewa C. NESTORY for their endless prayers and contributions on my research study, without forgetting my colleagues-class mates in Bachelor of Pharmacy whom we shared this academic difficulties.

Above all I will not forget to give my sincerely thanks to GOD who made all this possible.

CERTIFICATION

I certify that this work is an original research carried out by PAUL MAYENGO of the school of pharmacy Kampala International University- Western Campus, Ishaka-Bushenyi, UGANDA.

.....Date...../...../2010.

Dr. Ssemirembe Richard [BVM], Lecturer Pharmaceutical Agrovet- KIU.

SUPERVISOR

.....Date...../...../2010.

EXTERNAL EXAMINER

.....Date...../...../2010.

Associate Prof. Byarugaba Dominic

DEAN, SCHOOL OF PHARMACY-KIU

Terminologies:

Impregnate	Inoculate the culture medium using small round paper discs
Extraction	Removal of soluble materials from an insoluble residue either solid or liquid using liquid solvent(s)
Extract	Crude drug obtained from plants or animals or minerals
Antibiotics	Chemical substances used to inhibit/prevent the growth or kill bacteria
Mucronate	A blunt apex of a plant leaf (not sharp pointed)
"Kinu na Mche"	A local mortar and pestle made by wood used to pound hard materials to form powder

Abbreviations:

L. inermis	*Lawsonia inermis*
MHA	Mueller Hinton Agar
MICs	Minimum Inhibitory Concentrations
KIU-WC	Kampala International University-Western Campus
BPH	Bachelor of Pharmacy
E. coli	*Escheria coli*
S. aureus	*Staphyloccocus aureus*
mm	milli-meter
ml	milli-liter
mcg	microgram

ABSTRACT.

The purpose of this study was to evaluate the antibacterial activity, to determine the most efficiency extractive solvents and phytochemical profile of Hot water, Methanol alcohol and Lemon juice extract of *Lawsonia inermis* (henna) leaf.

The plant attracted the attention of researchers because of its use as anti-infective agent. The oily and alcoholic extracts of the plant *Lawsonia inermis* dried powdered leaves were separately obtained by maceration, while the aqueous was obtained by Infusion extraction method.

The bacteria organisms tested were *Staphylococcus aureus* and *Escherichia coli.* Extraction, Phytochemical screening and susceptibility test of the plant leaves were performed using standard procedures. The Phytochemical screening results revealed the presence of tannins, steroids and reducing sugars as the major bioactive constituents.

The Lemon juice extractive value was relatively much higher than the alcoholic and aqueous.

The result of susceptibility test showed that the extract had greater antibacterial effect against all the two test organisms with minimum inhibitory concentrations (MICs) used as compared with standard drugs.

Submitted.....

Reviewed.....

Internal defense.....

Accepted.....

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1.0 CHAPTER ONE

1.1 INTRODUCTION.

Lawsonia inermis (Henna) is a flowering plant 2.6m in height with an average dimension of a leaf 2.5-5cm (length) and x 2-3cm (width),the apex is mucunate, the margin is entire leaves are oppositely arranged to one another. (Singh M. et al, 2005, Traditional Methods of Cultivation and processing of Henna. Central Arid Zone Research Institute, India.pp 14, 21, Trease and Evance, 1989, Pharmacognosy, 13th edn,Chapter 22 pg. 214) It is in the family Lythraceae, that produces a dye molecule Lawsone, the molecule that has an affinity for binding with protein and this used to dye skin, hair, fingernails, leather, silk and wool. (Harborne SB and Baxter A, 2005, Phytochemical Dictionary, A handbook of bioactive compounds from plants; Taylor & Francis,London.). The dye molecule lawsone, is primarily concentrated in the leaf. It is an important medicinal plant of Indian systems of medicine (Ayurveda) commonly known as Mehndi (Trease and Evance, 1989, Pharmacognosy, 13th edn,Chapter 22 pg. 214). In some parts of the world, the plant is still the prime medicine used for many diseases treatment. (Stulberg D.L, Penrod M.A, Blatny R.A 2000, Common bacteria skin infections, pp. 119-124, http:/www.tatoo-me.com/science.htm, Hamem SS., 2002, Activity of some common pathogens in bacterial skin infections; thesis M.Sc. College of Education, Basra University. Iraq)

Lawsonia inermis is widely grown in various tropical regions of Asia, America and Africa at temperature between 35-45^{0}C soil pH 4.3-8.0. (Stulberg D.L, Penrod M.A, Blatny R.A 2000, Common bacteria skin infections, pp. 119-124,) The plant is scarcely grown in home gardens in the coast area of East Africa where the plant is locally known as **"Hina" in Kiswahili language** and has been used for adornment/body art (cosmetic). The plant is commercially cultivated in India, Si-Lanka, Pakistan, Yemen, Iran, Afghanistan, Sudan, Somalia, Libya and Bangladesh. (Trease and Evance, 1989, Pharmacognosy, 13th edn, Chapter 22 pg. 214)

As a medicinal plant *L. inermis* has been used as an astringent, antihypertensive and sedative agent. It has also been used in both internal and external against some pathogenic infections, the extracts showed antibacterial activity.

It exhibited anti infertility in animal and may induce or regulate menstruation. (http:/kingtutshop.com/Egyptian-Herb/henna.htn)

1.2 BACKGROUND

The earliest civilizations of medical and drug history indicates and believed that, the Babylonins, Assyrians, Sumerians, Semites, Ugaritics and Canaanites have been using *L. inermis* (henna) plant leaves as a source of a red-brown dye for body art (cosmetic). (Sofowora 1982)

There are numerous artifacts from Iraq, Palestine, Greece, Egypt, and Roma from 1400BC to 1AD that show women with henna patterns on their hands.

The early centre of the use of henna as a woman's adornment seems to have been in the Eastern Mediterranean where it grows wild. It was used by Canaanite women in pre-biblical times that spread it across North Africa between 1700 and 600BC.

Henna was used in Palestine from the earliest historical period and there are Roman records of henna being used by Jewish people living in Jerusalem during the historical period of **the birth of Christ**.

When **Islam** began in the 6 to 7th centuries AD, henna was incorporated into the customs of **Muslims** from the western Middle Eastern women's henna traditions that were wide spread and long established. The use of henna spread to other countries as Islam expanded, most of these countries are still continues celebrate the **"Night of the Henna"** as a beautiful and suitable ornament for women until present.

Since 1890, henna has been used in Europe for tinting the hair in form of Shampoo, also gives different sheds by mixing with leaves of other plants. As a dye for the skin or nail, powder may be mixed with water, sour milk, or red wine. (http:/kingtutshop.com/Egyptian-Herb/henna.htn)

1.3 PROBLEM STATEMENT

The cost and access to commercial antibacterial agents becomes harder to patients with skin bacterial diseases in society. Therapy with the same commercial agents is very expensive and may call for a variety of drugs some of them may not be available in many Pharmacies, yet many communities are exposed to expired drugs which fail to cure the ailments. Use of henna herbal is an effective substitute available and affordable treatment for numerous internal and external skin disorders.

1.4 HYPOTHESIS.

- The *Lawsonia inermis* leaf has antibacterial activity against some internal bacterial pathogens and external skin pathogens, is most effective than most commonly known antibacterial used in daily clinical practices,
- Tannins chemical constituents are present in *L. inermis* leaf extracts and aqueous based extracting method (Hot-water) is highly efficiency.
- There are (tannins) Phytochemical constituent of therapeutic value which occur in Extractable quantities.
- Various methods of extraction are of value

1.5 OBJECTIVES.

1.5.1 General objectives

To investigate the therapeutic potential, undertake the phytochemical screening and determine the most efficiency solvent in extraction of active constituents (extractive value) of *L.inermis* extracts.

1.5.2 Specific objectives

- To test the effectiveness of *L.inermis* extracts against different bacterial strains
- To establish the presence of antibacterial effect of the *lawsonia inermis* dried powder leaf extracts to detect the chemical constituents present in the *L.inermis* leaf.
- To determine the chemical constituents present in *L.inermis* leaf
- To address uses of some major constituents present in the leaf extract
- To determine the extractive value of the obtained yield

1.6 Justification of the Study

At the end of this study:

- Effective and affordable antibacterial remedy was obtained
- Chemical constituents present in the *L. inermis* were determined
- The efficient solvent (Lemon juice) and effective extracts (hot water) was identified

2.0 CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 About *L. inermis* (henna) plant.

Lawsonia inermis (Henna) a deseedios flowering plant has been used in herbal medicine for ages. The medicinal benefits of this plant have been discussed in only a few publications. (http:/kingtutshop.com/Egyptian-Herb/henna.htn) Studies have been done on preliminary pharmacognostical standardization of *Lawsonia inermis*–Linn seeds, (Trease and Evance, 1989, Pharmacognosy, 13th edn, Chapter 22 pg. 214). In order for indigenous plant to be exploited for medicinal purposes basic Phytochemical screening and bioassay must be undertaken as the first step towards the ultimate development of natural drug (Odebiyi and Sofowara, 1998) also is still appreciated that plants continued to be a major source of drugs and natural products on the basis of their therapeutics value(Lowin,1993).

It is well known that *L. inermis* plant have been used in traditional herbal medicine for many years in such a way that in some part of the world, herbs are still the prime medicines used in medical treatment. (The Scientific Literature on selected herb, Aromatic and Medicinal plants of the temperature zone, Archon books, pp.770, Hamden CT, Herbs: An index bibliography. Simon J., J.E, E.F, Chadwick and L.E Craker, 1984,)

The *L. inermis* shrub is a native to North Africa, Asia, and Australia, is naturalized and cultivated in the tropics of America, Egypt, India and parts of the Middle East. The species is sometimes classified as Lawsonia Alba -Lam. or Lawsonia ruba. The plant grows reaching a height of up to 6 meters; the plant has fragrant white or rose-red-flowers. (http:/www.tatoo-me.com/science.htm, The Scientific Literature on selected herb, Aromatic and Medicinal plants of the temperature zone, Archon books, pp.770, Hamden CT, Herbs: An index bibliography. Simon J., J.E, E.F, Chadwick and L.E Craker, 1984)

The reported life zone of *L. inermis* is 35 to 45°C with an annual precipitation of 0.2 to 4.2 meters with soil pH 4.3 to 8.0. (Singh M. et al, 2005, Traditional Methods of Cultivation and processing of Henna. Central Arid Zone Research Institute, India.pp 14,

21. , http:/www.tatoo-me.com/science.htm), The Scientific Literature on selected herb, Aromatic and Medicinal plants of the temperature zone, Archon books, pp.770, Hamden CT, Herbs: An index bibliography. Simon J., J.E, E.F, Chadwick and L.E Craker, 1984) *L.inermis* is planted today primarily as an ornament hedge, and is best known for its dried, ground leaves.

2.1.2 Antimicrobial activity of henna plant

Antimicrobial efficacy using bacterial cultures isolated from various skin diseases patients who attended dermatological clinics of common skin infections include cellulitis, impetigo, folliculitis and funicles and carbunches and noted that the most common cause of skin infections is *Staphylococcus aureus* which is frequently found in the nose and skin and its efficacy was compared with some commonly used antibiotics like Tetracycline, Ampicillin, Gentamycin and Ciprofloxacin.(http:/www.omjournal.org/originalarticles/FullText/2008/Antimi)

When comparing the minimum inhibitory concentration (MICs) of the extract with those of antibiotics some extracts showed pronounced antibacterial effects against isolated bacteria in-vitro than others, but no studies have been carried out to compare the extractive efficiency of different solvents as indicating in table one of this study results.

2.1.3 Henna as a medicinal plant

As a medicinal plant, *L.inermis* has been used for astringent, antihaemorrhagic, intestinal antineoplastic, cardiac inhibitory, antihypertensive, and sedative effects. It has been employed both internally and locally in jaundice, leprosy, small pox and infections of the skin. (http:/www.tatoo-me.com/science.htm, http:/kingtutshop.com/Egyptian-Herb/henna.htn, The Scientific Literature on selected herb, Aromatic and Medicinal plants of the temperature zone, Archon books, pp.770, Hamden CT, Herbs: An index bibliography. Simon J., J.E, E.F, Chadwick and L.E Craker, 1984

In Europe it has been used as a folk remedy against Amoebiasis, headache, bruises ranging burns to leprosy. (http:/kingtutshop.com/Egyptian-Herb/henna.htn, Blank T. et al, 1998, The body Shop Book of Wellbeing, Mind Body and Soul. Ebury press, London. Pp. 173-192)

Lawsonia inermis showed antibacterial, antifungal and ultraviolet light screening activity, it has showed ant infertility activity in animals and may induce menstruation. It is believed to have antiseptic properties as a cooling agent it is used for burning of skin. It also have great dandruff fighting ability, *L. inermis* is also used for rheumatic and arthritic pains. The antihaemorrhagic properties are attributed to lawsone. The naphthoguinone has emmenogogue and oxytocic actions. (http:/kingtutshop.com/Egyptian-Herb/henna.htn)

L. inermis features in Siddha system of medicine, as Siddlha physicians consider parts of henna to be astringent, detergent, deodorant, cooling and sedative.

2.1.4 Various parts of henna plant are used for treatment of different ailments

Fresh leaves are mixed with vinegar or lime juice is bandaged onto the soles of the feet to treat burning feet, a symptom of beriberi. Ground leaves applied to sore joints to ease rheumatism. The juice of the plant can be applied to the skin for headaches (rub), and the oil is applied to hair prevent it from going grey.

Its flower oil relieves muscular pains while its seeds are used as deodorant and regulates menstruation in female gynecological problems; *L.inermis* flower induces sleep, cure headaches and bruises.

Leprosy has been treated by *L.inermis* bark, as well as by an extract of leaves, flower and shoots. Bark has also been used to treat symptoms of jaundice and enlargement of the liver and spleen. It can be applied to the skin to treat eczema, scabies, fungal infection and burns. The Ayurvedic system uses the *L.inermis* leaves to treat vetiligo (pale patches on the skin where pigment is lost) and the seeds are used to cure fever, fruit oil is a folk remedy used in disorders causing hardening of the liver and diagram, and an ointment made from young fruit is used to prevent itching. (http:/www.tatoo-me.com/science.htm,

http:/www.tatoo-me.com/science.htm, The Scientific Literature on selected herb, Aromatic and Medicinal plants of the temperature zone, Archon books, pp.770, Hamden CT, Herbs: An index bibliography. Simon J., J.E, E.F, Chadwick and L.E Craker, 1984)

Oral administration of *L. inermis* leaf juice helps to reduce body weight as it dissolves breaks and flushes a way adipose / fat cells. (WWW.appliednutritional research.com)

2.1.5 Taxonomy (Scientific naming/classifications)

Kingdom – Plant

Division – Magnoliophyta

Class – Magnoliopsida

Order – Myrtales

Family – Lythraceae

Genus – Lawsonia

Species – *Lawsonia inermis*

Botanical name –*Lawsonia inermis* (http:/sen.wikipedia.org/wiki/Henna)

Other Local names of *L. inermis plant*:

Arabic : Henna

Kiswahili : Hina

Indian : Mehndi

Mexico : Mignorette, mendee

West Indies : Egyptian privef

(http:/www.tatoo-me.com/science.htm, http:/sen.wikipedia.org/wiki/

3.0 CHAPTER THREE

3.1 METHODOLOGY

3.2 Study design

The research study was carried out using plant (*Lawsonia inermis*) leaf which was air dried, ground then served to yield about 500g of fine powder then extracted, and used in the study.

3.3 Setting of the study.

The research conducted at Kampala International University (KIU) Western Campus -Bushenyi, western Uganda. The specimen of the leaf sample has been retained in the Pharmacy laboratory for reference purpose.

The Phytochemical screening and extracts residue weighing were assessed and determined in Pharmacy department while the antibacterial activity carried out in Microbiology department of KIU-western campus.

3.4 Plant identification and material collection:

In Ishaka Bushenyi-Uganda, the plant was identified by Mr. Byaruhanga Richard and proved by my supervisor Dr. Ssemirembe Richard and shown to Dr. Reddy - a Pharmacognosy Professor, both are lecturers at Kampala International University (W.C) Bushenyi Uganda.

Previously few leaves were collected from *L .inermis* shrubs growing naturally in Kabwohe Bushenyi – western Uganda in the month of July, 2009 but due to lack of access the plant in Bushenyi- Uganda and environmental condition instead leaves were obtained from a home/private garden, in Yombo Vituka village Temeke district in Dar-es-salaam city, Tanzania.

40 ripped lemon fruits and 5 liters of alcohol (methanol 99.5%) was bought for use as extracting solvents in this study. Distilled water was obtained from the Biochemical Department of KIU (WC). Other required materials, equipments and

instruments necessary for sensitivity test and detection of chemical constituents present in the plant leaf were requested and obtained from the respective university departments.

3.5 Sample Size Determination and Preparation.

A bout 3Kg of henna fresh leaves were collected during the day time around 10:00am; picked from the stems using hands cleaned by running tap water and then left to dry at room temperature for four days (96h). The dried leaves were ground to powder using a local pounding tool "Kinu na mche", sifted using a kitchen sieve packaged and stored in a cool place, dry and airtight containers.

3.6 Data collection technique

Data was started collected immediately from the beginning of the study by taking records of each procedure / activity being performed step by step using personal study log book until the end of the present research.

3.7 Data proving and analysis

The zone of inhibition of the extracts was measure using a calibrated ruler in millimeter with diameter of extract and standard drugs reference discs inclusive. Extract weighed and biological active constituents determined The results recorded..

3.8 Ethical Consideration.

The use of stock bacterial culture was requested from the Microbiology laboratory Technologist and the permission were given.

3.9 Inclusions and Exclusions criteria

Only fresh and healthy *L.inermis* leaves were collected dried and pound for extraction. Unhealthy, spoiled and damaged leaf was not sampled for the study.

3.10 Reliability/Validity

However the test organisms were obtained from the stock bacterial culture, the organisms selected for this study were previously tested for their effectiveness (alive or dead) before the main research being carried out

3.11 Extraction techniques. (Maceration /Infusion methods)

Three types of extracts were prepared in the present study; oily, alcoholic (by **maceration**) and hot-water (by **Infusion**) extracts. The oily extract was prepared by mixing 150g of dried powdered sample of *L. inermis* with 1500ml of lemon juice from freshly squeezed fruits for three days (72hs) while being stirred frequently in 24hs. The filtrate were then removed by applying a negative pressure first, the mixture was wrapped in the cotton cloth and squeezed slowly by slowly, then second, refiltered by using the cotton wool. The crude filtrate was poured into a known weighed beaker.

The alcoholic and water extracts were prepared by using the same procedure with exception that, alcohol (methanol 99.5%) and hot water (distilled) were used instead The crude extracts were then filtered by using cotton cloth first, then secondly refiltered by using Whatmann's filter paper No 42 [125mm].

Both filtrates were concentrated separately to obtain the residue by evaporation using hot- water- bath at 50°C. The crude residue was kept on the pharmacy laboratory bench in a dry and cool place for all the remaining solvent to evaporate, four days later, the weight of each sample were determined from each extract by using Electronic weighing balance.

3.12 Phytochemical screening procedures

The above mentioned extracts were subjected to preliminary phytochemical screening for detection of plant constituents the test for Tannins, phlobotannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides and reducing sugar were carried out as described

by Trease and Evans (1989), Sofowora (1993), Harborne (1973) and Nagawashi (2005). (The Henna Page – The Encyclopedia of Henna – Growing Henna)

Test for;

- **Tannins**

Small quantity of the extract was mixed with water and heated on water bath. The mixture was filtered and few drops of 0.1% ferric chloride were added to the filtrate. The brownish green colorations observed indicated the presence of tannins in the leaves.

- **Phlobatannins**

About 0.5g of the extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCL solution. No red precipitate was observed, indicating the absence of phlobotannins in the leaf.

- **Saponnins**

About 0.2g of the extract was shaken with 5ml distilled water and then heated to boil, formation of froths formed showed the presence of saponins

- **Flavonoids**

About 0.2g of the extract was dissolved in dilute NaOH, and HCl as added a yellow solution that turns colorless observed indicated the presence of flavonoids in the leaf.

- **Steroids**

2ml of acetic anhydride was added to 0.5g of extract with 2ml H_2SO_4. The colour changed from violet to blue in the plant showing the presence of steroids.

- **Terpenoids**

About 0.5g of the sample (extract) was dissolved in 1ml chloroform and 1ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration inter-phase formed indicated the presence of terpenoids.

❖ **Cardiac glycosides**

0.5g of the extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution, this was underplayed with 1ml of concentrated H_2SO_4. A brown ring inter-phase formed indicated a deoxysugar characteristic of cardenolides. A violet ring appeared below the brown ring, while in glacial acetic acid layer a greenish ring formed just gradually throughout the thin layer.

❖ **Reducing sugar**

0.5g extract was shaken with distilled water and filtered. The filtrate was boiled with drops of fehling's solution A and B for 2 minutes. An orange red precipitates appeared at the bottom of test indicating the presence of reducing sugar in the leaf.

3.13 Preparation of culture medium.

The Mueller Hinton sensitivity test Agar (MHA) was used to prepare the sensitivity test culture medium, the medium was prepared according to the manufacturer's instructions and sterilized by autoclave to 121°C for 15 minutes, left to cool to 50°C, then poured into petridishes of 90mm diameter each was filled with 25ml agar (to 4mm depth). (Monica Cheesbrough, 2004, District Laboratory Practice in Tropical Countries, Low price edition, Cambridge University press..- pp.136, P.A.Egwaikhide et al, 2008, Medwell journals, Studies on Bioactive Metabolites constituents and Antimicrobial Evaluation of leaf extracts of Eucalyptus *globules* Pp.44)

❖ Calculation for Preparation of the MHA medium

36g MHA powder to be dissolved in 1 liter distilled water (i.e. manufacturer's instructions, then to be sterilized at 121°C for 15minutes).

MHA medium preparation required to fill 10 petridishes (6 petridishes for testing medium and 4 petridishes were for reserve medium in case of contaminations). These were used to culture one out of two identified test organism. (i.e. 10 x 2=20 petridishes).

Each 90mm diameter petridishes required 25ml agar (i.e. 20 x 25ml = 500ml of total medium preparation adequate for both petridishes.) (Label on powdered culture media containers)

10ml added that may be lost due to evaporation during sterilization (500 + 10) ml = 510ml

Thus, if 36g MHA powder = in 1000ml distilled water

- ? - = in 510ml of MHA medium.

Then, $\frac{510 \times 36g}{1000}$ = 18.36g MHA powder

MHA powder needed to be dissolved in 510ml distilled water.

The mixture divided into 2 equal volumes in 250ml flasks boiled to dissolve before sterilization.

3.14 Preparation of the testing discs

The Disc diffusion Technique was used to investigate the antibacterial activity of *L.inermis* extracts. Test discs made by using filter paper cut in round-shaped using punching machine, soaked in MICs' extract solution for about 30min so that to absorb the extract then dried in a hot air oven for 20min at 50^{0}C, they were used as antibacterial testing strips. (Monica Cheesbrough, 2004, District Laboratory Practice in Tropical Countries Part 2, Cambridge University press, pp. 136-140)

3.15 Determination of the effectiveness of testing bacteria

❖ By Disc Diffusion Technique (Kirby Bauer Technique)

Two bacterial strains namely: *Staphylococcus aureus* and *Escherichia coli* were identified from Microbiology department (bacteria stock culture) of Kampala international university (W.C) and used in this study. The Discs diffusion Technique was used to investigate the antibacterial activity of *L.inermis* extracts.

Using a sterile inoculating wire loop, *S, aureus* bacteria strain was picked from the culture medium, 5ml of bacterial suspension made with nutrient broth in the sterile test tube. This preparation was inoculated onto one of the petridish containing MHA culture medium.

The petridish was shaken by swirling to ensure equal distribution of microorganisms onto the medium, then was allowed to stand so that to allow the suspension to sink in the medium. The discs impregnated onto the MHA culture medium which previously were inoculated with bacterial suspension of test organism.

The same procedure was repeated to determine the effectiveness of the second test organism, but this time the *E. coli* bacteria strain was used instead. Both two plates were incubated at 37°C for 18 hours. When observed, noted that all the two plates showed the growth. (Monica Cheesbrough, 2004, District Laboratory Practice in Tropical Countries, Low price edition, Cambridge University press. - pp. 136).

3.16 Studying the antibacterial activity of *L. inermis* leaf extracts.

By use of Disc diffusion technique (Kirby Bauer Technique) the antibacterial effect of three *L. inermis* extracts were tested against two above mentioned bacteria types (i.e. *S. aureus* and *E .coli*).

Onto each of the remaining eighteen out of twenty petridishes that containing the prepared Mueller Hinton sensitivity test Agar medium were inoculated with microorganisms suspension made by nutrient broth in sterile test tubes (using the same procedures as that were carried out on testing/checking for the effectiveness of test bacteria, the petridishes were then well numbered. By using a sterile straight forceps, the discs were impregnated to the bacterial culture medium with respect to the given numbers on the back of the petridishes at a distance of about 15mm from the petridish edge and 25mm from disc to disc. **Control experiments** using both extracting solvents and standard reference antibiotics were also set up. Both petridishes used for test & control experiments were incubated at 37°C for 24hours. (Monica Cheesbrough, 2004, District

Laboratory Practice in Tropical Countries, Low price edition, Cambridge University press. - pp.136)

3.17 Determination of zone of inhibitions and Minimum Inhibitory Concentration (MICs)

Inhibition of bacteria and the minimum inhibitory concentrations for each extract were assessed. Serial dilutions of different *L. inermis* extracts was conducted and MICs were obtained, then by using disc diffusion technique the zone size of inhibition of bacterial were assessed and measured using a calibrated ruler. (Label on powdered culture media containers)

❖ Serial dilution technique calculations

1. Test preparations were made by dissolving 10mg of each extract separately with 10ml of distilled water in a sterile test tubes (i.e. 10mg/10ml = mg/ml concentrations).
2. Serial dilution was then carried out as per diagrams below, starting with transferring of 1ml of mixture (preparation) from original tube to tube # 2 then another 1ml transferred to tube # 3 the same procedure repeated up to tube # 5.

❖ Calculations

Given C_1 = 10mg/10ml =1mg/1ml= 1000mcg/ml, is the concentration in tube #1

V_1 = 1ml

C_2 =?

V_2 = 1ml + 9ml = 10ml

Where C_1 = Concentration in tube # 1

V_1 = Volume of preparation picked from tube # 1

C_2 = Concentration in tube # 2

V_2 = Volume of mixture picked from tube 1 plus solvents in tube 2.

But $C_1 \times V_1 = C_2 \times V_2$

a) Concentration in tube 2 = mg /ml x ml = C_2 x 10ml

1mg = C_2 x 10ml

$C_2 = \frac{1mg}{10ml} = 0.1mg/ml = 100mcg/ml$

b) Concentration in tube 3

$100mcg/ml \times 1ml = C_3 \times 10ml$

$$C_3 = \frac{100mcg}{10ml} = 10mcg/ml$$

c) Concentration in tube 4

$10mcg/ml \times 1ml = C_4 \times 10ml$

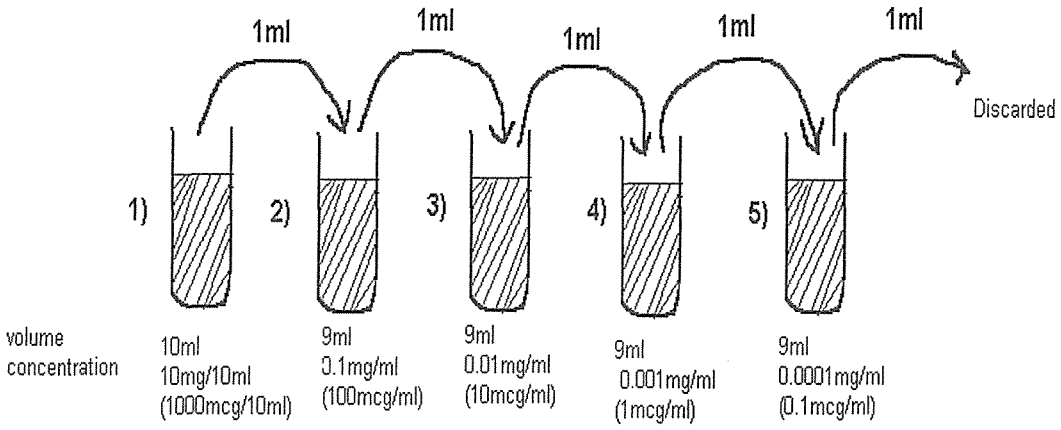
$$C_4 = \frac{10mcg}{10ml} = 1mcg/ml$$

d) Concentration in tube 5

$1mcg/ml \times 1ml = C_5 \times 10ml$

$$C_5 \ \frac{1mcg}{10ml} = 0.1mcg/ml$$

❖ **Serial dilution technique**



Key: 1, 2, 3, 4, and 5 = are test tubes

3.18 Comparing efficacy of henna extracts with standard antibiotics

Standard drugs; Tetracycline (10mcg), Ampicillin (10mcg), Ciprofloxacin (1mcg) and Chloramphecol (10mcg) antibacterial were used in this study for comparing with MICs of the antibacterial effect of *L.inermis* (henna) extracts. Mueller Hinton agar was used. Determination of MIC of henna extracts was carried out using serial dilution technique the zone of inhibition was measure using calibrated ruler.

4.0 CHAPTER FOUR

4.1 Results

4.1.1 Extractive value

Efficiency of different extractive solvents (extractive value) is indicated in Table 1 below.

Weight of dried crude powdered henna =150g
Weight of yield extracted by hot water =68.4g
% age yield due to hot water extraction method $\frac{68.4g}{150} \times 100 = \mathbf{45.6\%}$

Weight of dried crude powdered henna =150g
Weight of yield extracted by methanol 99.5% =27.9g
% age yield due to methanol extraction method $\frac{27.9g}{150} \times 100 = \mathbf{18.6\%}$

Weight of dried crude powdered henna =150g
Weight of yield extracted by lemon juice =86.0g
% age yield due to lemon juice extraction method $\frac{86.0g}{150} \times 100 = \mathbf{57.3\%}$

Table 1: Extractive values of *L. inermis* (leaf) of different solvents

Extraction method	Hot water (g)/%	Alcohol (g)/% Methanol 99.5%	Lemon juice (g)%
Cold	NE	27.9(18.6%)	86.0(57.3%)
Hot	68.4(45.6%)	NE	NE

Key: NE = Not Experimented (not done)

4.1.2 Phytochemical screening

The Phytochemical investigation of alcoholic, hot-water and lemon juice extract of *Lawsonia inermis* (leaf) were undertaken for the identification of the different type of chemical constituents present in the leaf. The plant studies showed that the leaf is rich mainly in Tannins, steroids and reducing sugar in all the extracts also terpenoids, and some saponins, flavonoids and cardiac glycosides were present but not in all extracts (Table 2).

Table 2: Phytochemical screening test results of *L. inermis* leaf extracts.

Test	Hot-water extract	Alcohol extract (99.5%Methanol)	Lemon juice(from fresh fruits)extract
Tannins	+	+	+
Phlobatannins	-	-	-
Saponins	-	+	-
Flavonoids	-	-	+
Steroids	+	+	+
Terpenoids	+	-	+
Cardiac glycosides	-	+	-
Reducing sugar	+	+	+

Key: + = present

\- = Absent

4.1.3 MICs with respective Zone of inhibition

When comparing the extracts' MICs effects with standard antibiotics, Hot water henna extracts showed pronounced antibacterial effects against *S .aureus* bacterial strain in-vitro but alcoholic and lemon juice henna extracts had relatively similar effects with those of antibiotics with a statistically significant difference between the effects of extracts and antibiotics (Table 3.)

Table 3: Minimum inhibitory concentration (MICs) with respective zone of inhibition (diameter in mm) against extractions, solvents and antibiotics used in the study.

Test organism (bacteria)	Grams stain	1. Extraction methods						2. Extraction solvents						3. Antibiotics							
		Hot water		Alcoholic		Lemon juice		Hot water		Methanol alcohol (99.5%)		Lemon juice (freshy squeezed)		Ampicillin		Tetracycline		Ciprofloxacin		Chloramphenicol	
		MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)	MICs (mcg/ml)	Inh.zone (mm)
S. aureus	+ve	100	**36**	100	**26**	100	**20**	1000	**Nil**	100	**8**	1000	**Nil**	10	**30**	10	**20**	1	**10**	10	**30**
E. coli	-ve	100	**14**	100	**20**	10	**7**	1000	**Nil**	100	**6**	1000	**Nil**	10	**Nil**	10	**40**	1	**30**	10	**Nil**

Key: Nil = No inhibitory zone was observed
S. aureus =*Staphylococcus aureus*
E. coli =*Escherichia coli*
MICs =Minimum Inhibition Concentrations (microgram per milliliter)

5.0 CHAPTER FIVE

5.1 Discussion

- The oily–based (lemon juice) *L. inermis* extractive value is higher than alcoholic and water extract indicating the superiority of oil-based *L. inermis* extraction process.

The general assessment shows that the extractive value result of lemon juice method is higher than that of hot water followed by alcoholic method. This indicated that the possibility of presence of considerable content of non-polar compounds in the leaf. As an advantage over other methods, possibly it penetrates easily and much into the henna plant cells and dissolves the constituents compares to other two solvents.

However, the hot water extract showed the highest inhibition zone size (36mm) diameter against *S. aureus* bacteria while alcohol extract showed the highest inhibition zone size (20mm) diameter against *E. coli* bacterial strain, the lowest MICs (10mcg/ml) was obtained from lemon juice against *E.coli* bacteria with inhibition zone size of 7mm diameter.

- **Phytochemical screening constituents application**

Tannins and flavonoids are known to have antibacterial and antifungal properties against *S. aureus & E. coli* bacteria strains (Khathem et al (2008), Altaway (1992), Futami(1991), existence of these compound implies that they can be used in treatment of ailments caused by these bacteria. (P.A.Egwaikhide et al, 2008, Medwell journals. Studies on Bioactive Metabolites constituents and Antimicrobial Evaluation of leaf extracts of *Eucalyptus globulus* Pp.44)

Steroids are class of compounds known for its pharmaceutical value to have relation with sex hormone, this may be a reason of why henna extract used to treat infertility in animal and induce menstruation since it serves as potent starting material in synthesis of these hormones (Sofowora and Okwu, 2001). (P.A.Egwaikhide et al, 2008, Medwell journals. Studies on Bioactive Metabolites constituents and Antimicrobial Evaluation of leaf extracts of Eucalyptus Globulus. Pp.44)

Saponins are class of compounds known to be effective in the treatment of syphilis and other venereal diseases (1993). (P.A.Egwaikhide et al, 2008, Medwell journals. Studies on Bioactive Metabolites constituents and Antimicrobial Evaluation of leaf extracts of *Eucalyptus globulus* Pp.44)

Reducing sugars are class of compounds known to propel metabolic processes in the body.

Terpenoids are class of compounds known and effective for raising cholesterol solubility in the body. (Sally Wehmeier, 2000, Oxford, Advanced Learner's Dictionary, 6th edn pp. 210.)

5.2 Conclusion

Lawsonia inermis (henna) plant leaf showed different range of extractive value with different solvents, however the oil based (lemon juice) method showed the highest extractive value/yield than aqueous and alcohol extraction methods.

The investigation of Phytochemical constituents in the henna leaf extract showed that the leaf is reach in tannins, steroids and reducing sugars which are useful as previously discussed under Phytochemical test results.

The leaf extracts has an in-vitro antibacterial activity against the tested bacterial strains which are responsible for the common internal and external wound and several other skin infections.

Hot-water extract have highest effect against *S. aureus* bacterial strain (susceptibility test), while alcohol extract showed the highest effect against *E.coli* bacterial strain with the same Minimum inhibitory concentrations (MICs). Lemon juice exhibited the least effective MIC compared to the other two solvents.

When compared with standard antibiotics Hot water henna extracts showed higher antibacterial activity while oily extracts showed relatively similar antibacterial effects like the commonly antibiotics which have been used in clinical practice. I conclude that henna has an in-vitro antibacterial activity against the tested bacterial strains.

❖ Limitations of the study:

The following aspects; lack of fund, lack of access to adequate research material (plant species) in nearby surroundings-Bushenyi, ineffectiveness of testing equipments (e.g. incubator in Histology Laboratory), lack of personal Computer, unpredictable/inconsistency power supply to operate working machines e.g. Autoclave, Incubator, Hot air Oven, Hot water bath are among aspects which delay this study.

5.3 Comments:

- However lack of modern equipments e g Chromatography equipments, lack of funds, attending usual class sessions were among the limitations that I faced during the time of conducting this study, there is a need for the University to have a special independent research laboratory which is fully equipped with basic tools for conducting research studies.
- I recommend that more research should be done on the pharmacological studies of antibacterial activity of *Lawsonia inermis* leaf, interpretation and standardization of the zone size of inhibition of the henna leaf extracts.
- Antifungal activity of the henna leaf should be investigated.
- Chromatographically, the leaf constituents should be isolated, identified and quantified for further pharmacological assessment activity.

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5.5 APPENDICES:

5.5.1 Picture 1 of *Lawsonia inermis* (Henna) plant

5.5.2 Picture 2 of *Lawsonia inermis* (Henna) plant

Thank you