

**A STUDY COMPARING THE ANTI-MICROBIAL
EFFECTIVENESS OF A WATER-BASED PREPARATION OF
HYDRASTIS CANADENSIS TO THAT OF AN ETHANOL BASED
PREPARATION ON THE *IN VITRO* GROWTH OF *CANDIDA*
ALBICANS AND *ESCHERICHIA COLI*.**

**BY
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A mini-dissertation submitted in partial compliance with the requirements for a
Master's Degree in Technology: Homoeopathy
at the Durban Institute of Technology.

I, Deena Naidoo do declare that this mini-dissertation represents my own work in both
conception and execution.

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DEDICATION

I would like to dedicate this work to my divine teacher and master Bhagawan Sri Sathya Sai Baba, it is with his guidance that I have completed my training as a homoeopathic physician.

I also thank my parents Siva and Nomie and my brother Mervin for the tremendous sacrifices that they have made for me these past six years of my life.

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ABSTRACT

The purpose of this study was to compare the effect of a water-based extraction of *Hydrastis canadensis* on the *in vitro* growth of *Candida albicans* and *Escheria coli*, to a control of distilled water in terms of the size of the zones of inhibition in the disc diffusion assay and turbidity of growth in the tube dilution method. These results were compared to the results obtained from similar experiments using an ethanol-based tinctures of *Hydrastis canadensis*.

Various studies have shown that ethanol-based herbal tinctures possess anti- microbial properties (Reid, 2002). Due to the fact that ethanol itself is anti- microbial, this study sought to determine whether the herb itself possesses anti- microbial properties. The anti- microbial effect of a water-based extraction of *Hydrastis canadensis* against *C. albicans* and *E. coli* were therefore assessed using the Kirby-Bauer Anti-microbial Sensitivity test. Parceval Laboratories Pty. (Ltd), using an adjusted version of method HAB 4a of the German Homeopathic Pharmacopoeia, prepared the water-based extraction. In addition to this, the tube dilution method was used to establish the Minimum Inhibitory Concentration (M.I.C) of the *Hydrastis canadensis*. A 62% ethanol tincture of *Hydrastis canadensis* was also tested on *E. coli* and *C. albicans*.

For this study a total of twelve plates were used, six Muller-Hinton Agar (M.H.A) and six Sabourauds Dextrose Agar (S.D.A) for the initial experiment. The blank susceptibility disc was inoculated with respective test and control substances, using a micropipette set at 10 µl. A total of two discs were placed equidistantly apart. The tube dilution method used a total of 20 test tubes. The organisms were arranged into two separate groups, with each group having 10 test tubes. Dilutions of *Hydrastis canadensis* (1:2, 1:3, 1:5 and 1:10) water extraction and ethanol tincture were used. The plates and test tubes were incubated at 37°C for 24 hours. The zones of inhibition formed on the plates were measured using Vernier callipers. The growth inhibition displayed in the tube dilution experiment was measured by comparing it to the Macfallans turbidity standard, which was set at 0.5 Macfallans.

A second experiment was conducted on *Hydrastis canadensis* water extraction after the researcher suspected contamination, when the results obtained from the tube dilution

method did not correspond to that hypothesized. It was found that the original water-based extraction of *Hydrastis canadensis* contained contamination.

A further test was also conducted to evaluate the efficacy of filter paper discs in holding 10 µl of the test and control substances compared to a 10 µl drop on the agar surface. This was proposed following the failure of *Hydrastis canadensis* water-based extraction and ethanol-based extraction to inhibit the *in vitro* growth of *C. albicans* and *E. coli* when using blank susceptibility disc. The results concluded that there was no significant difference between the drop and the disc, in terms of the size of the zones of inhibition both produced.

Non-parametric statistical analysis in the form of the Mann Whitney U Test was applied to perform the relevant inter-group analyses, and significance levels were set at 0.05. Data entry and analysis was done using SPSS for Window®, version 9.

The results obtained showed that the *Hydrastis canadensis* water extraction was ineffective in inhibiting the *in vitro* growth of both *E. coli* and *C. albicans* for both the disc diffusion and the tube dilution method. The *Hydrastis canadensis* ethanol tincture was also not effective in inhibiting the *in vitro* growth of *E. coli* and *C. albicans*, in the tube dilution method and the disc diffusion assay.

The results concluded that *Hydrastis canadensis* water extraction was ineffective in inhibiting the *in vitro* growth of both the *E. coli* and *C. albicans* when evaluated in terms of the disc diffusion assay and the tube dilution method.

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DEFINITIONS OF TERMS

Antibiotic Sensitivity Test – A test in which chemical substances capable of killing or inhibiting the growth of micro-organisms are added to suspensions of the intended microorganism and allowed to grow overnight in order to determine the bactericidal or bacteriostatic effect of the chemical substance. (Fong *et al.*, 1995: 407–433)

Cholecystitis – Term used for the inflammation of the gall bladder. (Roper, 1994: 56)

Dysphagia – Difficulty in swallowing. (Roper, 1994: 90)

Dysuria – Painful urination (Roper, 1994: 91)

Gram-stain – A bacteriological stain for differentiation of micro-organism. Those retaining the blue dye are Gram-positive, those unaffected by it are Gram-negative. (Roper, 1994: 121)

Haematemesis – Defined as the vomiting of blood. (Roper, 1994: 122)

Hypothyroidism – Defines those clinical conditions, which result from sub-optimal circulating levels of one or both thyroid hormones i.e. T3 or T4. (Roper, 1994: 138)

In vitro – Refers to biological experiments done in Petri plates (Petri dishes) or test tubes. (Fong *et al.*, 1995: 407-433)

In vivo – “in living”, refers to laboratory experiments testing chemical agents in a living organism. (Fong *et al.*, 1995: 407-433)

Leucoplakia – White thickened patch occurring on the mucous membranes i.e. inside of mouth, genitalia or on the lips. (Roper, 1994: 158)

Septicaemia – The persistence and multiplication of living bacteria in the blood (Roper, 1994: 241)

Tyramine – An amine found in foodstuffs, especially cheese. Tyramine has similar effects as adrenaline in the body. (Roper, 1994: 272)

Turbid – Cloudy state of a solution or substance. (Fong *et al.*, 1995:407-433)

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

Hydrastis canadensis has recently gained a reputation as an herbal antibiotic and immune system enhancer due to one of its active ingredients berberine. Berberine's action in inhibiting *C. albicans*, as well as other pathogenic bacteria, prevents the overgrowth of yeast that is a common side effect of antibiotic use. (Pizzorno and Murray, 1999: 775-777)

Pregnancy, diabetes mellitus and antibiotic treatment are the most common predisposing factors for *C. albicans* infection. Alcohol administration by means of medicinal or recreational to these patients is also undesirable or contra-indicated. Alcohol can cause problems in pregnancy such as bleeding, miscarriage, stillbirth and -premature birth. The World Health Organization (W.H.O.) suggests that there is really no safe level of alcohol consumption during pregnancy and abstaining completely is the safest option. (<http://www.adf.org.au/adp/index.html>) [Accessed 17 November 2003]

Numerous studies have been conducted to demonstrate the effectiveness of herbal extracts as anti-microbial agents. In one study conducted in Kenya in 2003, aqueous and ethanol extracts of 12 plant species, traditionally used in Kenya for treatment of ailments of infectious and/or inflammatory nature were screened for *in vitro* antibacterial and anti-inflammatory activities. Antibacterial activity was tested using the agar diffusion method. All the antibacterial activity was against Gram-positive bacteria with nine plant species showing some activity against *Staphylococcus aureus*. All the plant species showed some anti-microbial activities. In most cases, ethanol extracts caused higher inhibition than aqueous extracts. (Matu *et al.*, 2003: 35-37)

Budree (2003) demonstrated in his research that *Hydrastis canadensis* 62% v/v tincture exhibited an inhibitory effect on the *in vitro* growth of *C. albicans* producing a large zone of inhibition superceding the zone produced by the antibiotic substance Fluconazole, which was the positive control in his experiment. He also demonstrated that the 1:50 aqueous dilution of *Hydrastis candensis* 62 % v/v tincture was shown to be the Minimum Inhibitory Concentration (M.I.C). (Budree, 2003:139)

A study conducted in 1998 at the University of Pretoria, by the Faculty of Medicine Research Committee, in which 5 types of extractants were tested for the screening and isolation of anti-microbial components from plants namely, acetone; methanol; methylene dichloride; a mixture of chloroform, methanol and water; and water. The research found that after testing all parameters, no extractant was uniformly better than the other. (Eloff, 1998: 6) The choice of solvent, according to Eloff (1998), depends on what is intended with the extract. He concluded that when conducting an experiment to screen plants for anti-microbial components, the effect of extractant on the subsequent separation procedure is not important, but the extractant should not inhibit the bioassay procedure. (Eloff, 1998: 2)

In this study, the effectiveness of a water-based extraction of *Hydrastis canadensis* as an anti-microbial agent was tested against *C. albicans* and *E. coli*. These organisms have recently increased in terms of their infection rate, more so amongst the immuno-compromised patients. These organisms pose major problems to health care workers as they are becoming resistant to anti-microbial therapy. (Weinstein, 1998:102)

The study was conducted *in vitro* using both the disc diffusion assay and the tube dilution test. The zones of inhibition produced by both the test samples determined the anti-microbial sensitivity. The tube dilution method of measurement of microbial growth was compared to the Macfallans turbidity standard set at 0.5, which is equivalent to a 3+ growth. (Singh, 2003)

1.2 PROBLEM STATEMENT

The purpose of this study was to investigate the effectiveness of *Hydrastis canadensis* water-based extraction in inhibiting the *in vitro* growth of *E. coli* and *C. albicans*, in terms of the disc diffusion assay and the tube dilution test and to compare the results with similar experiments using ethanol tinctures.

1.3 SUB-PROBLEMS

1.3.1 Sub-problem one

To evaluate the effectiveness of *Hydrastis canadensis* water extraction in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in terms of the sizes of the zones of inhibition in the disc diffusion assay, and the turbidity of the solution in the tube dilution method.

1.3.2 Sub-problem two

To evaluate the effectiveness of *Hydrastis canadensis* 62% v/v ethanol tincture in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in terms of the sizes of the zones of inhibition in the disc diffusion assay, and the turbidity of the solution in the tube dilution method.

1.3.3 Sub-problem three

To compare the effectiveness of the *Hydrastis canadensis* ethanol tincture with that of the *Hydrastis canadensis* water extraction on the inhibition of the *in vitro* growth of *E. coli* and *C. albicans*.

1.4 HYPOTHESES

All hypotheses are stated in the null form.

1.4.1 Hypothesis one

It is hypothesized that *Hydrastis canadensis* water-based extraction will have no significant effect in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in terms of the size of the zones of inhibition in the disc diffusion assay and in the turbidity of the solution in the tube dilution method.

1.4.2 Hypothesis two

It is hypothesized that *Hydrastis canadensis* ethanol tincture will have no significant effect in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in terms of the size of the zones of inhibition in the disc diffusion assay and in the turbidity of the solution in the tube dilution method.

1.4.3 Hypothesis three

It is hypothesized that there will be no significant difference between the ethanol and water extraction of *Hydrastis canadensis*, in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in terms of the size of the zones of inhibition in the disc diffusion assay and in the turbidity of the solution in the tube dilution method.

1.5. DELIMITATIONS

1. This study was limited to one strain of *E. coli* and one strain of yeast namely *C. albicans*.
2. This study was limited to a single herb namely *Hydrastis canadensis*, which was tested in two forms, a water extraction and an ethanol tincture.
3. Only tincture produced in 62% (v/v) ethanol was used.
4. This study was limited to two methods of anti-microbial sensitivity namely disc diffusion assay and tube dilution method.
5. This was an *in vitro* study.

CHAPTER TWO

LITERATURE REVIEW

2.1 *HYDRASTIS CANADENSIS*

2.1.1 Family

Hydrastis canadensis is a plant that is classified under the *Ranunculaceae* family. (Hoffman, 1997: 204) - (See Appendix A)

2.1.2 Common name

Hydrastis canadensis is known by common names such as *goldenseal*, *yellow root*, *Indian tumeric*, *eye root*, *jaundice root*. (Pizzorno and Murray, 1999: 776)

2.1.3 History

Hydrastis canadensis (*Goldenseal*) is a plant that was first discovered and used by Native American Indian people for medicinal purposes. The discovery of the plant by white settlers was only after they had crossed the Appalachians. The plant being a native of heavily shaded woodlands in the midwestern United States, it is becoming rare due to over picking. It was used widely for curing infections and as a general tonic for the mucous membranes of the body and digestive tract. The Cherokee and other Indian tribes used *Hydrastis canadensis* in disorders of the eye and skin. (Pizzorno and Murray, 1999: 776)

2.1.4 Plant sections used

The dried rhizome and roots of the plants are used. (Hoffman, 1997: 204)

2.1.5 Collection

The three-year-old *Hydrastis canadensis* plant is picked in the autumn season, after the seeds are dried. The root and rhizome are carefully cleaned and allowed to dry. . (Hoffman, 1997: 204)

2.1.6 Chemical composition

Alkaloids isolated from *Hydrastis canadensis* include the following chemical compounds:

- Hydrastine (1.5 – 4.0 %)
- Berberine (0.5- 6.0%)
- Berberastine (2.0- 3.0%)
- Canadine
- Candaline
- Hydrastinine
- Meconin
- Chlorogenic acid
- Phytosterins
- Resins. (Pizzorno and Murray, 1999: 776)

2.1.7 Antibiotic activity

Hydrastis canadensis has recently gained a reputation as an herbal antibiotic and immune system enhancer. Berberine (the active ingredient in *Hydrastis canadensis*) has the ability to inhibit pathogenic bacteria e.g. *E. coli*, *Salmonella typhi* and *Vibrio cholerae*. It also prevents the overgrowth of yeast e.g. *C. albicans*. The anti-microbial activity of berberine increases with pH. At pH 8.0, it's anti-microbial activity *in vitro* is typically two to four times greater than it is at pH 7.0, which in turn is one to four times greater than at pH 6.0. (Pizzorno and Murray, 1999: 775-777)

2.1.8 Immune enhancing properties

Berberine contained in *Hydrastis canadensis*, has the ability to increase the amount of blood supply to the spleen, which then promotes the release of compounds such as tuftsin, a potent immune stimulator. The berberine also stimulates macrophage activity by the process of enhanced priming and triggering. (Pizzorno and Murray, 1999: 775-777)

2.1.9 Coagulating properties

Hydrastis canadensis is excellent in stopping bleeding from gushing wounds that are clean. It will aid the wound by stopping the bleeding and bringing the edges of the wound together. *Hydrastis canadensis* is however contra-indicated in dirty or infected wounds, which will only help by sealing in the dirt and the pus. (Wood, 1997: 300)

2.1.10 Anti-cancer properties

Berberine has the ability to kill tumor cells directly or by stimulating the white blood cells of the body against them. (Pizzorno and Murray, 1999: 775-777) A study that was conducted in 1990, demonstrating the antitumour activity against human and rat malignant brain tumor using berberine. It was shown that berberine used alone at a dose of 150mg/ml showed an average cancer kill of 91%. The combination of berberine and BCNU (the standard chemotherapeutic agent used for brain tumours) yielded a much more potent chemotherapeutic agent, exceeding the rate that the chemotherapeutic agent could achieve on it's own. (Rong-xun, 1990: 103., 658-665)

2.1.11 Clinical application

Diarrhea

Berberine containing plants are effective in treating diarrhea associated with infection by *E. coli*, *Shigella dysenteriae* (*shigellosis*), *Giardia lamblia* (*giardiasis*) and *Vibrio cholerae* (*cholera*). (Pizzorno and Murray, 1999: 775-777)

Complications related to the liver

Berberine has demonstrated in several clinical trials that it stimulates the secretion of bile and bilirubin. *Hydrastis canadensis* is effective in treating the hypertyraminemia of patients suffering from liver cirrhosis. Tyramine is responsible for some of the complications associated with chronic liver disease e.g. Hepatic encephalopathy and other cardiovascular and neurological complications. The total serum tyramine levels and its derivatives may induce cardiac and renal output dysfunction by lowering peripheral resistance, together with cerebral dysfunction. This information shows us the importance of *Hydrastis canadensis* in lowering tyramine levels and thereby preventing the complications of liver cirrhosis. (Pizzorno and Murray, 1999: 779)

As can be seen from the above paragraph, *Hydrastis canadensis* is useful for patients with liver disease. These patients however should not consume alcohol in any form, as it can be detrimental. (Pizzorno and Murray, 1999: 779) This research aimed to find a safer vehicle to administer *Hydrastis canadensis* and therefore investigated the anti-microbial properties of water-based extractions of *Hydrastis canadensis*.

2.1.12 Dosage

Herbalists prepare Hydrastis canadensis in three main ways:

1. Infusion (boiling water is poured over the dried herb);
2. Decoction (the plant is boiled in a pan);
3. Tinctures (the plant is soaked in alcohol which allows the active ingredients to leach out).

2.1.13 Manufacturing process of herbal tinctures

- **Collection and harvesting**

When plants are collected from their natural habitat, they are said to be "wild-crafted". When they are grown utilizing commercial farming techniques, they are said to be "cultivated". The mode of harvesting varies from hand labor to very sophisticated equipment. The mode is not as important as the time of harvesting. A plant should be harvested when the part of the plant being used contains the highest possible level of active compounds. Again, this is ensured by the use of proper analytical techniques. (Hopkins, 2002)

- **Drying process**

After harvesting, most herbs have moisture content of 60-80%, and cannot be stored without drying. Otherwise, important compounds would break down, or microorganisms could contaminate the material. The majority of herbs require relatively mild conditions for drying. Commercially, most plants are dried within a temperature range of 48 - 70 °C. During drying, the plant material must not be damaged, or suffer losses, that would prevent it from conforming to accepted composition standards. With proper drying, the herb's moisture content will be reduced to less than 14 %. (Hopkins, 2002)

- **Garbling Process**

Garbling refers to the separation of that portion of the plant to be used from other parts of the plant, dirt, and other extraneous matter. This step is often done during the collection process. Although there are machines that perform garbling, it is usually performed by hand. (Hopkins, 2002)

- **Grinding Process**

Grinding, or mincing, an herb means mechanically breaking down either leaves, roots, seeds, or other parts of a plant into very small units ranging from larger coarse fragments to fine powder. Grinding is employed in the production of crude herbal products as well as in the initial phases of extracts. Often the material has to be pre-chopped, or minced, before feeding it into a grinder. In the process of grinding, a number of machines can be used, but the most widely used is the hammer mill. These machines are simple in design. The hammers, arranged radially, follow the rotation of the shaft to which they are attached, breaking up the material that is fed into the machine from above. On the walls of the chamber is a grid, which determines the size of the material that is passed through it. Other types of grinders include knife mills and teeth mills. (Hopkins, 2002)

- **Evaluation of herbal preparation:**

- Organoleptic means the “impression of the organs”. This method of analysis involves the application of sight, odor, taste, touch, to identify the plant. This means that the first time an herb is brought into contact with the human senses, it can be readily identified. This method represents the simplest way of identifying an herb. (<http://www.viable-herbal.com/herbology1/herbs41.htm>) [Accessed 27 April 2004]
- There are herbs that are available in different forms e.g. powder or dried forms and therefore are only identified by microscopic examination. This method is also indispensable in identifying plants, because they display characteristic tissue structures and arrangement. The identification of parasites found on the herb e.g. molds, insects, faeces and fungi, is also made possible through this method therefore preventing these herbs from being used in the production of medicinal tinctures. (Hopkins, 2002)
- In the physical or crude plant identification method, the solubility, specific gravity, melting point, water content, degree of fiber elasticity, and other physical characteristics of the herb material are determined, as crucial elements in the identification of the plant. (<http://www.viable-herbal.com/herbology1/herbs41.htm>) [Accessed 27 April 2004].
- Various chemical / physical methods also are used to determine the percentage of active principles, alkaloids, flavonoids, enzymes, vitamins, essential oils, fats, carbohydrates, protein, ash, acid-insoluble ash, or crude fiber present. (Hopkins, 2002)
- The final analytical process requires more precise assays to determine quality. Sophisticated techniques, such as high-pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR), are often used to separate out molecules. The readings from these machines provide a chemical "fingerprint" as to the nature of chemicals contained in the plant or extract. These techniques are
- Invaluable in the effort to properly and fully identify herbs, as well as standardized extracts. The plant or extract can then be evaluated by various biological methods to determine pharmacological activity, potency, and

toxicity. (<http://www.viable-herbal.com/herbology1/herbs41.htm>) [Accessed 27 April 2004]

2.1.14 Herbal extracts

An extract is defined as a concentrated form of the herb obtained by mixing the crude herb with an appropriate solvent (such as alcohol and/or water). Most extracts that are produced by small manufacturers use maceration procedures. The simplest process consists of soaking the herb in the alcohol/water solution for a period of time (depending on the herb), and then filtering. Since tinctures are 1:5 concentrates; this means 80% of the bottle is alcohol & water, and only 20% herbal material. Larger manufacturers utilize more elaborate techniques to ensure that the herb is fully extracted and the solvent is reused. For example, a counter-current extraction process is often used. In this process, the herb enters into a column of a large percolator composed of several smaller columns. The material to be extracted is pumped through the different columns at a given temperature and flow speed, where it continuously mixes with solvent. The extract-rich solvent then passes into another column, while fresh solvent once again comes into contact with herbal material as it is passed into a new chamber. In this process, complete extraction of health promoting compounds can be performed. (<http://www.wala.de/english/GmbH/index.htm>) [Accessed 24 April 2004]

These forms (**tinctures, fluid extracts, and solid extracts**) are commonly used by herbal practitioners for medicinal effects:

- **Tinctures** are typically made using an alcohol and water mixture as the solvent. The herb is first soaked in the solvent for a specified amount of time, which usually depends on the herb itself. The soaking period is usually from several hours to several days in length, and some herbs may be soaked for much longer periods of time. The solution is then pressed out, and the tincture is collected as the end product. (<http://www.weleda.co.nz/news/2001-05.cfm>) [Accessed 27 April 2004]
- **Fluid extracts** are more concentrated than herbal tinctures. This is commonly a hydro-alcoholic mixture, but other solvents may be used e.g. (**vinegar, glycerin, propylene glycol, etc.**). Distilling off some of the alcohol, typically by using vacuum distillation and counter-current filtration usually makes commercial fluid extracts. The manufacturing of fluid extracts using old percolation method is still preferred by some manufacturers.

- A **solid extract** is produced by further concentration of a fluid extract as well as by other techniques such as thin layer evaporation. The solvent in this process is removed completely leaving behind a viscous extract (soft solid extract) or a completely dry solid extract (usually the part of the plant used e.g. leaves, roots, barks, stems.) The solid extract once dried is ground into a fine powder or coarse granules form. These solid extract forms can revert to a fluid extract or tincture once the respective solvent is added to it e.g. alcohol or distilled water. (<http://www.viable-herbal.com/herbology1/herbs41.htm>) [Accessed 24 April 2004]

Water-based extraction - Plant material (either root, leaves, stems or barks) of the herb used, is first weighed into a glass jar. After comminution or pulverizing, 1 part plant material is mixed with 10 parts heated distilled water in a container. The essences are then subjected to rhythmic light (i.e. the mixture is exposed to sunlight at different time intervals) and movement processes. After a week the essences are pressed and filtered. Thereafter it is pressed through 100 % cotton cloth and filtered through Whatman® filter paper number 1. Fieter, U. (parceval@adept.co.za) [Accessed 26 April 2004]. E-mail to Naidoo, D. (Dean20@webmail.co.za)

2.1.15 Adverse effects of alcohol

- **Introduction**

One of the difficulties in treating patients with alternative medicines that contain ethanol as a base substance is the religious prohibition. There are some patients who need treatment urgently but refuse due to the presence of alcohol in the medicines. The following religions are among the largest in the world, that prohibits alcohol in medicines to some degree.

- **Religious prohibition**

Islam

Islam is one of the religions that are very strict when it comes to the consumption of alcohol, in any form. They believe that any medicine containing alcohol, even in

minute quantities is prohibited for a *Muslim* to consume.

(<http://www.khomeini.com/gatewaytoheaven/PracticalLaws/HazarathAlikhamenei/MiscellaneousFatwas.htm>) [Accessed 05 May 2004]

Christianity

In the Christian faith there are many conflicting views on the use of alcohol for medicinal purposes, some Christians believe that it is strictly prohibited, as it alters the mental thought process in any quantity. The Bible talks about Christ refusing intoxicating wine even to relieve his pain and agony of the Cross, this to some Christians is a testimony of his strong disapproval of intoxicating beverages, even to relieve his pain. Proverbs 31:6 states that alcohol be administered to the sick as a pain reliever. This applied to our times, suggests that drugs may be given to a patient only on the order of a qualified physician.

(<http://www.biblicalperspectives.com/endtimeissues/eti26.pdf>)

[Accessed 05 May 2004]

Seventh day Adventist

The most devout Seventh Day Adventists are vegetarians, and consumption of coffee, tea, and alcohol are forbidden as they are regarded as substances altering the mind. (Bosley, 1995:24-25)

- **Pregnancy**

A study conducted at Michigan State University in America in 2001, reported that low doses of alcohol consumed pre-natally was linked to behavioural problems. Maternal alcohol consumption even at low levels was adversely related to child behavior, resulting in the identification of a dose-response relationship. The effect was observed at average levels of exposure of as little as 1 drink per week. Children with any prenatal alcohol exposure were 3.2 times as likely to have delinquent behavior scores in the clinical range compared with non-exposed children. The relationship between prenatal alcohol exposure and adverse childhood behavior outcome persisted after controlling for other factors associated with adverse behavioral outcomes. Pregnant women often ask clinicians if small amounts of alcohol intake are acceptable during pregnancy. These data suggest that no alcohol during pregnancy remains the best medical advice. (Sood *et al.*, 2001: 34)

2.2 CANDIDA ALBICANS

2.2.1 Classification

C. albicans belongs to the genus *Candida*, family *Cryptococcaceae*, within the *Fungi Imperfecti* (deuteromycetes) (Barlows *et al.*, 1991: 5)

Candida species are classified as yeast that is a fungi with a predominantly unicellular mode of development. The genus *Candida* as a whole comprises more than 150 species, whose main feature is the absence of any sexual form. They are classified in this genus by default rather than by design and the medically significant species represents only a minority subset of a large and widely disparate group. (Odds, 1994: 7)

2.2.2 Morphology and identification

C. albicans that are found in culture or tissue samples, grow as oval, budding yeast cells approximately 3-6 µm in size. In tissue sections *C. albicans* demonstrates yeast like forms (blastoconida), ‘pseudohyphae’, and true hyphae. (Kumar *et al.*, 2003: 492)

The formation of pseudohyphae occurs when the buds grow, but do not detach. This then allows for the formation of chains of elongated cells, that are ‘pinched’ or constricted at the separation points between cells. (Jawetz *et al.*, 2001: 550). Cream coloured colonies with a fermented or yeasty odour starts to grow on the agar after a period of 24 hours at 37°C or room temperature. The production of ‘chlamydospores’ is one of the diagnostic features of *C. albicans*. (Elmer, 1997: 1043)

Chlamydospores are thick walled cells that are subtended from the pseudohyphae of *C. albicans* by specialized cells called suspensor cells. The staining procedures for detection of most yeast in microbiological practice are the Gram stain and the potassium hydroxide “wet preparation”, with all yeast-staining gram positive. (Odds, 1994: 58)

The two microscopic features that are used to differentiate *C. albicans* from other species of *Candida* are the following:

- Following a period of incubation of approximately 90 minutes at 37°C in serum, the formation of true hyphae or germ tubes can be seen as the yeast cells of *C. albicans* replicates.
- The formation of chlamydospores, which are characteristically large and spherical, occurs following incubation on a nutritionally deficient media.

2.2.3 Epidemiology

C. albicans together with a few other yeast are common and harmless commensals of the mucous membranes and digestive tracts of normal healthy individuals.

(Greenwood *et al.*, 1994: 682)

2.2.4 Candidiasis

Candidiasis is the infectious disease manifestation of *C. albicans* and other yeasts of the genus *Candida*. The disease Candidiasis arises after the individuals immune resistance is decreased by illness, iatrogenic or physiological causes, which then allows for the overgrowth of their resident yeast flora. (Odds, 1994: 68)

Candidiasis occurs primarily in three different ways i.e.

- Mucocutaneous
- Cutaneous
- Systemic. (Elmer, 1997: 1044)

The most common of the above three types is the superficial mucocutaneous type, infecting the oral cavity. In superficial candidiasis (mucocutaneous and the cutaneous type), the *C. albicans* invades the epithelium or skin at the area where it is damaged or the weakest. The infection is limited to the local area of invasion. In systemic Candidiasis, *C. albicans* invades the bloodstream after the phagocytic cells of the host defences fail to contain the growth and dissemination of the yeast. *C. albicans*, once in the circulation can infect other organs for example, the kidney, the spleen, prosthetic heart valves and cause infection anywhere. (Jawetz *et al.*, 2001: 550)

2.2.4.1 Candidiasis of the oropharynx

Oral Candidiasis or Thrush is the most common clinical manifestation of Candidiasis in humans. (Elmer, 1997: 1046). This form of Candidiasis is characterized by the formation of white patches and plaques that follow a confluent pattern and resemble

milk curds. The areas that are mostly affected are the mucosae of the mouth, throat, gums and the surface of the tongue. The plaques in oral thrush are made up of necrotic material and desquamated, parakeratotic epithelia, invaded by the hyphae and yeast cells of *C. albicans* as they work their way into the stratum corneum of the epithelium (Odds, 1994: 119)

Oral thrush develops in most patients who suffer from Acquired Immuno- deficiency Syndrome (AIDS) and other risk factors include treatment with corticosteroids or antibiotics, infancy and old age, hyperglycaemia and systemic diseases e.g. hypothyroidism. (Jawetz *et al.*, 2001: 550). Chronic oral candidiasis may lead to extensive leucoplakia and angular cheilitis. (Greenwood *et al.*, 1992: 682) The clinical symptoms associated with oral thrush include pain, anorexia, nausea and vomiting. In some individuals incorrect fitment of dentures may cause oral candidiasis. This condition is called denture stomatitis and the symptoms include erythema and oedema of the portion of the palate that comes into contact with dentures. (Odds, 1994: 119)

2.2.4.2 Esophageal candidiasis

A common condition associated with AIDS patients and those that have haematolymphoid malignancies. (Kumar *et al.*, 2003: 492). The patient suffering from oesophageal candidiasis, presents clinically with dysphagia, retosternal pain and upper gastro-intestinal bleeding. Oesophageal candidiasis may also spread from a pre-existing oral thrush, especially in newborns. (Elmer, 1997: 1046)

2.2.4.3 Candidiasis of the gastro-intestinal tract

- **Gastric candidiasis**

C. albicans usually invades pre-existing gastric lesions e.g. gastric ulcers and sites of previous gastric resection. The candida thereafter produces 'thrush like' plaques, which can be seen by endoscopic examinations. The symptoms of the yeast infection are usually pain related to the stomach region together with vomiting and haematemesis. (Odds, 1994: 160)

- **Intestinal candidiasis**

This type of infection occurs predominately in individuals who have undergone major gastro-intestinal surgery or have cancer of the intestinal tract. (Elmer, 1997: 399)

- **Candida cholecystitis**

This rare type of Candidiasis *is* more likely due to the dissemination of the organism in the blood stream, and therefore the formation of abscesses in different organs of the body by the yeast. The pathology is related to the development of a candidal mass that blocks the common bile duct causing the cholecystitis. (Kumar *et al.*, 2003: 492).

2.2.4.4 Candidiasis of the respiratory tract

Candidiasis of the trachea and larynx can spread from infection in the pharynx. Infection of the lower respiratory tract with *C. albicans* *is* mostly a result of disseminated candidiasis, where the *C. albicans* *is* in the bloodstream. The incidence of candidiasis occurring primarily in the bronchial or pulmonary trees via the airways is extremely rare or undocumented. (Odds, 1994: 165)

2.2.4.5 Candida endocarditis

Patients who have undergone valve replacement surgery or have intracardiac indwelling catheters, following major cardiac surgery are at risk of developing this disease especially after the onset of an episode of septicaemia if neglected. *C. albicans* has displayed the ability to adhere to blood clots in an *in vitro* study and therefore poses the threat of dislodging with the blood clot, and causing infections at distant sites in the body. (Odds, 1994: 175).

2.2.4.6 Candidiasis of the genitalia

The invasion of the vaginal mucosae by *C. albicans* leads to the common condition referred to as *candida* vaginitis. This type of infection is a very common vaginal infection in females. The use of the oral contraceptive pill, diabetes and pregnancy are among the main contributing factors in developing this type of infection. (Kumar *et al.*, 2003: 492).

The symptoms of the above infection include severe intolerable itching and a thick yellow creamy discharge, which might have a foul odour. (Elmer, 1997: 1046)

2.2.4.7 Candidiasis of the urinary system

The aetiology for developing candidiasis of the urinary system is mostly the result of a pre-existing urinary tract infection, diabetes or spread of infection haematogenously from a distant site. The spread of infection from vaginal candidiasis is also possible especially in the immune compromised host. The symptoms present as a typical urinary tract infection i.e. Dysuria, urethritis. (Greenwood *et al.*, 1992: 323)

2.2.4.8 Cutaneous candidiasis

Cutaneous candidiasis can present in many different forms in the body e.g.

- Nails ('onychomycosis')
- Nail folds ('paronychia')
- Hair follicles ('folliculitis')
- Intriginous skin that are moist eg. armpits, web of fingers and toes ('intertrigo')
- Penile skin ('balanitis').
- 'Diaper rash' is a common condition that affects newborn babies, infecting the region of the perineum. (Kumar *et al.*, 2003: 492)

2.2.4.9 Systemic candidiasis

A condition also referred to as '*candidemia*', is mostly seen in patients who have undergone major surgery with indwelling catheters left behind post surgically for therapeutic drainage purposes, infectious spread from the gastro-intestinal or respiratory tract and intravenous drug abusers. (Jawetz *et al.*, 2001: 550)

According to Kumar (2003), patients that are at risk of developing this type of infection are those that have a suppressed immune system. This immune suppression according to him is a result of administration of corticosteroids and other immunosuppressive agents used in the treatment of various haematological and auto-immune diseases. The common types of infections are renal abscesses, myocardial abscesses and endocarditis, hepatic abscesses, endophthalmitis, brain abscesses, meningitis, and candida pneumonia. (Kumar *et al.*, 2003: 492)

2.2.5 H.I.V. and candida infections

Since the development of H.I.V., there has been a significant rise in the cases of candida infections in individuals affected. The most common type of candida infections seen today in H.I.V. Infections according to Berkow and Beers(2000) is oral and oesophageal candidiasis. Opportunistic infections of the vaginal mucosae are also experienced in H.I.V. positive females. The CD4 lymphocyte counts is not markedly reduced in candida vaginitis but in oral and oesophageal candidiasis a significant reduction in CD4 lymphocytes level is noted by some researchers. In the case of the oesophageal candidiasis the level was lower than $0.1 \times 10^9/L$. This then also allows us to use the progression of oral and oesophageal candidiasis, as a rough indicator of the H.I.V. infection status. (Elmer, 1997: 1046).

2.2.6 Laboratory diagnosis

The laboratory diagnosis of candidiasis is one that involves obtaining cultures from sputum, stool, urine, skin, mouth, vagina etc., as the *C. albicans* yeast makes up part of the normal flora of the body. The laboratory diagnosis of candidiasis is one that involves obtaining cultures from sputum, stool, urine, skin, mouth, vagina etc., as the *C. albicans* yeast makes up part of the normal flora of the body. The exercising of caution must be adhered to for this reason, and the clinician must diagnose the infection by using clinical symptoms and signs and excluding other etiologies together with the laboratory tests. (Berkow and Beers, 2000: 166)

The yeasts once obtained are cultured on nutrient rich agar e.g. Sabourauds Dextrose Agar (S.D.A) and incubated for 24 hours at 37°C. The organisms are then identified microscopically after gram-staining them, for the presence of germ tubes and chlamydospores, which identifies the organism *C. albicans*. (Jawetz *et al.*, 2001:552)

2.3 ESCHERICHIA COLI

2.3.1 Classification

According to Elmer (1997), *E. coli* belongs to the family Enterobacteriaceae, a large group of Gram-negative rods, and to the genus *Escherichia*. The Enterobacteriaceae are the most common type of Gram-negative rods that are found in clinical laboratories and have been linked to infections involving most tissues and organ systems in the body. The *E. coli*, despite this is also part of the normal commensal flora present in the gut of a healthy individual. (Jawetz *et al.*, 2001: 217)

2.3.2 Morphology and identification

- They appear microscopically as Gram-negative motile rods with peritrichous flagella or non – motile. The bacteria grow well on peptone or meat extract media without the addition of sodium chloride or other supplements and can survive aerobically or anaerobically. (Jawetz *et al.*, 2001: 217). They form smooth, colourless colonies 2-3 mm in diameter on the Muller Hinton agar plates. The bacteria incubation temperatures ranges from 14 - 45°C. (Greenwood *et al.*, 1992: 323).

There is difficulty at times from differentiating *E. coli* from other escherichiae members e.g. *Shigella* species. The reason for this is due to both sharing common features of gas production during the fermentation process and both appear very similar morphologically. In cases like this identification is left solely up to serological testing. (Elmer, 1997: 196).

2.3.3 Infections caused by *E. coli*

- **Urinary tract infection**

E. coli is regarded as the most common causative organism implicated in uncomplicated urinary tract infection (U.T.I), which does not require hospital admission. The bacteria have developed evolutionary methods of bypassing the host defence mechanism. (Greenwood *et al.*, 1992: 323). *E. coli* can reach the urinary tract in two ways:

- The blood stream (haematogenous spread)
- Ascending infection from lower urinary tract.

The most common route however is the spread from lower urinary tract.

The first step in the pathogenesis spread of lower urinary tract infection is the adhesion of the *E. coli* bacteria to the mucosal wall of the urethra, followed by the colonisation of the distal urethra. (Kumar *et al.*, 2003: 527). The ability of the bacteria to facilitate this action is due to the presence of its fimbriae, which allows a specially mediated adherence to the uroepithelial cells of the urethra. (Haslett *et al.*, 1999: 458-459)

Signs and symptoms:

The onset of symptoms and signs is very abrupt and is characterized by chills, flank pain, nausea and vomiting. (Berkow *et al.*, 2000: 1711). The above symptoms are associated with frequency of micturition and dysuria, and once the infection has spread to the bladder, the pain is of a suprapubic type experienced during and after voiding. The urine has a characteristic unpleasant odour and appears cloudy in specimens obtained from infected individuals. (Haslett *et al.*, 1999: 460).

- **Diarrhoea**

E. coli is an important aetiology of acute enteritis, especially in infants and immunocompromised patients. There are four main groups of *E. coli* species that cause acute enteritis and are classified according to their different mechanisms of infections. (Greenwood *et al.*, 1992: 326) .The five types are as follows:

- **Enterotoxigenic *E. coli* (ETEC)**

The symptoms of this strain of *E. coli* include a profuse watery type of diarrhoea called ‘travellers diarrhoea’, together with abdominal cramps, vomiting, nausea and dehydration. (Elmer, 1997: 197). The spread of this type of infection is by contact with contaminated water sources. (Mims *et al.*, 1998: 256).

- **Enteropathogenic *E. coli* (EPEC)**

The symptoms include low-grade fever, malaise, vomiting, and diarrhoea with a lot of mucus production and without the production of gross blood. (Elmer, 1997: 197). The epidemiological spread in this strain is by sporadic out breaks of infection in babies and children, especially in hospitals. (Greenwood *et al.*, 1992: 326)

- **Enteroinvasive *E. coli* (EIEC)**

The symptoms are dysentery type, with urgency, tenesmus, mucus and bloody stools. There is also an increase in leucocytes in the stool. (Greenwood *et al.*, 1992: 326). Infections are usually spread by food borne root rather than any animal or environmental reservoir. (Mims *et al.*, 1998: 256)

- **Enterohemorrhagic *E. coli* (EHEC)**

The patient presents with bloody diarrhoea, without fever and abdominal pain. Thrombocytopenia associated with haemolytic uraemic syndrome. (Longmore *et al.*, 1999: 250). The spread is usually by sporadic out breaks from, infected foods e.g. uncooked ground beef and un-pasteurised milk. (Mims *et al.*, 1998: 256)

2.4 PREVIOUS RESEARCH

Budree (2003) used *Hydrastis canadensis* 62% (v/v) ethanol tincture together with *Commiphora molmol* 86% (v/v) ethanol tincture and *Warburgia salutaris* 62% (v/v) ethanol tincture against *C. albicans* in the disc diffusion assay and the tube dilution method. His results showed that the *Hydrastis canadensis* 62% (v/v) ethanol tincture was the only tincture that inhibited the *in vitro* growth of *C. albicans* and achieved bigger zones of inhibition than the positive control fluconazole. It also achieved a Minimum Inhibitory Concentration of 1:50 dilution.

The possible weakness in his study, could be due to the following methodology errors:

- The use of Whatman® filter paper number 4 discs, which are not manufactured, to be used in disc diffusion studies. (Hewitt, 1989: 57)

- The filter paper discs were dipped into the herbal tinctures, therefore the amount of tincture placed on the disc was not known and could have varied between discs;
- The zones of inhibition were measured using a ruler and not a vernier caliper, a technique that was strongly advised against by Invernizzi (2002);
- The incorrect identification of the original source material. (Budree, 2003: 142)

Ramlachan (2002) used *Arctium lappa*, *Calendula officinalis* and *Echinacea purpurea* prepared in 62% (v/v) ethanol tincture against *C. albicans* as compared to the positive control nystatin in terms of the disc diffusion assay. She concluded from her results that the only tincture to produce statistically significant results was the *Arctium lappa* in 62% (v/v) ethanol. She also stated that the ethanol might have contributed to the anti-microbial effect of the tinctures at 18 hours of incubation and not the individual herbal constituents. The possible weakness in her study, could be due to the following methodology errors:

- The use of Whatman® filter paper number 3 discs, which are not manufactured, to be used in disc diffusion studies (Hewitt, 1989: 57);
- The zones of inhibition were measured using a ruler and not a vernier caliper;
- The incorrect identification of the original source material. (Ramlachan, 2002: 90)

Dhanraj (2001) evaluated the efficacy of *Echinacea angustifolia* prepared in 30% (v/v) and 62% (v/v) ethanol tincture in inhibiting the *in vitro* growth of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, *Escherichia coli* and *Enterococcus faecalis* using the disc diffusion assay.

The possible weakness in his study, could be due to the following methodology errors:

- The use of Whatman® filter paper discs, which are not manufactured, to be used in disc diffusion studies. (Hewitt, 1989: 57)
- The filter paper discs were dipped into the herbal tinctures, therefore the amount of tincture placed on the disc was not known and could have varied between discs.

- The incorrect identification of the original source material. (Dhanraj, 2001: 37-40)

Invernizzi (2002) evaluated the effectiveness of *Tulbagia Violacea* in herbal tincture and Homoeopathic dilution (1x and 6x) against Gram-positive and Gram-negative bacteria. Invernizzi found many flaws in the methodology of previous microbiological studies including his own. He highlighted the following flaws and also made appropriate recommendations to correct these flaws in future microbiological studies:

- The use of filter paper disc that are not manufactured for disc diffusion assay's. He recommended that industry approved disc that are used in such assay, replace the filter paper disc e.g. Blank susceptibility disc
- The use of a clear plastic ruler should not be used to measure the zones of inhibition, but rather a vernier caliper which will give a more accurate measurement.
- Dilution methods are also more sensitive than diffusion methods, and can yield a minimum inhibitory concentration in the event of positive anti-microbial results.
- The incorrect identification of the original source material. This error according to Invernizzi (2002) can be rectified by the researcher monitoring the manufacturing of the experimental herbal tinctures from the harvesting of the plant to the end product. (Invernizzi, 2002: 29)

CHAPTER THREE

METHODOLOGY

3.1 DATA

The research consists of two types of data i.e. Primary and secondary data.

3.1.1 Primary data

1. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extractions on *C. albicans*.
2. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extractions on *E. coli*.
3. Results of the experiment determining the effects of *Hydrastis canadensis* 62% v/v ethanol tincture on *C. albicans*.
4. Results of the experiment determining the effects of *Hydrastis canadensis* 62% v/v ethanol tincture on *E. coli*.
5. Results of the experiment determining the effects of distilled water on *C. albicans*.
6. Results of the experiment determining the effects of distilled water on *E. coli*.
7. Results of the experiment determining the effects of 62% ethanol tincture on *C. albicans*.
8. Results of the experiment determining the effects of 62% ethanol tincture on *E. coli*.
9. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:2 distilled water dilution on *C. albicans*.
10. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:3 distilled water dilution on *C. albicans*.
11. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:5 distilled water dilution on *C. albicans*.
12. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:10 distilled water dilution on *C. albicans*.

13. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:2 distilled water dilution on *E. coli*.
14. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:3 distilled water dilution on *E. coli*.
15. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:5 distilled water dilution on *E. coli*.
16. Results of the experiment determining the effects of *Hydrastis canadensis* water-based extraction 1:10 distilled water dilution on *E. coli*.
17. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:2 distilled water dilution on *C. albicans*.
18. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:3 distilled water dilution on *C. albicans*.
19. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:5 distilled water dilution on *C. albicans*.
20. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:10 distilled water dilution on *C. albicans*.
21. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:2 distilled water dilution on *E. coli*.
22. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:3 distilled water dilution on *E. coli*.
23. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:5 distilled water dilution on *E. coli*.
24. Results of the experiment determining the effects of 62% v/v *Hydrastis canadensis* 1:10 distilled water dilution on *E. coli*.

3.1.2 Secondary data

This data will be obtained from journal publications and articles from books.

3.2 CRITERIA GOVERNING ADMISSIBILITY OF DATA

Only data gathered from experiments conducted by the researcher at the Durban Institute of Technology: Steve Biko Campus, Department of Biotechnology microbiology laboratory was used. The validity of the information was verified by the laboratory's resident microbiologist, Mrs. Rena Briglal.

3.3 MATERIALS AND METHOD

3.3.1 Preparation of the water-based extraction of *Hydrastis canadensis*

Hydrastis canadensis was prepared by Parceval Pharmaceuticals (Pty) Ltd. (Cape Town) according to an adjusted method HAB 4a of the German Homoeopathic Pharmacopoeia. The *Hydrastis canadensis* radix – dried and finely milled (1mm sieve size) plant material was weighed into a glass jar. After comminution or pulverizing, 1 part plant material was mixed with 10 parts distilled water in a container. The essences were then subjected to rhythmic light and movement processes and then after a week the essences were pressed and filtered. Thereafter it was pressed through 100 % cotton cloth and filtered through Whatman® filter paper number 1. (Fieter, 2004)

3.3.2 Preparation of the ethanol tincture of *Hydrastis canadensis*

Hydrastis canadensis tincture (Batch no. 03RF70JM – expiry date: 11/2006) was prepared in 62% ethanol by Natura Homoeopathic Laboratories, according to method HAB 4a of the German Homoeopathic Pharmacopoeia. (Singh, 2004)

3.3.3 Preparation of distilled water control

Distilled water was obtained from the Homoeopathic Department Laboratory at Durban Institute Of Technology – Steve Biko Campus.

3.3.4 Preparation of 62% v/v ethanol control

The ethanol control that was used in this experiment was prepared at the Homoeopathic Department's Laboratory, Steve Biko Campus, Durban Institute of Technology. The 62% ethanol was prepared as follows: 650,68ml of 96% ethanol was mixed with 340.32ml of distilled water. (Naude, 2004)

3.3.5 Blank susceptibility disc

The use of blank susceptibility disc was used and not the traditional Whatman® filter paper number 4 discs, as recommended by Invernizzi (2002). The discs were purchased from Mast diagnostics (expiry date - 01/02/07- lot. no – 124986).

3.3.6 Preparation of the media

- **Sabourauds dextrose agar (S.D.A)**

The medium of choice for culturing the *C. albicans* is the Sabouraud's dextrose agar. All fungi need a protein and a carbohydrate source. Sabouraud's dextrose agar, the standard medium for the support of yeast and mould growth contains only dextrose and peptone as nutritional sources. (Cheesbrough, 1984.423-424) It was prepared according to the Oxoid® manual (1979) as follows:

1. 19,5g of Sabouraud's dextrose agar (manufacturer – SGHARLAV, batch no. – 9746 expiry date - 12/05), was weighed and added to 300ml (to make the twelve required plates) of distilled water in a screw top flask.
2. A magnetic stirrer was added to the bottom of the flask to aid the dissolving process. The mixture was shaken until the agar was thoroughly dissolved, (approximately five minutes).
3. The mixture was then autoclaved for 15 minutes at 121°C.
4. The flask was then allowed to cool in a beaker of cold water.
5. The agar was then poured into the agar plates to a depth of 4mm.
6. The agar was then allowed to further cool and solidify (approximately one hour).
7. The plates were checked for contamination by looking for growth in the zones of inhibition.

- **Muller Hinton Agar (M.H.A)**

The medium of choice for culturing *E. coli* is Mueller-Hinton agar. This is the routinely used agar in susceptibility testing. It was prepared according to the Oxoid® manual (1979) as follows:

1. 11,4g of Muller-Hinton Agar (manufacturer – BIOLAB-batch no – 1016417 expiry date - 09/2004) was weighed and added to 300ml of distilled water (to make the twelve plate required for this experiment) in a screw top flask.
2. A magnetic stirrer was added to the bottom of the flask to aid the dissolving process. The mixture was shaken until the agar was thoroughly dissolved.
3. The mixture was then autoclaved for 15 minutes at 121°C.
4. The flask was then allowed to cool in a beaker of cold water.
5. The agar was then poured into the agar plates to a depth of 4mm.
6. The agar was then allowed to further cool and solidify.
7. The plates were visually checked for contamination.

3.3.7 Preparation of the inoculum

- **Preparation of *C. albicans* inoculum**

- 1) Single colonies of *Candida albicans*, obtained from the Durban Institute of Technology Biotechnology Laboratory stock cultures, were used to inoculate a Sabouraud- dextrose agar plate and allowed to incubate for 24 hours at 37°C in an incubator.
- 2) A few individual colonies from the overnight culture were suspended in 10ml of Sabouraud- dextrose liquid medium using aseptic techniques.
- 3) The culture was swirled to allow even distribution of the culture.
- 4) 1ml of the liquid medium was aseptically pipetted into a test tube containing 9ml of saline solution.
- 5) The culture was then vortexed to enable adequate mixing.
- 6) The culture was made up to an organism suspension equivalent to McFarland 0.5 turbidity standard.

- **Preparation of *E. coli* inoculum**

- 1) A stock culture of *E. coli* was provided by the Department of Biotechnology, Durban Institute of Technology. A single colony was streaked onto a Mueller-Hinton agar plate and allowed to incubate for 24 hours at 37°C in an incubator.
- 2) A few individual colonies from the overnight cultures were suspended in 10ml of saline solution using aseptic techniques.
- 3) The culture was swirled to allow even distribution of the culture.
- 4) 1ml of the liquid medium was aseptically pipetted into a test tube containing 1 ml of saline solution.
- 5) The culture was then vortexed to enable adequate mixing.
- 6) The culture was made up to an organism suspension equivalent to McFarland 0.5 turbidity standard.

3.3.8 Innoculation of plates

C. albicans

The inoculation of six plates with *C. albican* was done using the following aseptic techniques:

1. A sterile cotton swab was dipped into the well-mixed saline test culture prepared above and excess inoculum was removed by pressing the saturated swab against the inner wall of the culture tube.
2. Using the swab, the entire agar surface of the plate was streaked, first in a horizontal direction and then vertically to ensure a uniform growth over the entire surface.

E. coli

The inoculation of six plates with *E. coli* was done using the following aseptic techniques:

- A sterile cotton swab was dipped into the well-mixed saline test culture prepared above and excess inoculum was removed by pressing the saturated swab against the inner wall of the culture tube.
- Using the swab, the entire agar surface of the plate was streaked, first in a horizontal direction and then vertically to ensure a uniform growth over the entire surface.

3.3.9 Impregnation and placement of disc on the inoculated media

The blank susceptibility discs were placed over the agar surface by means of sterile forceps. Each disc was gently pressed down to ensure that the discs adhered to the agar surface. There was a total of two discs on each plate. A pre-measured amount of 10 µl of the test substances and the controls was pipetted onto separate discs using a micropipette.

3.3.10 Incubation of plates

Inoculated plates were incubated in an inverted position for 24 hours at 37°C. After incubation, the plates were examined for the presence of growth inhibition, which was indicated by a clear zone surrounding each disc. The susceptibility of an organism to a substance was determined by the size of the zone, which was measured using vernier calipers. Each test for both groups was conducted using three replicates to ensure consistency and provide statistically viable data.

3.3.11 Preparation of *Hydrastis canadensis* dilutions

Water-based extraction

- **Preparation of *Hydrastis canadensis* 1:2 dilution**

1ml of *Hydrastis canadensis* water-based extraction was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 1 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

- **Preparation of *Hydrastis canadensis* 1:3 dilution**

1ml of *Hydrastis canadensis* water-based extraction was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 2 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

- **Preparation of *Hydrastis canadensis* 1:5 dilution**

1ml of *Hydrastis canadensis* water-based extraction was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 4 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

- **Preparation of *Hydrastis canadensis* 1:10 dilution**

1ml of *Hydrastis canadensis* water-based extraction was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 9ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

Ethanol tincture

- **Preparation of *Hydrastis canadensis* 1:2 dilution**

1ml of *Hydrastis canadensis* 62% v/v tincture was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 1 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

- **Preparation of *Hydrastis canadensis* 1:3 dilution**

1ml of *Hydrastis canadensis* 62% v/v tincture was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 2 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004)

- **Preparation of *Hydrastis canadensis* 1:5 dilution**

1ml of *Hydrastis canadensis* 62% v/v tincture was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 4 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004).

- **Preparation of *Hydrastis canadensis* 1:10 dilution**

1ml of *Hydrastis canadensis* 62%v/v tincture was pipetted using a sterile micropipette to a 10 ml screw-top clear vial containing 9 ml of distilled water. The bottle lid was fastened and the bottle was gently agitated to mix the dilution evenly. (Naude, 2004)

3.3.12 Tube dilution method

1. Organisms of *C. albicans* and *E. coli* were each transferred into five test tubes containing 2ml of distilled water, and the standard number of organisms was measured using the Macfallans turbidity standard, which was set at 0.5 Macfallans. The method was as follows:
 - The 2ml of distilled water was pipetted into a test tube.
 - A loopful of the respective organisms were transferred to each of the five test tube and the turbidity brought up to the Macfallans standard of 0.5, which was compared to a pre-measured test tube containing 0.5 Macfallans standard.
 - The mouth of the test tube was flamed in order to prevent contamination and the lid was replaced.
 - A total of twenty test tubes were made, with ten test tubes used for testing each organism in a group.
2. 2ml of the dilutions of 1:2, 1:3, 1:5, 1:10 were respectively transferred into each of the five test tubes with one test tube acting as a control in each group, containing only the 0.5 Macfallans number of organisms.

The test tubes were incubated at 37°C for 24 hours. (Singh, 2003)

3. The results were recorded by comparing the turbidity of the experimental test tube with 0.5 Macfallans turbidity as follows:
 - 0.5 Macfallans – 3+ growth (DENSE)

0	NO GROWTH
1+	SCANT
2+	MODERATE
3+	DENSE
4+	VERY DENSE

3.3.13 Determination of Minimum Inhibitory Concentration

- 8 test tubes, each containing 2ml of *E. coli* liquid culture with an organism suspension equivalent to McFarland 0.5 turbidity standard were prepared.
- 8 test tubes, each containing 2ml of *C. albicans* liquid culture with an organism suspension equivalent to McFarland 0.5 turbidity standard were prepared.
- 2ml of *Hydrastis canadensis* water-based extraction 1:2 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:3 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:5 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:10 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:2 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:3 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:5 dilution was pipetted into one of the *E. coli* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:10 dilution was pipetted into one of the *E. coli* liquid culture tubes.

- 2ml of *Hydrastis canadensis* water-based extraction 1:2 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:3 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:5 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* water-based extraction 1:10 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:2 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:3 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:5 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- 2ml of *Hydrastis canadensis* ethanol tincture 1:10 dilution was pipetted into one of the *C. albicans* liquid culture tubes.
- All 16 tubes were incubated for 24 hours at 37°C.
- The tubes were inspected for growth inhibition using McFarlands 0.5 turbidity standard as a comparison.

3.3.14 Test for contamination (procedure)

The above test was conducted after contamination was suspected following the results obtained from the tube dilution test of the *Hydrastis canadensis* water-based extraction.

1. A SDA and a MHA plate were obtained from the Durban Institute of Technology Department of Microbiology Laboratory
2. Each plate was inoculated with the water-based extraction of *Hydrastis canadensis* in the following manner:
 - A sterile cotton swab was dipped into the *Hydrastis canadensis* water-based extraction and excess inoculum was removed by pressing the saturated swab against the inner wall of the culture tube.
 - Using the swab, the entire agar surface of the plate was streaked, first in a horizontal direction and then vertically to ensure a uniform growth over the entire surface.
3. The plates were incubated in an inverted position for 24 hours at 37 °C.
4. The plates were visually examined for the presence or absence of growth.
5. The colonies that were present on each plate were fixed onto slides by the following method:
 - A loopful of organisms was mixed in a drop of water that was placed on a slide.
 - The mixture was smeared to form a thin layer of organisms of between 15-20mm in diameter.
 - The smear was left to air-dry.
 - The slide was rapidly passed through a flame with the smear side up for purposes of heat fixation.
 - The validity of this process was confirmed by placing the slide on the back of the hand, thereby checking that the slide was not overheated resulting in damage caused to the organisms.
6. The slides were then subjected to the Gram staining method as follows.
 - The slides were flooded completely with crystal violet for 60 seconds.
 - The crystal violet was washed off with clean water.
 - Excess water was tipped off the slide, which was thereafter completely flooded with iodine.
 - The iodine was washed off with clean water.
 - The slide was decolourised with acetone-alcohol for a few seconds and washed off promptly with clean water.
 - The slide was thereafter flooded with the neutral safranin for 1 minute.
 - The safranin was washed off with clean water.

- The excess water was tipped off and the slides were left on a draining rack to air-dry.
7. The slides were examined microscopically, first on 45x magnification, and then 100x oil immersion lens to look for bacteria and cells (Cheesbrough, 1993: 31).

3.4 DROP TEST PROCEDURE

The above test was conducted after obtaining no growth inhibition of the *C. albicans* with both the water-based extraction and the ethanol tincture. This was contradictory to the results obtained by Budree (2003). The variable that differed between this study and Budree's, was that he used discs punched out from Whatman® filter paper number 4 in the disc diffusion assay. This study utilised blank susceptibility disc after following recommendations of Invernizzi (2002) and the drop test therefore aimed to reproduce a part of Budree's study, in order to evaluate the ability of the disc punched out from the filter paper to hold 10µl of test substance. The procedure was as follows:

1. Two SDA and two MHA plates were obtained from the Durban Institute of Technology Department of Microbiology Laboratory.
2. The SDA plates were inoculated with *C. albicans*, and the MHA plates were inoculated with *E. coli* as follows:
 - a. A sterile cotton swab was dipped into the well-mixed saline test culture prepared above and excess inoculum was removed by pressing the saturated swab against the inner wall of the culture tube.
 - b. Using the swab, the entire agar surface of the plate was streaked, first in a horizontal direction and then vertically to ensure a uniform growth over the entire surface.
3. Whatman® filter paper number 4 was used. The filter paper was punched out into discs of 5mm in diameter. These discs were placed in a jar and autoclaved at 121°C for 15 minutes to ensure sterilization (Hewitt and Vincent, 1989: 57).
4. Each of the four plates was divided in halves with a permanent marker. Each half was labelled as either experiment or control.
5. Two Whatman® filter paper number 4 discs were placed side by side on the SDA and MHA plates, one disc on the experiment side, and one on the control side by means of a sterile forceps. 10µl of *Hydrastis canadensis* 62% (v/v)

ethanol tincture was pipetted onto the experimental filter paper discs. 10µl of 62% (v/v) ethanol was pipetted onto the control filter paper discs.

6. 10µl of *Hydrastis canadensis* 62% (v/v) ethanol tincture was pipetted (dropped) directly onto the experimental sides of the remaining MHA and SDA plates. 10µl of 62% (v/v) ethanol was pipetted (dropped) onto the control sides of the remaining MHA and SDA plates.
7. The plate was incubated in an inverted position for 24 hours at 37°C.
8. The plates were visually examined for growth inhibition by measuring the zones of inhibition using Vernier callipers.

3.5 DATA ANALYSIS

Statistical methods

Procedure for disc diffusion assay

Procedure 1.1

Intergroup comparison between *Hydrastis canadensis* water-based extraction and distilled water on *E. coli*.

The Mann-Whitney U test is a simple non-parametric test that compares the medians of two independent samples (Daniel, 1999: 678). This test was used to compare *Hydrastis canadensis* water-based extraction and distilled water on *E. coli*.

•Hypothesis testing

The null hypothesis H_0 , states that there is no difference in the diameter of the zone of inhibition between *Hydrastis canadensis* water-based extraction and distilled water on *E. coli* / *C. albicans* with respect to the variable comparison at the $\alpha = 0.05$ level of significance. The alternative hypothesis H_1 , states that there is a difference at the same level of significance.

$$H_0: M_1 = M_2$$

$$H_1: M_1 \neq M_2$$

• Decision rule

At $\alpha = 0.05$ level of significance, the null hypothesis is rejected if $P < \alpha$ where P is the observed significance level or probability value. Otherwise the null hypothesis is accepted at the same level of significance.

Reject H_0 if $P < \alpha$

Accept H_0 if $P \geq \alpha$

P is the observed significance level or probability value (Fisher and van Belle, 1993: 315).

Procedure 1.2

Intergroup comparison between *Hydrastis canadensis* water-based extraction and distilled water on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* water-based extraction and distilled water on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.3

Intergroup comparison between *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.4

Intergroup comparison between *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.5

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.6

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.7

Intergroup comparison between *Hydrastis canadensis* water-based extraction and 62% (v/v) ethanol on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* water-based extraction and 62% (v/v) ethanol on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.8

Intergroup comparison between *Hydrastis canadensis* water-based extraction and 62% (v/v) ethanol on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* water-based extraction and 62% (v/v) ethanol on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.9

Intergroup comparison between distilled water and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare distilled water and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.10

Intergroup comparison between distilled water and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare distilled water and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.11

Intergroup comparison between distilled water and 62% (v/v) ethanol on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare distilled water and 62% (v/v) ethanol on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 1.12

Intergroup comparison between distilled water and 62% (v/v) ethanol on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare distilled water and 62% (v/v) ethanol on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure for tube dilution method

Procedure 2.1

Intergroup comparison between 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Hypothesis testing

The null hypothesis H_0 , states that there is no difference in turbidity between *Hydrastis canadensis* water-based extraction and distilled water on *E. coli* / *C. albicans* with respect to the variable comparison at the $\alpha = 0.05$ level of significance.

The alternative hypothesis H_1 , states that there is a difference at the same level of significance.

$$H_0: M_1 = M_2$$

$$H_1: M_1 \neq M_2$$

- Decision rule

At $\alpha = 0.05$ level of significance, the null hypothesis is rejected if $P < \alpha$ where P is the observed significance level or probability value. Otherwise the null hypothesis is accepted at the same level of significance.

Reject H_0 if $P < \alpha$

Accept H_0 if $P \geq \alpha$

P is the observed significance level or probability value (Fisher and van Belle, 1993: 315).

Procedure 2.2

Intergroup comparison between 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.3

Intergroup comparison between 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.4

Intergroup comparison between 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.5

Intergroup comparison between 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.6

Intergroup comparison between 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.7

Intergroup comparison between 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure 2.8

Intergroup comparison between 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare the 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

- Hypothesis testing

As per procedure 2.1

- Decision rule

As per procedure 2.1

Procedure for drop test vs disc diffusion method

Procedure 3.1

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.2

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.3

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.4

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.5

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol drop on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol drop on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.6

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol drop on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol drop on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.7

Intergroup comparison between 62% (v/v) ethanol disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare 62% (v/v) ethanol disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.8

Intergroup comparison between 62% (v/v) ethanol disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare 62% (v/v) ethanol disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.9

Intergroup comparison between 62% (v/v) ethanol disc and 62% (v/v) ethanol drop on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare 62% (v/v) ethanol disc and 62% (v/v) ethanol drop on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.10

Intergroup comparison between 62% (v/v) ethanol disc and 62% (v/v) ethanol drop on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare 62% (v/v) ethanol disc and 62% (v/v) ethanol drop on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.11

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture drop and 62% (v/v) ethanol drop on *E. coli*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture drop and 62% (v/v) ethanol drop on *E. coli*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

Procedure 3.12

Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture drop and 62% (v/v) ethanol drop on *C. albicans*.

The Mann-Whitney Test (Wilcoxon inversion test) was used to compare *Hydrastis canadensis* 62% (v/v) ethanol tincture drop and 62% (v/v) ethanol drop on *C. albicans*.

- Hypothesis testing

As per procedure 1.1

- Decision rule

As per procedure 1.1

CHAPTER FOUR

RESULTS

This chapter covers the results obtained from statistical analysis of the data obtained.

4.1 *Hydrastis canadensis* water-based extraction disc diffusion assay

4.1.1 Procedure 1.1 - 1.2

(See Table 4.1)

Table 4.1 Intergroup comparison between *Hydrastis canadensis* water-based extraction and distilled water on *E. coli* and *C. albicans* (Mann-Whitney Test).

	1.1 <i>E. coli</i>	1.2 <i>C. albicans</i>
P-Value	1.000	1.000

- Interpretation of results for Procedure 1.1

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and distilled water on *E. coli*.

- Interpretation of results for Procedure 1.2

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and distilled water on *C. albicans*.

4.1.2 Procedure 1.3 - 1.4

(See Table 4.2)

Table 4.2 Intergroup comparison between *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli* and *C. albicans*.

	1.3 <i>E. coli</i>	1.4 <i>C. albicans</i>
P-Value	0.317	1.000

- Interpretation of results for Procedure 1.3

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *E. coli*.

- Interpretation of results for Procedure 1.3

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and *Hydrastis canadensis* 62% (v/v) ethanol tincture on *C. albicans*.

4.1.3 Procedure 1.5 – 1.6

(See Table 4.3)

Table 4.3 Intergroup comparison between *Hydrastis canadensis* water-based extraction and 62% (v/v) ethanol control on *E. coli* and *C. albicans*.

	1.5 <i>E. coli</i>	1.6 <i>C. albicans</i>
P-Value	0.317	1.000

- Interpretation of results for Procedure 1.5

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and the 62% (v/v) ethanol control on *E. coli*.

- Interpretation of results for Procedure 1.6

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* water-based extraction and the 62% (v/v) ethanol control on *C. albicans*.

4.1.4 Procedure 1.7 – 1.8

(See Table 4.4)

Table 4.4 Intergroup comparison between distilled water control and 62% (v/v) ethanol control on *E. coli* and *C. albicans*.

	1.7 <i>E. coli</i>	1.8 <i>C. albicans</i>
P-Value	0.317	1.000

- Interpretation of results for Procedure 1.7

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between distilled water control and 62% (v/v) ethanol control on *E. coli*.

- Interpretation of results for Procedure 1.8

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between distilled water control and 62% (v/v) ethanol control on *C. albicans*.

4.1.5 Procedure 1.9 – 1.10

(See Table 4.5)

Table 4.5 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *E. coli* and *C. albicans*.

	1.9 <i>E. coli</i>	1.10 <i>C. albicans</i>
P-Value	1.000	1.000

- Interpretation of results for Procedure 1.9

P = 1.000

$\alpha = 0.05$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *E. coli*.

- Interpretation of results for Procedure 1.10

P = 1.000

$\alpha = 0.05$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *C. albicans*.

4.1.6 Procedure 1.11 – 1.12

(See Table 4.6)

Table 4.6 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *E. coli* and *C. albicans*.

	1.11 <i>E. coli</i>	1.12 <i>C. albicans</i>
P-Value	1.000	1.000

- Interpretation of results for Procedure 1.11

P = 1.000

$\alpha = 0.05$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *E. coli*.

- Interpretation of results for Procedure 1.12

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture and 62% (v/v) ethanol control on *C. albicans*.

4.2 Tube dilution method

4.2.1 Procedure 2.1 – 2.2

(See Table 4.7)

Table 4.7 Intergroup comparison between the 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *E. coli* and *C. albicans*.

	2.1 <i>E. coli</i>	2.2 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 2.1

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Interpretation of results for Procedure 2.2

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:2 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:2 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

4.2.2 Procedure 2.3 – 2.4

(See Table 4.8)

Table 4.8 Intergroup comparison between the 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *E. coli* and *C. albicans*.

	2.3 <i>E. coli</i>	2.4 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 2.3

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Interpretation of results for Procedure 2.4

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:3 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:3 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

4.2.3 Procedure 2.5 – 2.6

(See table 4.9)

Table 4.9 Intergroup comparison between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli* and *C. albicans*.

	2.5 <i>E. coli</i>	2.6 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 2.5

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Interpretation of results for Procedure 2.6

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

4.2.4 Procedure 2.7 – 2.8

(See table 4.10)

Table 4.10 Intergroup comparison between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli* and *C. albicans*.

	2.5 <i>E. coli</i>	2.6 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 2.5

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Interpretation of results for Procedure 2.6

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:5 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:5 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

4.2.5 Procedure 2.9 – 2.10

(See table 4.11)

Table 4.11 Intergroup comparison between the 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *E. coli* and *C. albicans*.

	2.7 <i>E. coli</i>	2.8 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 2.7

P = 0.317

$\alpha = 0.05$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *E. coli*.

- Interpretation of results for Procedure 2.8

P = 0.317

$\alpha = 0.05$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in turbidity between the 1:10 dilution of *Hydrastis canadensis* 62% (v/v) ethanol tincture and 1:10 dilution of *Hydrastis canadensis* water-based extraction on *C. albicans*.

4.3 Disc vs drop diffusion assay

4.3.1 Procedure 3.1 – 3.2

(See Table 4.12)

Table 4.12 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *E. coli* and *C. albicans*.

	3.1 <i>E. coli</i>	3.2 <i>C. albicans</i>
P-Value	0.317	1.000

- Interpretation of results for Procedure 3.1

P = 0.317

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between the *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *E. coli*.

- Interpretation of results for Procedure 3.2

$$P = 1.000$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between the *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol disc on *C. albicans*

4.3.2 Procedure 3.3 – 3.4

(See Table 4.13)

Table 4.13 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli* and *C. albicans*.

	3.3 <i>E. coli</i>	3.4 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 3.3

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between the *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

- Interpretation of results for Procedure 3.4

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between the *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

4.3.3 Procedure 3.5 – 3.6

(See Table 4.14)

Table 4.14 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol control drop on *E. coli* and *C. albicans*.

	3.5 <i>E. coli</i>	3.6 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 3.5

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol control drop on *E. coli*.

- Interpretation of results for Procedure 3.6

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol tincture disc and 62% (v/v) ethanol control drop on *C. albicans*.

4.3.4 Procedure 3.7 – 3.8

(See Table 4.15)

Table 4.15 Intergroup comparison between 62% (v/v) ethanol control disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli* and *C. albicans*.

	3.7 <i>E. coli</i>	3.8 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 3.7

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between 62% (v/v) ethanol control disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *E. coli*.

- Interpretation of results for Procedure 3.8

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between 62% (v/v) ethanol control disc and *Hydrastis canadensis* 62% (v/v) ethanol tincture drop on *C. albicans*.

4.3.5 Procedure 3.9 – 3.10

(See Table 4.16)

Table 4.16 Intergroup comparison between 62% (v/v) ethanol control disc and 62% (v/v) ethanol control drop on *E. coli* and *C. albicans*.

	3.9 <i>E. coli</i>	3.10 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 3.9

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between 62% (v/v) ethanol control disc and 62% (v/v) ethanol control drop on *E. coli*.

- Interpretation of results for Procedure 3.10

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between 62% (v/v) ethanol control disc and 62% (v/v) ethanol control drop on *C. albicans*.

4.3.6 Procedure 3.11 – 3.12

(See Table 4.17)

Table 4.17 Intergroup comparison between *Hydrastis canadensis* 62% (v/v) ethanol control drop and 62% (v/v) ethanol control drop on *E. coli* and *C. albicans*.

	3.11 <i>E. coli</i>	3.112 <i>C. albicans</i>
P-Value	0.317	0.317

- Interpretation of results for Procedure 3.11

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol control drop and 62% (v/v) ethanol control drop on *E. coli*.

- Interpretation of results for Procedure 3.12

$$P = 0.317$$

$$\alpha = 0.05$$

The null hypothesis is accepted since $P \geq \alpha$. Thus, there is no significant difference in diameter of the zones of inhibition between *Hydrastis canadensis* 62% (v/v) ethanol control drop and 62% (v/v) ethanol control drop on *C. albicans*.

4.4 TEST FOR CONTAMINATION

The test revealed that there was indeed contamination present in the water-based extractions of *Hydrastis canadensis*. The following organisms were identified following microscopic examination under the 100x oil immersion lens:

- **M.H.A Plate**
 - : **Gram-positive bacilli (Dark purple)**
 - : **Gram-positive cocci (Dark purple)**
 - : **Gram-negative cocci (Pale to dark red)**

- **S.D.A Plate**

: Gram-positive budding yeast (Dark purple)

: Gram-negative bacilli (Pale to dark red)

CHAPTER FIVE

DISCUSSION

5.1 HYDRASTIS CANADENSIS WATER EXTRACTION

The results of this study demonstrated that the *Hydrastis canadensis* water-based extraction had no anti-microbial effect on the *E. coli* and the *C. albicans*, in terms of the zones of inhibition in the disc-diffusion assay. No growth inhibition was exhibited, resulting in a p-value of 1.000 when compared to the negative control using the Mann-Whitney U test.

Statistically, none of the dilutions of *Hydrastis canadensis* water-based extraction exhibited significant growth inhibition of *E. coli* and *C. albicans* in terms of turbidity in the tube dilution tests. The absence of any significant statistical difference between the anti-microbial effect of the sample tested, and the distilled water negative control, was due to the use of a small sample size and the use of herbal water extractions of the herb (*Hydrastis canadensis*). This method of herbal preparation effectively removed the ethanol component found in herbal tinctures, thereby eliminating the superfluous anti-microbial effect that the ethanol may have on the *E. coli* and the *C. albicans*.

5.2 HYDRASTIS CANADENSIS 62%(v/v) ETHANOL TINCTURE

The results indicate that the *Hydrastis canadensis* 62% (v/v) tincture had no anti-microbial effect on the *E. coli* and the *C. albicans*, in terms of the zones of inhibition in the disc-diffusion assay. No growth inhibition was exhibited, resulting in a p-value of 0.317 when compared to the negative control using the Mann-Whitney U test.

These results are similar to the findings of Ramalachan (2002) and Invernizzi (2002), who both reported on the failure of herbal tinctures in 62% (v/v) ethanol in inhibiting the *in vitro* growth of micro-organisms. These results however contradict the findings of Budree (2003), who reported that *Hydrastis canadensis* 62% (v/v) ethanol tinctures was effective in inhibiting the *in vitro* of *C. albicans*.

Statistically, none of the dilutions of *Hydrastis canadensis* 62% (v/v) tincture exhibited significant growth inhibition of *E. coli* and *C. albicans* in terms of turbidity in the tube dilution tests. The absence of any significant statistical difference between

the anti-microbial effect of the sample tested, and the ethanol negative control, was possibly due to the use of a small sample size.

5.3 HYDRASTIS CANADENSIS WATER-BASED EXTRACTION AND HYDRASTIS CANADENSIS 62% (v/v) TINCTURE.

The results indicate that there is no significant difference between the *Hydrastis canadensis* 62% (v/v) tincture and the *Hydrastis canadensis* water-based extraction used in this study, in terms of the zones of inhibition in the disc-diffusion assay. A p-value of 0.317 for *E. coli* and 1.000 for the *C. albicans* was obtained when *Hydrastis canadensis* 62% (v/v) tincture was compared to the *Hydrastis canadensis* water-based extraction using the Mann-Whitney U test.

5.4 GENERAL DISCUSSION

The possible failure of this study to obtain statistically significant results, could be a result of one of the following flaws in experimental method:

- The choice of sample size was too small to have a statistically significant result.
- The contamination of the water-based extraction of *Hydrastis canadensis*, which was proved true by the test for contamination carried out subsequently after the unusual results obtained from the tube dilution test.
- The incorrect identification of the original source material.
- The utilization of incorrect methodology, in the manufacturing process of the water-based extractions.
- Essential oils and other anti-microbial components could not be extracted from the *Hydrastis canadensis* herb by the water-based extraction method.
- Plate with heavy microbial growth may have been used and this therefore influenced the size of the zones of inhibition.
- It should be noted however that plates which displayed minute quantities of single colony growth in the zones of inhibition were recorded as having a 0 inhibitory effect. This was adopted in order to eliminate the researcher from recording false results.

The *Hydrastis canadensis* water-based extraction may prove to be an effective anti-microbial agent when tested *in vivo*. This may be due to the herb possessing properties that influence the body's immune system, by stimulating and enhancing it.

5.5 DISC AND DROP TEST

The results indicate that there is no statistically significant difference between the *Hydrastis canadensis* 62% (v/v) tincture disc and *Hydrastis canadensis* 62% (v/v) tincture drop on the *in vitro* growth of *E. coli* and the *C. albicans*, in terms of the sizes of the zones of inhibition in the disc-diffusion assay. A p-value of 0.317 was obtained for both *E. coli* and *C. albicans* when the *Hydrastis canadensis* 62% (v/v) tincture disc was compared to the *Hydrastis canadensis* 62% (v/v) tincture drop using the Mann-Whitney U test.

The results indicate that there is no statistically significant difference between the 62% (v/v) ethanol control disc and 62% (v/v) ethanol control drop on the *in vitro* growth of *E. coli* and the *C. albicans*, in terms of the sizes of the zones of inhibition in the disc-diffusion assay. A p-value of 0.317 was obtained for both *E. coli* and *C. albicans* when the 62% (v/v) ethanol control disc was compared to the 62% (v/v) ethanol control drop using the Mann-Whitney U test.

CHAPTER SIX

6.1 CONCLUSION

The purpose of this study was to evaluate the effectiveness of *Hydrastis canadensis* water-based extraction, in inhibiting the *in vitro* growth of *E. coli* and *C. albicans*, in terms of the size of the zones of inhibition in the disc diffusion assay and the turbidity of the contents of the test tube in the tube dilution method. These results were then compared to similar results obtained from using the *Hydrastis canadensis* 62% (v/v) ethanol tincture.

Hydrastis canadensis water-based extraction was ineffective in inhibiting the *in vitro* growth of both *E. coli* and *C. albicans* for both the disc diffusion assay and tube dilution method.

Hydrastis canadensis 62% (v/v) ethanol tincture was not effective in inhibiting the *in vitro* growth of *E. coli* and *C. albicans* in the disc diffusion assay and tube dilution method.

6.2 RECOMMENDATIONS

1. This study should be repeated using water-based extractions produced by the Wala method that is sterilized by gamma radiation.
2. This study should be repeated using a larger sample size in order to obtain a statistically significant result.
3. Studies should be done on fluid and solid herbal extracts of *Hydrastis canadensis*.
4. A study should be done to evaluate the manufacturing process of herbal tinctures used in practice, in terms of the quality control adopted from the time the herb is harvested to the completion of the end product.
5. *Hydrastis canadensis* produced by the method stated above in point 1, should be tested *in vivo* for anti-microbial effectiveness, by means of a controlled clinical trial.
6. Studies investigating the effect of filter paper discs as opposed to blank susceptibility discs in the disc diffusion assay should be carried out in the future.

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APPENDIX A



APPENDIX B

RAW DATA

TUBE DILUTIONS GROUP A (H₂O)

E COLI

1:2 - 3+

1:3 - 3+

1:5 - 2+

1:10 - 1+

Distilled water control 3+

CANDIDA ALBICANS

1:2 - 3+

1:3 - 3+

1:5 - 2+

1:10 - 1+

Distilled water control 3+

TUBE DILUTIONS GROUP B (ROH)

E COLI

1:2 - 0

1:3 - 0

1:5 - 0

1:10 - 0

62% (v/v) ethanol control - 0

CANDIDA ALBICANS

1:2 - 0

1:3 - 0

1:5 - 0

1:10 - 0

62% (v/v) ethanol control - 0

PLATES GROUP A (H₂O)

E COLI

TEST CONTROL

P1 0

P2 0

P3 0

PLATES GROUP B (ROH)

E COLI

TEST CONTR

P1 8.00 mm 8.00 mm

P2 7.50 mm 7.50 mm

P3 0.00 mm 0.00 mm

CANDIDA ALBICANS (H₂O)

P1 0

P2 0

P3 0

CANDIDA ALBICANS (ROH)

P1 0 0

P2 0 0

P3 0 0

0.5 MACFALLANS = 3+ GROWTH

DISC SIZE 7mm

VISUAL TURBIDITY

	0 NO GROWTH
1+	SCANT
2+	MODERATE
3+	DENSE
4+	VERY DENSE

APPENDIX C

CANDIDA ALBICANS DISC

HYDRASTIS	8.5mm
CTL1	8.5mm
CTL2	8.5mm

DROP TEST

HYDRASTIS	0 - INITIAL 11mm
CTL1	0
CTL2	0

E-COLI DISC

HYDRASTIS	8.5mm
CTL1	10mm
CTL2	8mm

DROP TEST

HYDRASTIS	0- INITIAL 10mm
CTL1	0-INITIAL 11mm
CTL2	0-INITIAL10.5mm

APPENDIX D

Tube dilution results for E-coli (H2O v.s.ROH)

Mann-Whitney Test

Ranks

	Substance	N	Mean Rank	Sum of Ranks
dil1	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil2	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil3	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil4	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		

Test Statistics^b

	dil1	dil2	dil3	dil4
Mann-Whitney U	.000	.000	.000	.000
Wilcoxon W	1.000	1.000	1.000	1.000
Z	-1.000	-1.000	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance

Plate e-coli results

Mann-Whitney Test

Ranks

	Substance	N	Mean Rank	Sum of Ranks
Test	1.00	1	1.00	1.00
	2.00	1	2.00	2.00
	Total	2		
Control	1.00	1	1.00	1.00
	2.00	1	2.00	2.00
	Total	2		

Test Statistics^b

	Test	Control
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance

plate candida results

Mann-Whitney Test

Ranks

	Substance	N	Mean Rank	Sum of Ranks
Test	1.00	1	1.50	1.50
	2.00	1	1.50	1.50
	Total	2		
Control	1.00	1	1.50	1.50
	2.00	1	1.50	1.50
	Total	2		

Test Statistics^b

	Test	Control
Mann-Whitney U	.500	.500
Wilcoxon W	1.500	1.500
Z	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance

Tube dilution results for E-coli (H2O v.s.ROH)

Mann-Whitney Test

Ranks

	Substance	N	Mean Rank	Sum of Ranks
dil1	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil2	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil3	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil4	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		

Test Statistics^b

	dil1	dil2	dil3	dil4
Mann-Whitney U	.000	.000	.000	.000
Wilcoxon W	1.000	1.000	1.000	1.000
Z	-1.000	-1.000	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance

Tube results for Candida (H2O v.s ROH)

Mann-Whitney Test

Ranks

	Substance	N	Mean Rank	Sum of Ranks
dil1	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil2	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil3	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		
dil4	1.00	1	2.00	2.00
	2.00	1	1.00	1.00
	Total	2		

Test Statistics^b

	dil1	dil2	dil3	dil4
Mann-Whitney U	.000	.000	.000	.000
Wilcoxon W	1.000	1.000	1.000	1.000
Z	-1.000	-1.000	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a

NPar Tests - Inter Group Analysis

Mann-Whitney Test - 1:2 dilution - Ethanol v.s Water

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance used

Mann-Whitney Test - 1:3 dilution - ethanol v.s water

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance used

Mann-Whitney Test - 1:5 dilution - ethanol v.s water

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance used

Mann-Whitney Test- 1:10 dilution (ethanol vs. water)

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Substance used

NPar Tests - Inter group analysis - disc v.s drop method

Mann-Whitney Test - Disc test v.s disc control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.500
Wilcoxon W	1.000	1.500
Z	-1.000	.000
Asymp. Sig. (2-tailed)	.317	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Disc test v.s Drop test

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Disc test v.s Drop control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test – Disc test vs. Drop control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Disc control v.s Drop control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Drop test v.s Drop control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.000
Wilcoxon W	1.000	1.000
Z	-1.000	-1.000
Asymp. Sig. (2-tailed)	.317	.317
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

NPar Tests - Inter group analysis - Plates

Mann-Whitney Test - Water test v.s Water control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.500	.500
Wilcoxon W	1.500	1.500
Z	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Water test v.s Ethanol test

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.500
Wilcoxon W	1.000	1.500
Z	-1.000	.000
Asymp. Sig. (2-tailed)	.317	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Water test v.s Ethanol control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.500
Wilcoxon W	1.000	1.500
Z	-1.000	.000
Asymp. Sig. (2-tailed)	.317	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test-Water test vs. Ethanol control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.500
Wilcoxon W	1.000	1.500
Z	-1.000	.000
Asymp. Sig. (2-tailed)	.317	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Water control v.s Ethanol control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.000	.500
Wilcoxon W	1.000	1.500
Z	-1.000	.000
Asymp. Sig. (2-tailed)	.317	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

Mann-Whitney Test - Ethanol test v.s Ethanol control

Test Statistics^b

	E.COLI	CANDIDA
Mann-Whitney U	.500	.500
Wilcoxon W	1.500	1.500
Z	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000 ^a	1.000 ^a

a. Not corrected for ties.

b. Grouping Variable: Test substance

APPENDIX E

TUBE DILUTION TEST

(*Hydrastis canadensis* 62% (v/v) ethanol tincture)



TUBE DILUTIONS (*E. coli*)

↓ ↓ ↓ ↓ ↓

CONTROL 1:2 1:3 1:5 1:10

TUBE DILUTIONS (*C. albicans*)

↓ ↓ ↓ ↓ ↓

CONTROL 1:2 1:3 1:5 1:10

TUBE DILUTION TEST

(*Hydrastis canadensis* water-based extraction)



TUBE DILUTIONS (*E. coli*)

↓ ↓ ↓ ↓ ↓

CONTROL 1:2 1:3 1:5 1:10

TUBE DILUTIONS (*C. albicans*)

↓ ↓ ↓ ↓ ↓

CONTROL 1:2 1:3 1:5 1:10

Anti-microbial effects of *Hydrastis canadensis* water-based extraction on *C. albicans*

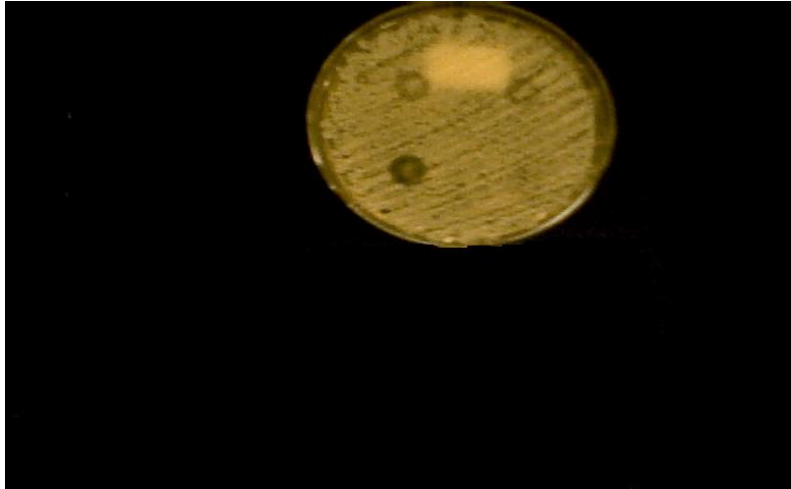


Anti-microbial effects of *Hydrastis canadensis* water-based extraction on *E. coli*.



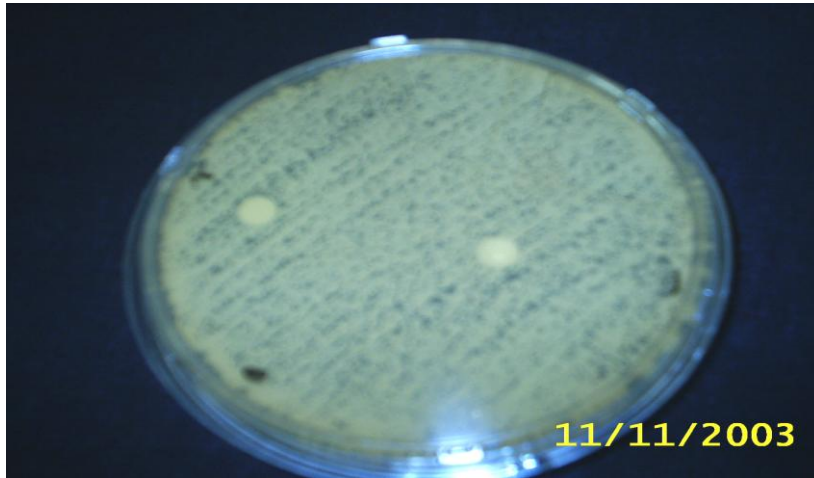
DROP TEST vs. DISC TEST (DROP)

Hydrastis canadensis 62% (v/v) ethanol tincture on *C. albicans*



DROP TEST vs. DISC TEST (FILTER PAPER DISC)

Hydrastis canadensis 62% (v/v) ethanol tincture on *C. albicans*



TEST FOR CONTAMINATION

Hydrastis canadensis water extraction streaked on a S.D.A plate (brownish plate) and a M.H.A (clear plate)

