Phytochemical Screening, Antimicrobial and Cytotoxicity Studies of *Heritiera fomes*

By

Rifah Tabassum
ID: 16146029

A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.)

Department of Pharmacy
Brac University
March 2020

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Rifah Tabassum
16146029

Approval

The project titled "Phytochemical Screening, Antimicrobial and Cytotoxicity Studies of *Heritiera fomes*" submitted by Rifah Tabassum of Spring, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 27th February 2020.

Examining Committee:

Supervisor:
(Member)

Farhana Alam Ripa
23/02/2020

Dr. Farhana Alam Ripa
Assistant Professor, Department of Pharmacy
Brac University

Program Coordinator:
(Member)

Professor Dr. Hasina Yasmin
Department of Pharmacy
Brac University

Departmental Head:
(Chair)

Professor Dr. Eva Rahman Kabir
Chairperson, Department of Pharmacy
Brac University

Ethics Statement

This study does not include any kind of animal or human trial.

Abstract

The present study was conducted to find out phytochemical constituents, cytotoxic property and antimicrobial effect of available parts of *Heritiera fomes* by using different solvents during extraction. The phytochemical screening was performed following Standard Method. Presence of cytotoxicity was tested by *in-vitro* brine shrimp lethality bioassay with different concentrations (400µg/mL-1200µg/mL). Antimicrobial activity was tested by disk diffusion method with concentrations of 500µg/mL and 1000µg/mL. Alkaloids, steroids, resins were some of the chemical constituents identified during phytochemical screening. The plant indicated antimicrobial activity in dose-dependent manner with highest effect against *Shigella dysenteriae* and *Shigella boydii* when Ethyl Acetate was present as solvent. The mortality rate due to cytotoxic effect was observed below 50% and maximum cytotoxicity level was found in root extracts. Although experimented extract showed antimicrobial effect presence of which responsible bioactive compounds behind it was not investigated, thus further work regarding this can be done.

Keywords: *Heritiera fomes;* Phytochemical screening; Brine shrimp lethality bioassay; Antimicrobial activity; Disk diffusion method

Dedication

To my family, teachers and friends

Acknowledgement

Firstly, I would like to give my gratitude to Almighty Allah for his continuous blessings and help to complete the work. After that, I would like to give special thanks to my supervisor Dr. Farhana Alam Ripa, Assistant professor, Department of Pharmacy, for her continuous support. Her guidance, inspiration and patience from the very beginning till the last day of my work helped me to complete my project.

I would also like to thank Dr. Eva Rahman Kabir, Professor and Chairperson, Department of Pharmacy, Brac University for her cooperation and support throughout the project.

Last but not the least, I would like to give thanks to all the lab officers and staffs who helped me a lot during the project by giving the continuous guidance, support and especially for their cooperative attitude.

Finally, I would like to thank my family, friends, and teachers for their motivation, patience and support during the project.

Table of Contents

Declaration.....**ii**

Approval ..... **iii**

Ethics Statement.....**iv**

Abstract.....**v**

Dedication .....**vi**

Acknowledgement.....**vii**

Table of Contents ..... **viii**

List of Tables .....**xi**

List of Figures.....**xii**

List of Acronyms ..... **xiii**

Chapter 1 Introduction.....**14**

1.1 Use of Medicinal Plants around the Globe 15

1.2 Medicinal Plants of Bangladesh 16

1.3 Phytochemical Screening of Medicinal Plants 17

1.4 *In-vitro* Brine Shrimp Lethality Bioassay.....17

1.5 Antimicrobial Activity Test of medicinal plants 18

1.6 Taxonomic classification of *Heritiera fomes* 18

1.7 Morphology of *Heritiera fomes*.....19

1.8 Habitat of *Heritiera fomes*.....20

1.9 Literature Review 21

1.10 Traditional Uses of *Heritiera fomes* 21

1.11 Rational of the Study 22

1.12 Aim of the Study 22

1.13 Objectives of the Study 22

Chapter 2 Methodology 23

2.1 Collection and Identification of *H.Fomes* 23

2.2 Process of Extraction 23

2.3 Preparation and drying of the plant parts 23

2.4 Extraction 24

2.5 Phytochemical Screening of *H. fomes* 26

2.6 *In-vitro* Brine Shrimp Lethality Bioassay 29

2.7 Disk Diffusion Method for Testing Antimicrobial Activity 30

Chapter 3 34

Results 34

3.1 Phytochemical Screening of Ethanolic Extract of *Heritiera fomes* 34

3.2 Images of the Phytochemical Screening of Extracts of *H.fomes* 34

3.2 *In-vitro* Brine Shrimp Lethality Bioassay 36

3.3 Antimicrobial Activity Test by Disk Diffusion Method 38

Chapter 4 42

Discussion 42

Chapter 5 44

Conclusion **44**

Chapter 6 ..... **45**

Future Works **45**

References **1**

List of Tables

Table 1 Names of the gram positive and gram negative bacteria used during the test.....31

Table 2 Screening of Ethanolic Extract of Heritiera fomes.....34

Table 3 Assay of In-vitro Brine Shrimp Lethality Test.....36

Table 4 Result of the concentration of 500µg/disc of Leaves extract of *Heritiera fomes*.....38

Table 5 Result of the concentration of 1000µg/disc of Leaves extract of *Heritiera fomes*.....39

Table 6 Result of the concentration of 500µg/disc of Barks extract of *Heritiera fomes*.....39

Table 7 Result of the concentration of 1000µg/disc of Barks extract of *Heritiera fomes*.....40

Table 8 Result of concentration of 500µg/disc of Root extract of *Heritiera fomes* 40

Table 9 Result of concentration of 1000µg/disc of Root extract of *Heritiera fomes* 41

List of Figures

Figure 1 *Heritiera fomes* in its natural habitation.....19

Figure 2 Distribution Map of *Heritiera fomes* (Kathiresan, 2010).....20

Figure 3 Steps of preparation and drying.....23

Figure 4 Step by step procedure of Extraction.....24

Figure 5 Screening of Steroid and Alkaloid accordingly.....34

Figure 6 Screening of Tannins.....35

Figure 7 Screening of Resins and Terpenoids 35

Figure 8 Sample of the Zone of Inhibition Test for Antimicrobial Activity 38

List of Acronyms

HE	Ethanol extract of *H.fomes*
HEA	Ethyl acetate extract of *H.fomes*
HPET	Petroleum ether extract of *H.fomes*
HC	Chloroform extract of *H.fomes*

Chapter 1

Introduction

Man has thrived from the ancient age to find the cure of his diseases and plants have always been the source of his findings. Medicinal plants and herbs have always thus been in use for treating diseases and eradicating human sufferings. (Rates, 2000) In the present date also the presence of medicinal plants can be seen in the Pharmaceutical world as almost 80% of medicines of today's world are directly or indirectly obtained from them. (Imran Mahmud, 2014)

A medicinal plant can be termed as a plant which can be used to relieve, prevent or cure a disease or have the property to change a physiological and pathological process. It can be any kind of a plant which can act as a source of drugs or their precursors. Any medicine manufactured in the crude form or transformed into any pharmaceutical formulation after obtaining from any kinds of plants is termed as Herbal Medicine. (Rates, 2000) Medicinal plants, herbs and essential oils possess a large amount of secondary metabolites which work as potential sources of drug and have therapeutic importance. Advantages of using medicinal plants like the economic benefits, effective outcomes and their easy availability make them the better alternative choice over the synthetic sources for therapeutic purposes. (Diversity, 2001) Another important advantage of using natural sources like plants is its safety. These benefits allowed the medicinal use of plants to be age-old tradition. History indicates that the association of plants and medicines had been drawn from ancient times as 4000–5000 B.C. Chinese are thought as the first ones to use natural herbal preparations as medicines to treat various diseases. The Indian practice of the indigenous system of medicine known as Ayurveda is termed as one of the ancient medical ways for healing people.

Widely used drugs of now like Morphine, Vincristrine, Aswagandha, Quinidine, Atropine, Reserpine, Digoxin and many more are all derived from plant sources. The folk medicine practitioners of medicine treat around 90% patients in Bangladesh, 85% in Burma and 80% in India (P. Prakash, 2005). Since, the rural people of Bangladesh cannot always reach to modern medicinal facilities the traditional healers are there primary doctors. These healers termed as “Kaviraj” use medicinal plants for their regular treatment in different forms. (Md. Shahadat Hossan, 2010) In the past few years an increasing interest in the alternative therapies and therapeutic use of natural products are seen. 80,000 species among the 2, 50,000 plant species of higher order recorded till now in the world are used for medicinal purpose. (Jain, 2016) Worldwide prescribed drugs contain 25% medicinal plant based drugs and among the 252 drugs listed as basic drugs by the World Health Organization (WHO) 11% are of plant origin and a large number of the synthetic drugs are also obtained from natural precursors. Moreover above 60% of anti- infectious and anti-tumor drugs of the ones available in the market and the ones under clinical trial are from natural sources directly or indirectly. (Rates, 2000)

1.1 Use of Medicinal Plants around the Globe

In drug discovery natural compounds can work as the lead compounds and aid in designing new formulations along with synergizing the effect of conventional medicines. Around the world different countries use the concept of medicinal plants in their healthcare system due to its number of benefits. (Jain, 2016) Countries in the East like China and India had built a well-established and well-renowned herbal medicines industry. The European countries for instance France and Germany also have their own shares of herbal medicines. About 50% medicines sold on medical prescriptions in Germany are phytochemical products. On the

other hand in North America, phytochemical and natural products are sold as under the tag of “health foods”. Following the European countries the Latin American countries are also focusing towards natural sourced medicinal products. (Rates, 2000)

Still the more diversified prospect of using higher order plants as the sources of new drugs is yet to be explored. A smaller percentage of the large numbered plants in the earth have been properly studied and their pharmacological properties have been completely unfolded as in many cases, only pharmacological screening or preliminary studies are performed. (Diversity, 2001)

1.2 Medicinal Plants of Bangladesh

Bangladesh, being a South Asian country lying within the tropical zones has the perfect environment and geographical location to have a lush and rich cultivation of a number of medical plant species. As a result, there exists a prestigious heritage of the use of medicinal plants in treating diseases in this country. (Shaikh Jamal Uddin, 2008) There is an important status of the use of medicinal plants and herbal products in the socio-cultural, medicinal and spiritual arena of the rural and tribal society of Bangladesh. Among the listed 2000 species of medicinal plants available within this sub-continent around 500 had been in use in this country as “traditional/folk medicine”. (Ghani, 1998) *Clerodendrum viscosum* locally known as Bhant, *Dillenia indica* locally known as Chalta, *Clitoria ternate* locally known as Aparajita, *Diospyros peregria* locally known as Gab, *Dipterocarpus turbinatus* locally known as Garjan, *Ecbolium Viride* locally known as Nilkhanta, *Glinus oppositifolius* locally known as Gima, *Saraca asoca* locally known as Ashok, *Glycosmisper taphylla* locally known as Daton and *Jasminum sambac* locally known as Beliphul are few commonly used medicinal plants in Bangladesh for conditions such as hypotensive, ulcers, white leprosy, asthma, diarrhea, gout, common cold, jaundice and many more. (Snigdha Bardhan, 2018) Generally these medicinal

plants are taken in the crude form or made into edible liquid forms by Kavirajes in Bangladesh who are the medicinal practitioners of folk medicine. These Kavirajes usually have to rely on formulations produced from medicinal plants for treating the common ailments. (Mohammad Ali, 2011)

1.3 Phytochemical Screening of Medicinal Plants

The Phytochemical Screening of medicinal plants refers to extraction, identification and screening of the medicinally active compounds generally found in plants. For instance few bioactive substances which are derived from plants include alkaloids, flavonoids, carotenoids, glycosides, tannins, antioxidants and other phenolic compounds (N. Gandhiraja, 2009). Naturally a set of phytochemicals are present in plants and they hold a number of biological significances by maintaining an efficient role in protecting plants against various pathogenic microbes. Through different methods of separation these components are extracted and identified for research purposes on the medicinal plants. (Lone, 2013)

1.4 *In-vitro* Brine Shrimp Lethality Bioassay

Cytotoxic activity of medicinal plants is tested by a number of methods. The Brine Shrimp Lethality Bioassay is one of those. It is comparatively a simpler and higher throughput Cytotoxicity test of various bioactive chemicals in the tested compounds. This method is based on the capacity of the tested sample on killing the simple organism, *Artemia salina* known as Brine Shrimp in *In-vitro* condition. The process is widely in practice to evaluate the toxicity of a number of substances like pesticides, natural plant extracts and even heavy metals. The method is also the primary toxicity screening for continuing in detailed researches on animal models. (Alluri V. Krishnarajua, 2005)

1.5 Antimicrobial Activity Test of medicinal plants

Antimicrobial Activity tests for medicinal plants are defined as the qualitative methods used to measure the ability of their extracts to inhibit the selected microbial growth and the antibiotic resistance. (N. Gandhiraja, 2009) With the passage of time infections grew to be more serious level and the scenario is worsening because of the recent antibiotics resistance standing as an obstacle in giving full therapeutic solutions. In such conditions plants can play a vital role as they already produce different secondary metabolites during creating their self defense mechanisms against fungal and bacterial infections. Plant-derived drugs can thus be useful to discover new antimicrobial agents having new novel mechanisms of actions with fewer side effects. (Shaikh Jamal Uddin, 2008) The Zone of Inhibition Test or the Kirby-Bauer Test is used in this study.

1.6 Taxonomic classification of *Heritiera fomes*

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Malvales

Family: Sterculiaceae

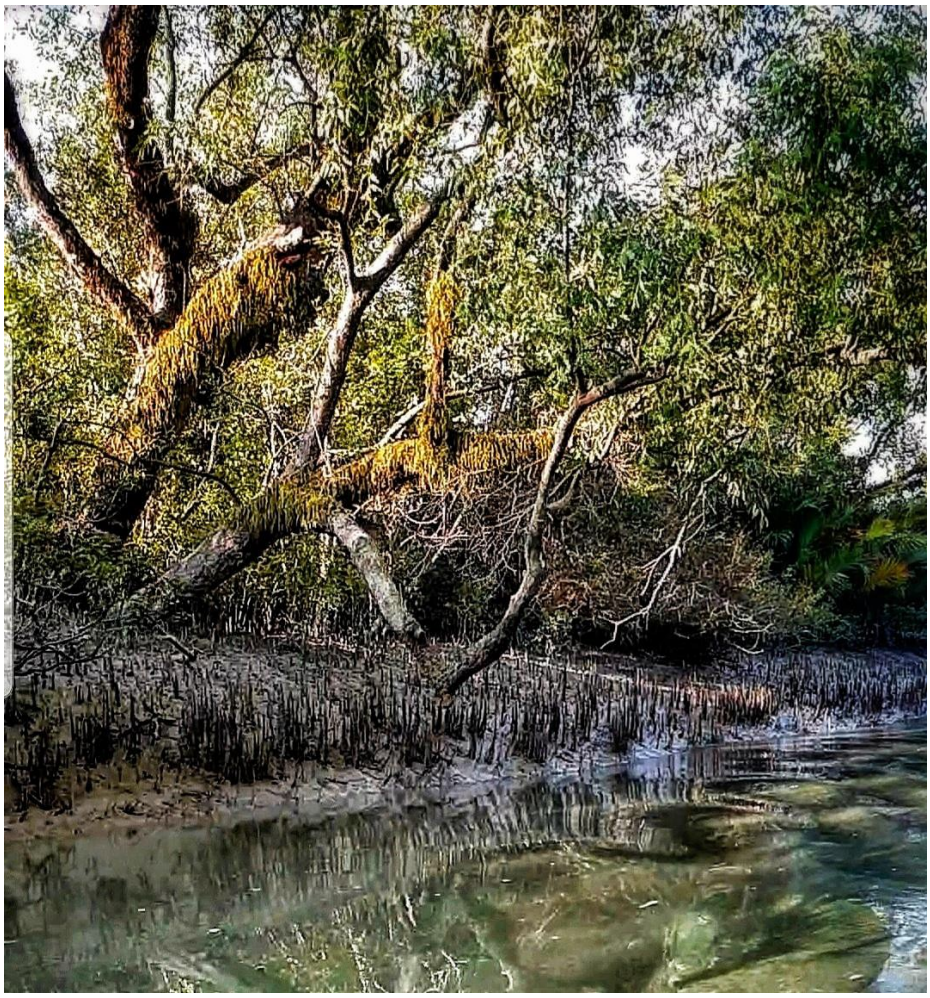

Figure 1 Heritiera fomes in its natural habitation

1.7 Morphology of *Heritiera fomes*

The full grown plants of *H.fomes* species grow up to 25 m normally. This species of *Heritiera* have pneumatophores, a specialized form of aerial roots to help them exchange gas in the swampy environment. (Kathiresan, 2010) Longitudinally splintered barks are seen in these plants with dark green leaves in grouped form near the twigs. Near the month of March and April its unisexual flowers are seen being arranged in panicles. The fruit carpel's length generally varies between 3.81cm and 5.08 cm, which fall to the land during the month of July and August after being ripen. (Md. Aslam Hossain, 2013)

1.8 Habitat of *Heritiera fomes*

Heritiera fomes is a common inhabitant of the largest mangrove forest known as Sundarban (0.57 million ha), located at the south-western side of Bangladesh and spreads towards the coast of the Bay of Bengal and West Bengal of India. (Md. Qumruzzaman Chowdhury, 2008) Areas having heavy annual rainfall of around 1600 mm to 5334 mm, with a warm and humid climate of 7.22 °C to 37.78 °C are the suitable habitat of *H.fomes*. The geographical habitat of this species contains a hostile environment with high salinity, low and high tidal waves, high humidity and temperature. Along with these abiotic stress factors there exists biotic stress also from the insects and microorganisms. These factors contributed to form highly developed and evolved morphological and many physiological adaptations within the plants of *Heritiera fomes* species. These physiological alterations resulted in the synthesis of many novel compounds which offer protection against the biotic and abiotic stressful environment. Such compounds can act as secondary metabolites and their biological and medicinal properties can be used to fulfill the health care needs of man. (Aritra Simlai, 2013)

Figure 2 Distribution Map of Heritiera fomes (Kathiresan, 2010)

1.9 Literature Review

Previous investigations showed that the plants of *Heritiera fomes* bear the antioxidant, antihyperglycemic, anticancer, antimicrobial and antinociceptive properties. Different chemical constituents for example tannins, steroids, glycosides, flavonoids, gums, reducing sugars were found present in these plants by phytochemical analyses. The ethno botanical properties of *H.fomes* were analyzed and different therapeutical activities were estimated. (Imran Mahmud, 2014) The plant is traditionally recommended for gastrointestinal diseases, gout, diabetes mellitus and many other diseases. (Md. Shahadat Hossan, 2010) Experiments conducted previously have indicated low toxicity of the *H.fomes* species as it showed less than 50% lethality rate during the *in-vitro* Brine Shrimp Lethality Test conducted with the Ethanolic extracts from its plants within the concentrations of 10 to1000 μg/ml. (Md. Aslam Hossain, 2013) Studies have determined antimicrobial effect by *H.fomes* against the Gram positive bacteria: *K.rhizophilia, B. subtilis and S. aureus* and against the Gram-negative bacteria: *E. coli, P.aeruginosa*. Moreover, inhibiting property against the growth of *C. albicans,* a kind of yeast was found, following the disk diffusion method to test antimicrobial activities. (Helle Wangensteena, 2009)

1.10 Traditional Uses of *Heritiera fomes*

Locally this plant has been used as a traditional medicine due to its pharmacological activities. Different parts of the plant such as leaves, roots and stems are used in different edible forms for the treatment of skin diseases like abscess, acne, eczema, scabies, rash or sores, again for hepatic disorders such as jaundice or hepatitis, diabetes and most commonly used for gastrointestinal diseases including dysentery, diarrhea, acidity, indigestion, stomachache, constipation or bloating. Moreover it is also used to cure fever or minor pain. (Md. Aslam Hossain, 2013)

1.11 Rational of the Study

Heritiera fomes is naturally abundant in the humid and salty areas of Bangladesh, especially in the Sundarbans. The trees are used commonly as timber although both previous and recent studies indicated the presence of many active phytochemical constituents in the plants. Investigation over these chemical compounds and their natural ability to survive in an abiotic and biotic stressful environment can extend the horizon of novel drugs and significant therapeutical advancement in the health care sector of Bangladesh. This study was conducted with the purpose of scrutinizing and examining the presence of these phytochemical constituents, cytotoxicity effect and the Antimicrobial Activity of *H.fomes* against both Gram Positive and Gram Negative Bacteria.

1.12 Aim of the Study

The aim of the study is to screen out the phytochemical constituents, examine cytotoxicity and antimicrobial activity of *H.fomes.*

1.13 Objectives of the Study

The objectives of this study are:

- To screen out the phytochemical constituents of *H. fomes.*
- To investigate the presence of toxicity in leaves, barks and roots of *H.fomes.*
- To examine the Antimicrobial activity of *H.fomes.*

Chapter 2 Methodology

2.1 Collection and Identification of *H.Fomes*

For the experimental investigation leaves, bark and root of *Heritiera fomes* was collected from Sundarban, Bagerhat district, Bangladesh in June of 2019. The mentioned parts of the plant were brought separately and submitted to the National Herbarium of Bangladesh (NHB), located in Mirpur, Dhaka for authentication. They identified the body parts of *Heritiera fomes* provided the DACB ACCESSION NO.: - 50664.

2.2 Process of Extraction

Many steps are included in the process of extraction; those can be divided into two parts:

a. Preparation and drying of the plant parts

b. Extraction

2.3 Preparation and drying of the plant parts

Leaves, barks and roots were washed carefully with water to remove the dirt or dust particles. Afterwards the washed parts were spread out over clean papers for natural drying under the sun for two weeks.

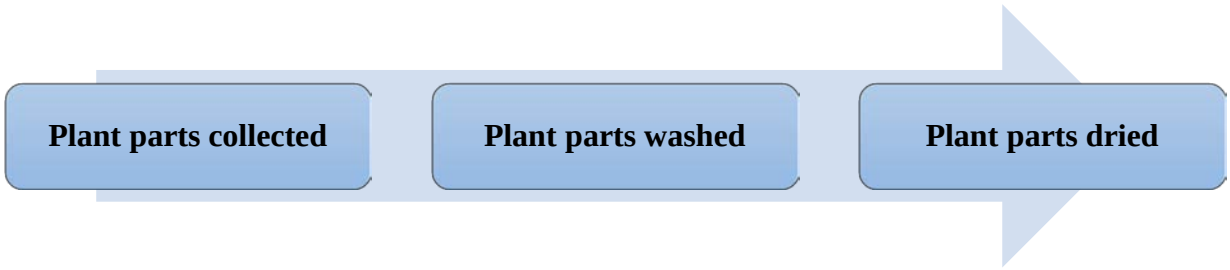

Figure 3 Steps of preparation and drying

2.4 Extraction

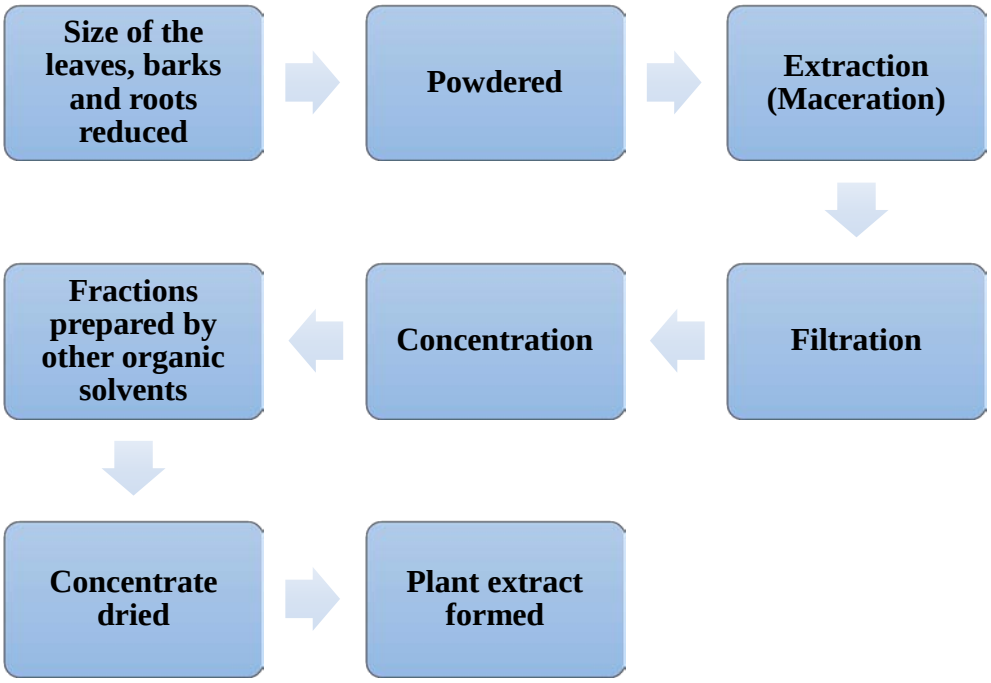

Figure 4 Step by step procedure of Extraction

2.4.1 Size Reduction

The dried out parts are now cut down into smaller pieces to reduce their sizes. The leaves are shredded; the barks and the roots are broken down into small pieces of two or three inches length. Next, these smaller pieces are granulated into fine powders individually and weighed. Three clean air tight plastic containers were labeled with necessary information and the crude powders were stored in them.

2.4.2 Maceration of extraction

Afterwards, maceration of extraction process was performed where ethanol was used as the organic solvent. 250gm of each powdered leaves, bark and root was weighed and completely soaked into 1L of ethanol in separate beakers for five days at normal room temperature (22-25 °C). Regular stirring was done to soak the plant parts properly in the solvent.

2.4.3 Filtration

After that, the ethanol soaked solutions were first filtered using a clean cotton cloth and then filtered using the Filter papers to obtain a clear solution with the crude extract.

2.4.4 Concentration

Half of the filtered crude extracts were separated and stored into separate beakers. The rest half of the three separate crude extracts were transferred to the rotary evaporator to obtain the concentrated filtrate by removing the ethanol.

2.4.5 Drying of concentrate

In order to protect the concentrate from any microbial augmentation during the time of drying, Laminar Air Flow (LAF) was used to evaporate the solvent. The concentrated dried forms of the extracts were then collected in ethanol washed three separate petri-dishes within the Laminar Air Flow (LAF). The petri-dishes were then safely enclosed using aluminum foil and stored for further investigation.

2.4.6 Separation

The previously stored half of the ethanol soaked extract solutions were individually then mixed with Petroleum Ether (PET) first and separated using a separating funnel. The extracts dissolved in Petroleum Ether (PET) were then stored in sealed glass vile. Next, the ethanol dissolved parts from the previous separating funnel were mixed with Ethyl Acetate in another

separating funnel and the extracts dissolved in Ethyl Acetate were stored in sealed glass vile. Finally, Chloroform was added to the remaining ethanol dissolved extracts and the extracts dissolved in Chloroform were collected from the separating funnel and stored accordingly. As a result, based on the specific gravity of the four organic solvents: Ethanol, Petroleum Ether (PET), Ethyl Acetate and Chloroform four fractions of each three (leaves, barks and roots) in total 12 fraction extracts were separately prepared.

2.5 Phytochemical Screening of *H. fomes*

Alkaloid, steroid, tannin, saponin, resin, tarpenoid and glycoside phytochemical screening was done on the crude extracts to figure out the qualitative chemical composition of *H.fomes.*

2.5.1 Detection of Alkaloid

Two tests were performed with the filtrate ethanol extracts of leaves, barks and roots separately to detect the presence of alkaloid; following are mentioned about their processes:

Wagner's Test

3ml of the filtrate solution of ethanol leaves, barks and roots were taken in 3 different test tubes and marked. Next 1ml of Hydrochloric acid was added to the test tubes and shaken well. Then the Wagner's reagent (a combination of iodine and potassium iodide was freshly made) was added drop wise to each test tube. A reddish brown precipitate was formed in each of the test tubes indicating that alkaloid was present in the leaves, barks and roots of *H.fomes*. (Thilagavathi.T, 2015)

Mayer's Test

10ml of Mayer's reagent was freshly prepared by dissolving 0.1358 g of mercuric chloride and 0.5g of Potassium Iodide in 10ml distilled water. 3ml of the filtrate solution of ethanol leaves, barks and roots were taken in 3 different test tubes and marked accordingly. Next, the

prepared Meyer's reagent was added drop wise to the test tubes which resulted in a cream colored precipitation at bottom of the test tubes. This indicated the presence of alkaloid in the body parts of *H.fomes.* (Neelapu Neelima, 2011)

2.5.2 Detection of Steroid

For the detection of steroid in the parts of *H.fomes* Libermann-Burchard test was performed.

Libermann-Burchard Test

For this test the Libermann-Burchard reagent was first freshly prepared by combining 5ml of Acetic Anhydride with 5ml of concentrated Sulphuric Acid and adding 50ml of Ethanol while cooling it in ice cold water. Then the solution was heated at 100°C for 5 to 10 minutes. Next, 10 mg of the concentrated ethanol extract of leaves, barks and roots were taken separately from the petri-dishes and were dissolved in 0.5ml of hot Acetic Anhydride in test tubes. Then 0.5ml filtrate Chloroform was added into the separate test tubes. After the extracts were fully dissolved the freshly prepared Libermann-Burchard reagent was added drop wise which resulted in the formation of a blue-green ring around the test tubes of bark and root extracts. This indicated that steroid was present in the barks and roots of *H.fomes.* (Neelapu Neelima, 2011)

2.5.3 Detection of Tannin

For this test 1ml of filtrate ethanol solution of the leaves, barks and roots extract was added with 2ml of distilled water in separate test tubes. Next 2-3 drops of Ferric Chloride was added into the solution which resulted in the formation of green to blue-green color in the test tube of leaves indicating that Catechin Tannin was present in it. And a dark blue to bluish black color was seen in the test tubes of barks and roots indicating that Gallic Tannin was present in there. (Faraz Mojab, 2003)

2.5.4 Detection of Saponin

For detecting saponin, 1ml of the filtrate ethanol solution of the leaves, barks and roots extract were taken in test tubes and 1ml of distilled water was added to them. Next the test tubes were shaken vigorously and kept still to observe the stable persistent forth for 20mins. Barks and roots of *H.fomes* contain saponin at high amount compared to the leaves as a very foaming texture was seen after they were shaken. (N. Gandhiraja, 2009)

2.5.5 Detection of Resin

In order to detect resin, 1ml of the filtrate ethanol solution of the leaves, barks and roots extract was taken and few drops of Acetic Anhydride was added. Then 1ml of concentrated Sulphuric Acid was added. Orange to yellow colorization was seen in all the test tubes indicating the presence of resin in the parts of the *H.fomes* plant. (Faraz Mojab, 2003)

2.5.6 Detection of Tarpenoid

The detection of the tarpenoid was done by the Salkowski Test where 5ml of the filtered ethanol extract of the 3 parts of the *H.fomes* plant was added to 2ml of Chloroform. Then 3ml of concentrated Sulphuric Acid was carefully added to them which produced a reddish brown colorization at the interface in the test tubes. This indicated the presence of tarpenoid in the parts of *H.fomes* plant. (Neelapu Neelima, 2011)

2.5.7 Detection of Glycosides

The glycoside detection was done by the Keller-Killiani Test, taking 4ml of the filtered ethanol extracts of leaves, barks and roots individually in 3 test tubes. Next, few drops of Glacial Acetic Acid and few drops of Ferric Chloride were added to the extract containing test tubes. Then few drops of concentrated Sulphuric Acid were added. This was supposed to give a reddish brown colorization at the junction of two layers where the upper layer would

be of bluish-green color. Since the mentioned phenomenon was not observed it can be stated that glycoside is not present in the body parts of *H.fomes.* (Thilagavathi.T, 2015)

2.6 *In-vitro* Brine Shrimp Lethality Bioassay

2.6.1 Culture of Brine Shrimp

Brine shrimp eggs were cultured in a round bottom flask where 3L of artificial seawater (40mg/L of NaCl) was taken and a temperature of roughly 25°C to 28°C was maintained for 36 hours. Then the newly hatched phototropic nauplii were collected by applying light to a specific area of the round bottom flask and a pipette.

2.6.2 Preparation of Stock Solutions of Leaves

In order to perform the Cytotoxicity test using Brine Shrimp at the very beginning 4 stock solutions of the 4 fraction extracts of the leaves of *Heritiera fomes* were prepared. 32mg from each fraction was weighed and dissolved in 200µl of pure Dimethyl Sulfoxide (DMSO). Then the volume was made up to 20 ml with prepared sea water. The concentration of this stock solution was then 1600µg/ml.

2.6.3 Preparation of Stock Solutions of Barks

Likewise, 4 stock solutions of the 4 fraction extracts of the barks of *Heritiera fomes* were prepared. 32mg from each fraction was weighed and dissolved in 200µL of pure Dimethyl Sulfoxide (DMSO) and the volume was made up to 20ml with prepared sea water. The concentration of this stock solution was then 1600µg/ml.

2.6.4 Preparation of Stock Solutions of Roots

4 fraction extracts of the roots of *Heritiera fomes* were also taken and the 4 stock solutions were prepared by taking 32mg from each fraction and dissolving them in 200µl of pure

Dimethyl Sulfoxide (DMSO). Then the volume was made up to 20 ml with prepared sea water. The concentration of this stock solution was also 1600µg/ml.

2.6.5 Preparation of the Samples for the test

Samples from each stock solution were prepared having the concentrations: 1200µg/ml, 800µg/ml and 400µg/ml.

2.6.6 Preparation of Control for the test

For the control group of the Cytotoxicity test, solutions of Vincristin Sulfate having the concentrations of 1200µg/ml, 800µg/ml and 400µg/ml were prepared.

2.6.7 Experimental Procedure

The 36 samples prepared were taken in 36 different test tubes in the amount of 2.5ml in each and 2.5ml of seawater containing 10 nauplii were added to them. The test tubes were then wrapped in aluminum foil papers and kept in normal room temperature for 24 hours. After 24 hours the lethality bioassay was done where the number of dead nauplii was counted of each sample.

$$\% \text{ Mortality} = (\text{Number of dead nauplii} / \text{Number of nauplii taken}) \times 100$$

2.7 Disk Diffusion Method for Testing Antimicrobial Activity

The antimicrobial property test was done by the 4 fraction extracts of the leaves, barks and roots of the *H.fomes* following the Disk Diffusion Method.

2.7.1 Materials and Apparatuses

1. Nutrient Agar Medium
2. Filter paper discs

3. Petri-dishes
4. Micropipette
5. Sterile forceps
6. M.H. Agar
7. Screw cap test-tube
8. Autoclave
9. Spirit burner
10. Incubator
11. Laminar air flow hood
12. Nose mask and hand gloves

2.7.2 Microorganisms used for the test

Both gram positive and gram negative bacteria were used to perform the antimicrobial test.

Table 1 Names of the gram positive and gram negative bacteria used during the test

Gram Positive Bacteria	Gram Negative Bacteria
• *Bacillus subtilis* • *Staphylococcus aureus*	• *Escherichia coli* • *Salmonella typhi* • *Salmonella paratyphi* • *Shigella dysenteriae* • *Shigella shiga* • *Shigella boydii* • *Pseudomonas aeruginosa* • *Klebsiella pneunomiae*

2.7.3 Sterilization procedure

All the needed devices for the test including the beakers, cottons, conical flasks and forceps were sterilized by the autoclave and stored in an aseptic place before use. All the works were performed under the laminar air flow hood and the UV light was lit before starting of the experiment for 1 hour in order to maintain the control environment. One time useable sterilized petri-dishes were used to perform the experiment.

2.7.4 Sample Preparation

In total 12 stock solutions were prepared for the test from which sample solutions from each having the concentrations of 500µg/ml and 1000µg/ml were prepared.

2.7.5 Experimental Procedure

i. 2.5 g of nutrient broth was weighed and dissolved in 100 ml of distilled water to prepare the broth for culturing the bacteria.
ii. Next 10 conical flasks were taken with 10ml of the prepared broth and 10 mentioned bacterial strains were added. These conical flasks were stored in the incubator in shaking position for the next 24 hours at 37 ° C temperature.
iii. After 24 hours, the conical flasks were taken out from the incubator and were kept in a controlled environment for later use.
iv. The agar medium was prepared next by using 7.6 g of M.H. agar and it was dissolved in 200 ml of distilled water.
v. The prepared agar was then placed on patriotic plates and kept for cooling in room temperature.

vi. After the agar medium was solidified in the petri-dishes, the prepared bacterial strains of the conical flasks were spread out carefully on them by the cotton bars.

vii. Kanamycin discs were used as the standard in all the petri-dishes and placed carefully.

viii. Likewise the samples' discs prepared and blanks were introduced in the petri-dishes and kept in the incubator at a temperature of 37 ° C for next 24 hours to provide the optimal environment for the bacterial growth.

ix. After 24 hours, the petri-dishes were collected from the incubator and the inhibition zone of the standard and the sample discs were measured.

Chapter 3

Results

3.1 Phytochemical Screening of Ethanolic Extract of *Heritiera fomes*

Table 2 Screening of Ethanolic Extract of Heritiera fomes

Class of Compounds	Name of the Test	Present (+)/Absent(-)
Alkaloid	Wagner's Test	+
	Mayer's Test	+
Steroid	Libermann-Burchard Test	+
Tannin	Ferric Chloride Test	+
Saponin	Foam Test	+
Resin	Acetic Anhydride Test	+
Terpenoid	Salkowski's Test	+
Glycoside	Keller-Killiani Test	-

3.2 Images of the Phytochemical Screening of Extracts of *H.fomes*

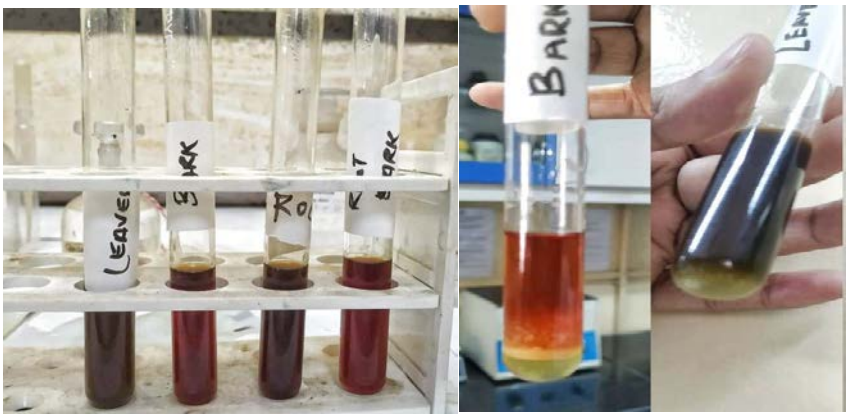

Figure 5 Screening of Steroid and Alkaloid accordingly

Figure 6 Screening of Tannins

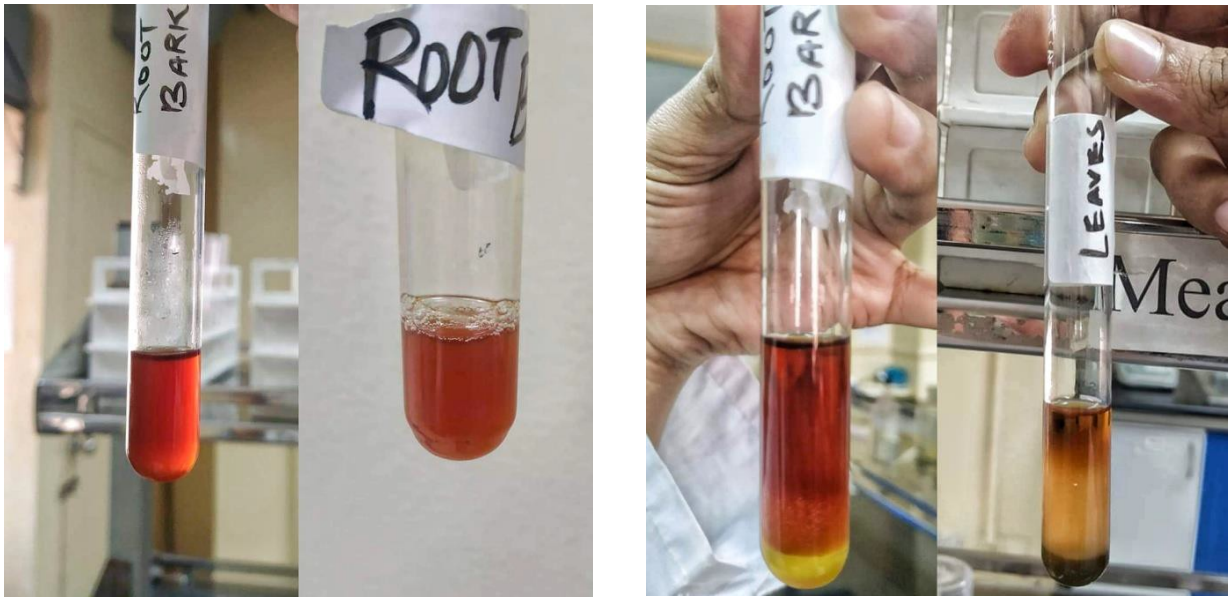

Figure 7 Screening of Resins and Terpenoids

3.2 *In-vitro* Brine Shrimp Lethality Bioassay

Table 3 Assay of In-vitro Brine Shrimp Lethality Test

Plant Parts	Organic Solvents	Concentrations	%Mortality	% Mortality of Control (Vincristin Sulfate)
Leaves	Ethanol	1200µg/ml	30	100
		800µg/ml	10	100
		400µg/ml	0	90
	Petroleum Ether	1200µg/ml	20	100
		800µg/ml	10	100
		400µg/ml	0	90
	Ethyl Acetate	1200µg/ml	30	100
		800µg/ml	20	100
		400µg/ml	0	90
	Chloroform	1200µg/ml	30	100
		800µg/ml	20	100
		400µg/ml	10	90
Barks	Ethanol	1200µg/ml	10	100
		800µg/ml	10	100
		400µg/ml	0	90
	Petroleum Ether	1200µg/ml	10	100
		800µg/ml	0	100
		400µg/ml	0	90
	Ethyl Acetate	1200µg/ml	30	100

		800µg/ml	10	100
		400µg/ml	0	90
	Chloroform	1200µg/ml	40	100
		800µg/ml	30	100
		400µg/ml	10	90
Roots	Ethanol	1200µg/ml	30	100
		800µg/ml	20	100
		400µg/ml	0	90
	Petroleum Ether	1200µg/ml	20	100
		800µg/ml	20	100
		400µg/ml	10	90
	Ethyl Acetate	1200µg/ml	30	100
		800µg/ml	20	100
		400µg/ml	20	90
	Chloroform	1200µg/ml	40	100
		800µg/ml	40	100
		400µg/ml	30	90

3.3 Antimicrobial Activity Test by Disk Diffusion Method

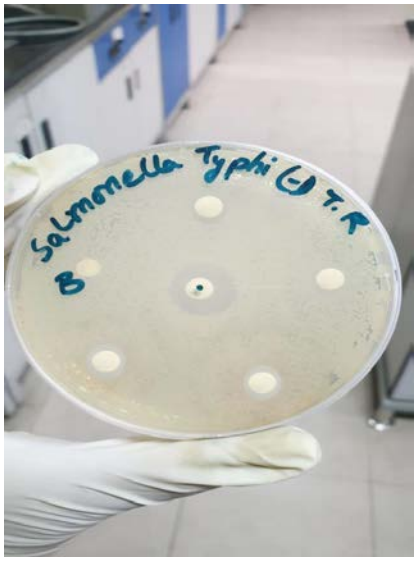

Figure 8 Sample of the Zone of Inhibition Test for Antimicrobial Activity

Table 4 Result of the concentration of 500µg/disc of Leaves extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	14mm	12mm	15mm	18mm
S.typhi	17mm	12mm	12mm	18mm
S.paratyphii	14mm	11mm	13mm	18mm
S.dysenteriae	16mm	15mm	13mm	19mm
S.sonnei	14mm	10mm	12mm	17mm
S.boydii	16mm	12mm	11mm	18mm
B.subtilis	12mm	10mm	13mm	16mm
S.aureus	12mm	11mm	11mm	16mm
P.aeruginosa	11mm	12mm	10mm	13mm
K.pneomoniae	10mm	10mm	12mm	17mm
Kanamycin	25mm	27mm	23mm	30mm

Table 5 Result of the concentration of 1000µg/disc of Leaves extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	18mm	15mm	16mm	20mm
S.typhi	20mm	19mm	15mm	23mm
S.paratyphii	20mm	19mm	15mm	23mm
S.dysenteriae	23mm	19mm	20mm	24mm
S.sonnei	21mm	17mm	19mm	23mm
S.boydii	23mm	19mm	20mm	24mm
B.subtilis	20mm	19mm	17mm	23mm
S.aureus	21mm	17mm	16mm	26mm
P.aeruginosa	19mm	17mm	16mm	23mm
K.pneomoniae	18mm	18mm	16mm	20mm
Kanamycin	25mm	27mm	23mm	30mm

Table 6 Result of the concentration of 500µg/disc of Barks extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	12mm	14mm	15mm	17mm
S.typhi	16mm	12mm	11mm	18mm
S.paratyphii	12mm	13mm	13mm	18mm
S.dysenteriae	17mm	15mm	11mm	20mm
S.sonnei	16mm	14mm	11mm	19mm
S.boydii	15mm	10mm	12mm	19mm
B.subtilis	16mm	11mm	12mm	19mm
S.aureus	12mm	12mm	13mm	17mm
P.aeruginosa	13mm	11mm	10mm	17mm

K.pneomoniae	11mm	11mm	13mm	15mm
Kanamycin	29mm	30mm	21mm	33mm

Table 7 Result of the concentration of 1000µg/disc of Barks extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	16mm	18mm	18mm	20mm
S.typhi	20mm	18mm	16mm	21mm
S.paratyphii	20mm	18mm	16mm	21mm
S.dysenteriae	22mm	20mm	18mm	24mm
S.sonnei	22mm	21mm	19mm	24mm
S.boydii	22mm	20mm	18mm	24mm
B.subtilis	19mm	17mm	16mm	23mm
S.aureus	16mm	15mm	17mm	20mm
P.aeruginosa	19mm	17mm	15mm	22mm
K.pneomoniae	16mm	18mm	18mm	21mm
Kanamycin	29mm	30mm	21mm	33mm

Table 8 Result of concentration of 500µg/disc of Root extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	12mm	11mm	14mm	18mm
S.typhi	12mm	13mm	11mm	19mm
S.paratyphii	11mm	11mm	13mm	19mm
S.dysenteriae	14mm	11mm	13mm	25mm
S.sonnei	18mm	14mm	15mm	19mm
S.boydii	16mm	12mm	16mm	19mm

B.subtilis	12mm	11mm	12mm	18mm
S.aureus	11mm	12mm	10mm	17mm
P.aeruginosa	11mm	12mm	10mm	15mm
K.pneomoniae	12mm	13mm	10mm	15mm
Kanamycin	27mm	22mm	19mm	32mm

Table 9 Result of concentration of 1000µg/disc of Root extract of Heritiera fomes

Microorganisms	**HE**	**HC**	**HPET**	**HEA**
E.coli	17mm	15mm	18mm	22mm
S.typhi	19mm	20mm	17mm	23mm
S.paratyphii	19mm	20mm	17mm	23mm
S.dysenteriae	20mm	19mm	18mm	22mm
S.sonnei	25mm	20mm	20mm	23mm
S.boydii	20mm	19mm	18mm	22mm
B.subtilis	18mm	16mm	19mm	24mm
S.aureus	19mm	17mm	16mm	19mm
P.aeruginosa	18mm	15mm	15mm	20mm
K.pneomoniae	19mm	17mm	14mm	23mm
Kanamycin	27mm	22mm	19mm	32mm

Chapter 4

Discussion

There are a variety of ways to use plants as therapeutic resources. Many medicinal plants are used as crude extracts or like raw materials in the pharmaceutical industries. The pharmacological property bearing part can be extracted and used in the forms of tinctures, powders, fluid extracts, pills or capsules. And the most prominent way of using the plant resources economically is following the proper extraction and purification procedure, which can isolate the selected compounds and can be taken as active drugs. (Jain, 2016) For this reason phytochemical screening of the plant is very crucial in the primary stage of plant based researches as it identifies the constituents present in it and gives a direction for the study. The phytochemical screening of *H.fomes* performed in this study indicated the presence of alkaloids, tannins, resins, steroids, terpenoids and saponins. Though glycoside was indicated by various studies presence of it was not found by the experiment conducted in here.

A low mortality rate is observed in the fractions of the extracts of the *H.fomes* indicating a very low Cytotoxicity of the plant. Though the mortality rate is 0% in most of the fractions at lower concentrations, Chloroform present fraction shows a comparatively higher mortality rate than the rest. With the increase of concentration of the samples the rate of mortality also increased gradually which shows that the cytotoxic property of *H.fomes* is very less but dose- dependent. From the calculated zone of inhibition, it is observed that different parts such as leaves, barks and roots of *H.fomes* fractioned and extracted using four organic solvents: Ethanol, Petroleum Erher, Ethyl Acetate and Chloroform, showed successive inhibiting property against the selected microorganisms in all the concentrations. It can, thus be stated from the experiment that *H.fomes* has antimicrobial activity. Analyzing and comparing the results of the zone of inhibition test of antimicrobial activity it is seen that the inhibiting property of microbial growth increases with the

increasing concentration of the fractions, proving it to be dose- dependent. Moreover, comparatively a higher antimicrobial activity is seen when the leaves, barks and roots of *H.fomes* is fractioned and extracted using the organic solvent, Ethyl Acetate and the lowest is seen when Petroleum Ether was used.

Chapter 5

Conclusion

Heritiera fomes being one of the prominent local plants of Bangladesh still lacks behind in being properly and efficiently used. Though it is used in mechanical works and as timbers, its therapeutic effects are yet to be given purpose and used in synthesizing novel drugs.

Concluding with the findings of this study, *H.fomes* species plants can be recommended to be used to synthesize Antimicrobial drugs. Moreover as the Cytotoxicity level of the plant is very low severe toxic effects may be absent in controlled concentrations.

Chapter 6

Future Works

The various phytochemical compounds present in *Heritiera fomes* can be extracted and examined to figure out their pharmacological effects individually. The phenolic constituents present in the species can be extracted in bulk amount and can be used to produce Cancer preventing drugs. The bioactive components responsible behind the antimicrobial effect can be investigated and extracted for further work.

Alongside, other screening procedures can be conducted to find out its other chemical constituents. Its Antimicrobial activity against other microorganisms including Viruses can be investigated. Other Pharmacological Activities of the plant can be examined using efficient methods.

References

Arya, D., Meena, M., Neha, G., & Vidya, P. (2014). In vitro anti-inflammatory and anti- arthritic activity in methanolic extract of Cocculus hirsutus (L.) Diels. In vivo and In vitro. *International Journal of Pharmaceutical Sciences and Research*, *5*(5), 1957–1962. https://doi.org/10.1017/CBO9781107415324.004

Banerjee, S., Biswas, S., Chanda, A., Das, A., & Adhikari, A. (2014). Evaluation of phytochemical screening and anti-inflammatory activity of leaves and stem of Mikania scandens (l.) wild. *Annals of Medical and Health Sciences Research*, *4*(4), 532. https://doi.org/10.4103/2141-9248.139302

Basak, U. C., Das, A. B., & Das, P. (1996). Chlorophylls, carotenoids, proteins and secondary metabolites in leaves of 14 species of mangrove. *Bulletin of Marine Science*, *58*(3), 654–659.

Elkhamlichi, A., El Hajaji, H., Faraj, H., Alami, A., El Bali, B., & Lachkar, M. (2017). Phytochemical screening and evaluation of antioxidant and antibacterial activities of seeds and pods extracts of Calycotome villosa subsp. Intermedia. *Journal of Applied Pharmaceutical Science*, *7*(4), 192–198. https://doi.org/10.7324/JAPS.2017.70428

Gundidza, M. (1985). Phytochemical screening of some Zimbabwean medicinal plants. *The Central African Journal of Medicine*, *31*(12), 238–239.

Hossain, M., Panthi, S., Asadujjaman, M., Khan, S., Sadhu, S., & Ferdous, F. (2013). Phytochemical and Pharmacological Assessment of the Ethanol Leaves Extract of Heritiera fomes Buch. Ham. (Family-Sterculiaceae). *Journal of Pharmacognosy and Phytochemistry*, *2*(3), 95–101.

Jeong, S. H., Kim, J., & Min, H. (2018). In vitro anti-inflammatory activity of the Artemisia Montana leaf ethanol extract in macrophage RAW 264.7 cells. *Food and Agricultural Immunology*, *29*(1), 688–698. https://doi.org/10.1080/09540105.2018.1439454

Karthikeyan, R., & Koushik O, S. (2016). Anti-Inflammatory Activity of Ethanolic Extract of Flowers Hymenocallis littoralis (Jacq.) Salisb. By HRBC Membrane Stabilization

Method. *Translational Biomedicine*, *7*(2), 1–5. https://doi.org/10.21767/2172-0479.100060

Kim, H. J., Park, C. G., Varghese, R., Lee, J. Y., Kim, Y. O., & Sung, G. H. (2017). In-vitro antioxidative, antiinflammatory properties of Aurea helianthus leaf extract a Korean traditional medicinal plant. *Saudi Journal of Biological Sciences*, *24*(8), 1943–1947. https://doi.org/10.1016/j.sjbs.2017.11.046

Leelaprakash, G., & Mohan Dass, S. (2011). Invitro anti-inflammatory activity of methanol extract of enicostemma axillare. *International Journal of Drug Development and Research*, *3*(3), 189–196.

Nateque Mahmood, M., Prasad Dhakal, S., & Keast, R. (2014). The state of multi-purpose cyclone shelters in Bangladesh. *Facilities*, *32*(9/10), 522–532. https://doi.org/10.1108/f- 10-2012-0082

Patra, J. K., & Thatoi, H. (2013). Anticancer activity and chromatography characterization of methanol extract of Heritiera fomes Buch. Ham., a mangrove plant from Bhitarkanika, India. *Oriental Pharmacy and Experimental Medicine*, *13*(2), 133–142. https://doi.org/10.1007/s13596-013-0113-7

Paun, G., Neagu, E., Moroeanu, V., Albu, C., Ursu, T. M., Zanfirescu, A., Negres, S., Chirita, C., & Radu, G. L. (2018). Anti-inflammatory and antioxidant activities of the Impatiens noli-tangere and Stachys officinalis polyphenolic-rich extracts. *Brazilian Journal of Pharmacognosy*, *28*(1), 57–64. https://doi.org/10.1016/j.bjp.2017.10.008

Probha, N. S. (2017). *Investigation of in-vitro antioxidant and cytotoxic potential of methanolic extract of Oroxylum indicum*. *February*.

Simlai, A., & Roy, A. (2013). Biological activities and chemical constituents of some mangrove species from Sundarban estuary: An overview. *Pharmacognosy Reviews*, *7*(14), 170–178. https://doi.org/10.4103/0973-7847.120518

S.s, S., & J.a, J. W. (2017). In vitro assays to investigate the anti-inflammatory activity of herbal extracts: A Review. *Jayasuriya et Al. World Journal of Pharmaceutical Research*, *6*(December), 131. https://doi.org/10.20959/wjpr201717-10058

Ullah et al. (2014). anti-inflammatory activity of ethanolic extract of Curcuma zedoaria rhizome. *BMC Complementary & Alternative Medicine*, 1–12.

Upadhyay, V. P., & Mishra, P. K. (2008). Population status of mangrove species in estuarine regions of Orissa coast, India. *Tropical Ecology*, *49*(2), 183–188.

Zaman, K. A., & Khalid, A. A. (2015). Free radical scavenging activity of some bangladeshi medicinal plants. *Pharmacologyonline*, *3*(2015-DECEMBER), 29–32.

Phytochemical Screening, Antimicrobial and Cytotoxicity Studies of Heriteria fomes

ORIGINALITY REPORT

16%	3%	4%	15%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to BRAC University Student Paper	7%
2	Submitted to NCC Education Student Paper	1%
3	Submitted to Covenant University Student Paper	1%
4	Anupama Singh, Vikas Anand Saharan, Anil Bhandari. " Pharmacognostic standardization with various plant parts of ", Pharmaceutical Biology, 2013 Publication	1%
5	Submitted to Federal University of Technology Student Paper	1%
6	Submitted to Our Lady of Fatima University Student Paper	<1%
7	www.ajol.info Internet Source	<1%

Submitted to Higher Education Commission