Evaluation of Analgesic Activity of Different Parts of *Heritiera fomes* on Swiss Albino Mice

By

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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.
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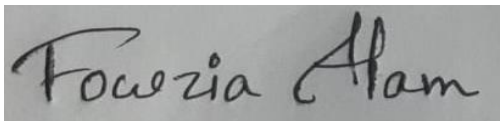

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Approval

The thesis titled "Evaluation of Analgesic Activity of Different Parts of *Heritiera fomes* on Swiss Albino Mice" submitted by Fowzia Alam of Spring, 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on March, 2022.

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Ethics Statement

Ethical Permission has been achieved from the Department of Pharmacy, Jahangirnagar University.

Abstract

Heritiera fomes is a common mangrove species carrying many novel phytochemicals and is traditionally used for its therapeutic activities. This study inspected the analgesic activity of different parts of ethanolic crude extracts of *H. fomes* using Swiss albino mice. The investigation included two methods- acetic acid- induced writhing method and formalin induced pain test. The crude extracts were administered in mice at a dose of 250mg/kg and 500mg/kg body weight to calculate percent inhibition and was compared with standard indomethacin drug. All the results were statistically significant ($P<0.01$) and the ethanol extract of *H. fomes* leaves and roots showed effective therapeutic result of 84.67% and 83.54% inhibition. Other extracts also showed assuasive analgesic activity in a dose dependent manner compared to the standard indomethacin drug. To conclude, overall investigation indicated that the ethanol extracts of different parts of *H. fomes* carry potential analgesic therapeutic agents to control pain.

Keywords: *Heritiera fomes*, Analgesic, Acetic-acid, Formalin, Ethanol.

Dedication

I want to dedicate this project to my lovable parents and respective teachers for their continuous love and assistance.

Acknowledgement

All praise to Almighty Allah, and I would like to commence by expressing my gratitude to Him for the continuous blessings.

Then, I would like to convey special thanks to my supervisor, Dr. Farhana Alam Ripa, Assistant professor, School of Pharmacy, for her continuous support throughout the project and giving me the opportunity to work on it. From the start to the end, her instruction, advice, and patience have been a great help for me to complete my project. I would also want to express my gratitude towards our Honorable Dean and Chairperson, Dr. Eva Rahman Kabir, School of Pharmacy, Brac University for her cooperation and support to complete the research.

Finally, I want to show my heartiest gratification to the lab officers and staffs for providing their immense assistance as well as cooperative attitude to complete the work successfully. I would conclude by thanking my family and friends for their motivation and support throughout my work.

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List of Acronyms

WHO	World Health Organization
BNH	Bangladesh National Herbarium
THP	Traditional Health Practitioners
IUCN	International Union for Conservation of Nature
NMR	Nuclear Magnetic Resonance
IC_{50}	Drug concentration required for 50% inhibition in vitro
DPPH	2,2 diphenyl-1-picrylhydrazyl
TLC	Thin Layer Chromatography
EAC	Ehrlich Ascites Carcinoma
EHFB	Ethanol extract of *H. fomes* barks
EHFR	Ethanol extract of *H. fomes* root
EHFL	Ethanol extract of *H. fomes* Leaves
ICDDRB	International Centre for Diarrheal Disease Research, Bangladesh
BDH	British Drug House
CHPL	Certified Health IT Product List
SEM	Standard Error of Mean
NSAID	Non-steroidal anti-inflammatory drug

Chapter 1

Introduction

From the ancient time, discovering medicinal properties in plants to alleviate diseases is an old treatment method. There are boundless evidences from different sources of finding healing power in nature. Acknowledging the importance of medicinal plants is the outcome of the struggles of countless years against ailments leading humans to trace medicinal agents in leaves, flowers, barks, stems, roots, seeds, fruits, and many other parts of the plants. Currently, science is considering the use of plant origin drugs in modern pharmacotherapy and developing drugs from medicinal plants. This has brought many opportunities and increased the capability of the pharmacists and physicians to expertise in the research field (Srivastava, 2018). Medicinal plants have been a central component of health care for centuries to develop many human cultures all over the world. Both traditional medicines and modern medicines use medicinal plants as valuable resources for drug development. According to WHO, about 80 percent of the world's population; particularly people living in the vast rural areas of developing countries depends on herbal medicines for their primary health care (Mamedov, 2012) and more than 50 major drugs developed from tropical plants are available in today's global market (Hosseinzadeh et al., 2015).

Besides, WHO has highly encouraged and recommended the application of medicinal agents from plants on account of their safety, high potency, easy availability and cost effectiveness in conventional and modern medicines. In Bangladesh, more than 1000 plant species are reported to contain medicinal properties and approximately 455-747 plants have been explained along with their curative properties for various diseases (Alamgir and Fatema, 2014). The south-western part of Bangladesh (Khulna, Satkhira, Bagerhat and Patuakhali) is blessed with the globe's enormous mangrove woodland, "Sundarban" that contains a great

diversity of medicinal plants. In Khulna division, around 33 medicinal plants have been detected and documented at Bangladesh National Herbarium (BNH) with their therapeutic effects to be used as folk medicines. Being one of the vastly grown tree species of Sundarban, "*H. fomes*" commonly known as "Sundari" tree is also included in the list (Mollik et al., 2009).

1.1 Use of Traditional Medicine in Bangladesh

According to WHO, traditional medicine is the sum total of knowledge, skill, and practices established from theories, beliefs, and experiences familiar to different cultures, used in the health care system to treat and prevent illness. Medicinal plants are considered one of the most predominant fields of traditional medicine. Bangladesh has a vast collection of medicinal plants since it is located in the largest deltaic plain- Ganges- Brahmaputra delta and largely contains flood plains with a sub-tropical monsoon climate. A distinctive geophysical position, continuous supply of deltoid freshwater and the appropriate climate make the country blessed with a large variety of plant species (Mukul, Biswas and Rashid, 2017). It is predicted to possess more than 6000 plant species like bryophytes, pteridophytes, gymnosperms, angiosperms, algae, and ferns and around 455-747 of which are claimed to contain healing agent (Flora, 2021).

Medicinal plants used in traditional medicine naturally occur in the forests, coastal areas, bushes and surplus land along the canal and in some other places in Bangladesh. Homoeopathic, ayurvedic, unani and the traditional medical systems are four kinds of available traditional medicine systems existing in the country. Those who practice the folk or traditional medical system are known as Kavirajes (Ghani, 1998) who mainly work with the indigenous medicinal plants for treatment. Rural people depend on them for their treatment purpose since the medicines are readily available, effective and cheap. Bangladesh has more

than 87000 villages having at least one or two practicing Kavirajes in most of them. Adequate amount of information can be gained from their knowledge of medicinal plants to carry on scientific researches to develop safe and effective drugs. Besides, previous ethnomedicinal studies also indicate a positive outcome of the medicinal plants used by different Kavirajes for the treatment of a particular disease (Rahman et al., 2001). Some of the common medicinal plants which are used traditionally in Bangladesh are mentioned below-

Table 1: List of some medicinal plants of Bangladesh (Rahman et al., 2001)

Botanical Name (Family)	**Local Name**	**Pharmacological activity**	**Traditional Use**
Adhatoda vasica (Acanthaceae)	Basak	Antitussive	Cough
Allium cepa (Liliaceae)	Piyaj	Diuretic, Fibrinolytic, Antidiabetic	Diuresis
Azadirachta indica (Meliaceae)	Neem	Antibacterial, Antifungal	Infection
Artabotrys odoratissimus (Annonaceae)	Katchampa	Antimicrobial	Cholera
Allium sativum (Liliaceae)	Rasun	Antidiabetic, Anti-inflammatory	Diabetes
Aegle marmelos (Rutaceae)	Bael	Antidiarrhoeal	Diarrhoea
Persicariea stagnina (Polygonaceae)	Biscatali	Analgesic	Pain
Moringa oleifera (Moringaceae)	Sajna	Antihypertensive	Stomachic
Momordica charantea (Cucurbitaceae)	Karolla	Antidiabetic	Diabetes

Bambosa longispiculata (Gramineae)	Bans	Hypoglycemic	Constipation

1.2 Classification of Plant drugs

Medicinal plants are the oldest natural root of crude drugs or natural drugs. Spermatophytes or seed bearing plants are the major source of these drugs and angiosperm phylum is the superior one. These natural drugs are classified into different systems in the following manner based on different characteristics to identify them easily –

Table 2: Classification of Natural Drugs from Plants (Shah & Seth, 2010)

Classification	Characteristics
1.Alphabetical classification	Drugs are organized in alphabetical order by using their English or Latin names. This system is used in the Pharmacopoeias.
2.Taxonomical classification	It is based on their botanical classification. (kingdom, subkingdom, class, division, family, order, genus and species)
3.Morphological classification	Classified based on the morphological characteristics or the part of the plant that is used as a drug, e.g. leaves, roots, bark, etc.
4.Pharmacological classification	Indicates the pharmacological action and therapeutic properties of the drug, e.g. antimicrobial, analgesic, emetic, etc.
5.Chemical classification	Grouping the drugs based on their principal

	chemical constituents, e.g. alkaloid, glycosides, tannins, etc.
6.Chemotaxonomical classification	This classification depends on the chemical similarity of a taxon.

1.3 Significance of Herbal Medicine as Analgesic Drug

Any substances that can relieve pain or algesia can be termed as an analgesic (painkiller) which alleviates pain following various mechanisms and acts either centrally or peripherally. Since the prehistoric era, pain has always been a matter of concern for humans which lead them to discover medicaments from natural sources, mainly from plants. Medicinal plants have a great abundance of potential phytochemicals providing analgesic activities and exhibit lesser side effects than synthetic drugs. Therefore, using traditional medicinal plants with analgesic effects has achieved worldwide approval and has become a significant field to explore for research work. So far, innumerable amount of medicinal plants and their derived phytochemicals have been evaluated for their analgesic effects (Rauf et al., 2017). Some examples of medicinal plants having analgesic activity are shown below-

Table 3: Some Plants with anti-nociceptive activity (Akram et al., 2013)

Medicinal Plants	**Family**
Syzygium jambos	Myrtaceae
Phyllanthus amarus	Euphorbiacea
Rubus hirtus	Rosaceae
Papaver somniferum	Papaveraceae
Alpinia zerumbet	Zingiberaceae
Teucrium polium	Lamiaceae

Pinus densiflora	Pinaceae
Urtica urens	Urticaceae
Helicteres isora	Malvaceae
Piper solmsianum	Piperaceae

1.4 Description of Sample Plant: *H. fomes*

In this study the selected plant to investigate pharmacological activities is *H. fomes* which is a remarkable mangrove woody plant. It grows naturally in the river deltas and coastal lands of tropical and subtropical areas of the world. For several years, this species has been utilized by the coastal people for various purposes including healthcare. Additionally, a significant amount of evidences and investigations stated that this plant holds many potential effective phytochemicals of great pharmacological importance (Mahmud et al., 2014). This study deals with the investigation of analgesic activities of this plant by collecting crude extracts of different parts of the plants.

Figure 1: H. fomes (Mahmud et al., 2014)

(A)

(B)

Figure 2: Bark (A) & Leave (B) of H. fomes (Mahmud et al., 2014)

(A)

(B)

Figure 3: Root (A) & Twigs (B) parts of H. fomes (Mahmud et. al., 2014)

1.4.1 Taxonomical classification of *H. fomes*

Local Name: Sundari

Binomial Name: *Heritiera fomes*

Kingdom: Plantae

Order: Malvales

Family: Malvaceae

Subfamily: Sterculioidea

Genus: Heritiera

Species: *H. fomes*

(V. P. Upadhyay1, May 19, 2008)

1.4.2 Botanical Features of *H. fomes*

Habitation

H. fomes occurs abundantly in the earth's most enormous mangrove forest called "Sundarban", situated at the south-western segment of Bangladesh. This plant is locally called by the name "Sundari" and it is believed that the "Sundarban" derived its name from this tree because of its high abundance in the forest. It occupies 52.7% of the total zone and the standing volume is 63.8%. Besides Bangladesh and India, it also exists in the coastal areas of some countries like Myanmar, Thailand, and Malaysia (Mahmud et al., 2014).

Salinity

The mangrove forest has continuous inter-relation with the saline seawater because of its location near the sea. *H. fomes* have their own salinity features for its growth and distribution in the forest. It prefers very low saline condition (5–15 psu) in contrast to the other species. The mangrove forest is comprised of 6017 square km of land area of which 1874 square km is covered by the rivers. The fresh river water and the sea water have caused sedimentation to the soil in order to develop the land region of the forest. So, there remains a different saline concentration level and *H. fomes* can survive in this environment through adaptation process. Moreover, the ecological condition of the forest like the temperature (20.4° C-31.5° C), annual rainfall (1640-2000 mm.) etc. are also suitable conditions for the growth of *H. fomes* (Mahmud *et al.*, 2014).

Morphology

H. fomes is a medium sized plant that has a growth of 25m. Usually, the color of the leaves are dark green and the petioles are short (1 cm). This plant contains pneumatophores that begin to grow at the age of 3. Among this species, this is the only plant having pneumatophores. The pneumatophores can grow up to 50 cm. They are one kind of excess part of the roots growing out of soil surface for gaseous exchange. It has hard, heavy and sustainable wood and the color of sapwood and heartwood are pinkish grey and radish dark brown respectively. The flowers of this plant are unisexual which are arranged in panicles and made of 5 stamens. The stamens are combined together to form a cylindrical shape called pistilloid. March and April are the months of its flowering. The fruits have a light green color and contains a single seed. During June and July the seed ripens and falls to land (Mahmud et al., 2014).

1.4.3 Some Phytochemical constituents

H. fomes is a prominent mangrove species with a great potentiality to utilize for the treatment purpose. It has many chemical constituents paving the way for the discovery of various drug compounds. The phytochemical screening of different parts of *H. fomes* (leaves, barks, stems etc.) shows the following result-

Table 4: Some Phytochemical constituents of H. fomes (Mahmud et al., 2014)

Plant Sections	**Phytochemical Constituents**
Leaf	chlorophyll a (0.25%), chlorophyll b (0.09%), carotenoids (0.11%), polyphenols (39.45%), tannins (21.12%), proteins (29.22%)
Extraction of leaves	Saponins, reducing suger, tannins, glycosides, alkaloids, flavonoids, gums, steroids.
Cortex	Tannin, large amount of proanthocyanidins (condensed tannins)
Stem Bark	Pentameric, trimeric and hexameric procyanidins
Nuclear Magnetic Resonance (NMR) spectroscopy (chloroform extract)	Stigmasterol, β-Sitosterol, Sitostenone

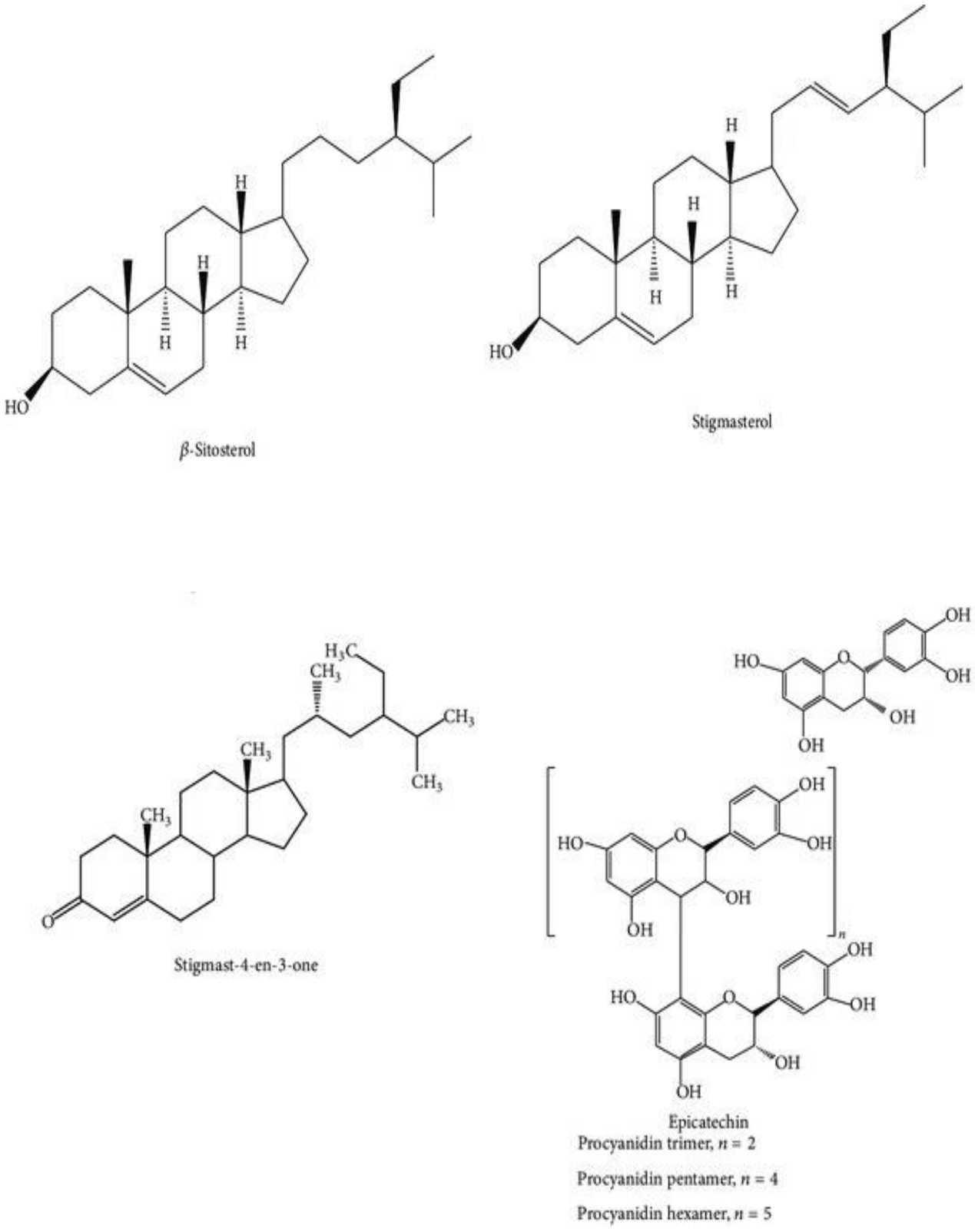

Figure 4: Some phytochemical constituent structures of H. fomes (Mahmud et al., 2014)

1.4.4 Ethnomedicinal report on *H. fomes*

Ethnomedicine is the other name of traditional medicine which is a subclass of medical anthropology. It deals with the relevant written data and the people who are passing information about their knowledge, practice and experience to next generation for several centuries. Anthropological research to discover drug depends on the scientific ethnomedical studies. Having the vast variety of species of fauna and flora, Sundarban, the mangrove forest in Bangladesh has become a remarkable field of practicing ethnomedicine.

Among the diverse species of this forest, *H. fomes* is a notable mangrove tree for its remarkable use in traditional medicine. The regional kavirajes of the southern part of Bangladesh prescribe this plant for various treatment purposes. The rural people use the leaves, roots, and stems to treat many health conditions like GI diseases, ailments of epidermis and liver dysfunctions. Rural people use the bark part to treat goiter and diabetes. Pain and fever are also treated traditionally using this plant.

The inhabitants of these areas cannot get easy access to modern medicine and so they depend on these plants for their health care. Unfortunately, the IUCN Red List Categories and Criteria stated that, plants of Sundarban are facing extinction from many regions due to coastal development, surplus harvesting, deviation of water supply and alteration in salinity of the land. This matter must be taken into consideration for the preservation of this great biodiversity as well as to utilize ethno-botanical and ethno-medicinal knowledge (Mahmud et al., 2014).

Table 5: Use of H. fomes in Traditional Medicine (Mahmud et al., 2014)

Parts of the Plants	**Medicinal Uses**
Seeds and Leaves	Diseases like dysentery, acidity, constipation, diarrhea, dysentery, colic, stomachache, lack of appetite etc. can be cured.
Stem bark	Traditionally used for acne, itch, adscess, infections, eczema, scabies, dermatitis, rash, warts, sores and scars.
Cortex	Goiter, diabetes
Twigs	Oral infection, to control pain in tooth

1.4.5 Pharmacological activities of *H. fomes*

H. fomes is enriched with many phyto-constituents which have been investigated in other trees showing a broad scale of therapeutic activities. Plants containing saponins are reported to exhibit biological activities like spermicidal, antimicrobial, anti-inflammatory, and cytotoxic activities. This plant also contains flavonoids which possesses antioxidant activity and can scavenge different radicals by blocking the steps involved in arachidonate cascade. Polyphenols are considered main components to perform many protective mechanisms against diseases which can be found in *H. fomes* leaves. Polyphenols can work as antimicrobial, free radical scavengers, and anticancer agents as well as can hinder human platelet aggregation.

Phytochemicals like tannins and proanthocyanidins of plants have several biological activities like antibacterial, antiherpetic, cytotoxic, antineoplastic, and anthelmintic. Herbivores and

parasites can be attacked by them. Proanthocyanidins are mainly flavonoid polymers having the potentiality against diarrheal diseases. There are many studies which indicate that some flavonoids like catechins, proanthocyanidins, and proanthocyanidin-rich extracts have therapeutic antidiarrheal agents (Mahmud et. al., 2014).

Some experimented pharmacological activity of different parts of *H. fomes* are mentioned below-

Table 6: Some observed pharmacological activity of H. fomes (Mahmud et. al., 2014)

Plant parts extracts in different solvents	**Observed Pharmacological activity**
EtOH extracts of leaves.	Antioxidant, Antimicrobial
80% EtOH crude, $CHCl_3$, EtOAc extract of stem bark	Antioxidant, Antimicrobial
MeOH extract of bark	Antihyperglycemic
MeOH extract of both leaf and stem powder	Anticancer activity, chromatography characterization
EtOH extracts of pneumatophores	Comparative antibacterial activity

1.5 Rationale of the Project

To minimize side effects of the synthetic medicaments and drug resistance, it has become essential for the scientists and researchers to discover new effective therapeutic compounds from natural source like plants. *H. fomes* is one of the most common medicinal plants used in traditional medicine for several years. Folk medicine practitioners have been using this plant

for various health conditions including pain. Therefore, the purpose of this study is to validate the claim of traditional health practitioners scientifically in the laboratory and to detect the analgesic activities of this plant. This study evaluates the anti-nociceptive effects of *H. fomes* leaves, stem barks and roots crude extract in Swiss Albino Mice which can serve as a basis for further inspection on this plant to develop safer and effective analgesic drug compound from nature.

1.6 Aim of the research

This research aims to inspect the in vivo assessment of analgesic activities of various concentrates of *H. fomes* leaves, stem barks and roots extracts.

1.7 Objectives of the project

- To identify the different parts of *H.fomes* having the medicinal value of analgesic property.
- To validate the claim of possessing medicinal value and the traditional use of this plant.
- The main objective is to create an estimation of the analgesic actions of the different crude extracts of *H. fomes* on an animal model.

1.8 Literature Review

There are many previously conducted researches on *H.fomes* plant which reveal the importance of therapeutic efficacy of this plant and justify its uses in folk medicine by the

healthcare practitioners and rural people. The studies indicate its potentiality to show multiple therapeutic activities, including antimicrobials, antioxidants etc. For instance-

- The study observed that the crude extract of *H. fomes* plant can increase the pancreatic secretion of insulin thereby decreasing body glucose level.
- Effective outcome of IC_{50} antioxidant activity was found by performing Qualitative and quantitative antioxidant screening of leaves extract of *H. fomes*. Here, DPPH assay was followed for quantitative assay and thin layer chromatographic (TLC) technique was followed for qualitative assay.
- There are reports to show protective germicidal mechanisms against *P. aeruginosa, S. aureus, B. subtilis, K. rhizophil* ,by the bark extracts of *H. fomes*
- Moreover, leaves and stems extracts have been demonstrated to contain agents to cure cancer opposed to B16 mouse melanoma providing 40 percent inhibition (in vitro) and EAC on Swiss albino mice (in vivo).
- Antihyperglycemic and antimicrobial actions can also be found in the other species of this genus.(Mahmud et. al., 2014).

Chapter 2

Methodology

There are mainly three steps in the extraction procedure- assembling the plant segments, drying and crushing into coarse powder and then extraction.

2.1 Collection and Identification of the plant (*H. fomes*)

Here, the selected subject of the project is *H. fomes* plant. The fresh leaves, stem bark and root of the plant *H. fomes* were chosen for pharmacological investigation and were collected in December, 2021 from Sundarban, Bagerhat district, Bangladesh. Afterwards, the plant was identified and authenticated by a taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka (**DACB Accession No: 50664**).

2.2 Preparation of Plant Samples

At first, the collected fresh leaves, stem barks and roots of the plant *H. fomes* were washed and cleaned accurately by using running tap water to eradicate the dust particles. After that, rinsing was done using distilled water. The leaves, barks and roots were separated and cut into small pieces and then dried in sun shed for 2 weeks. Then, the dried parts of the plants were pulverized into a coarse powder with a laboratory electric blender. Thus the powdered samples for the project were ready. Finally, airtight containers were used to keep the powdered plant samples to prevent contamination and were stored in a cool, dark and dry place.

2.3 Extraction Process

First of all, 250 gm. powder was measured from previously prepared powdered leaves, stem barks, roots. After that, they were immersed in 1L ethanol at a certain room temperature (22-

25 °C) for a week in three individual glass vessels and they were shaken and stirred occasionally. Afterwards, two layers of phase were seen in the vessels where the superior phase possessed the ethanol solution and the bottom phase contained the sediment. Then, filtration as well as elimination of the sediments of these sample solutions was done using a neat cotton cloth. Afterwards, by using filter papers, the acquired solutions were filtered once more. The filtrates went through fractional separation in order to procure three types (leave, stem bark and root) of extracts. Here, the solvent is ethanol. After the filtration, the filtrates were assembled individually and the total portion was divided into two portions. Then, by using a rotary evaporator (100 rpm), one of the portions was concentrated. Normal room temperature was maintained to evaporate the filtrates and the concentrated parts of the plant were taken in 3 individual petri-dishes. In the end, three separate vials were used to collect the extracts and the ethanolic crude extracts of *H.fomes* roots, barks and leaves were named as EHFR, EHFB and EHFL respectively.

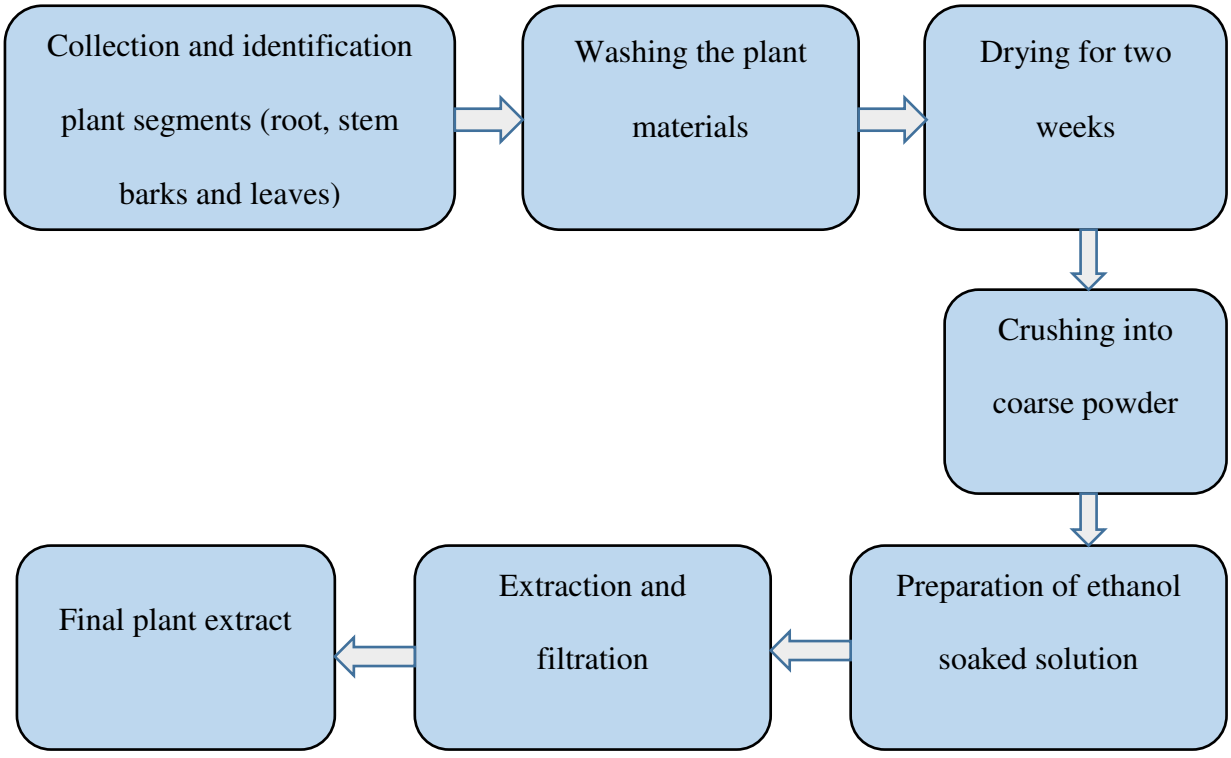

Figure 5: Steps of extraction method of different parts of H. fomes

2.4 Drugs

"Indomethacin" drug of Square Pharmaceuticals Ltd, Bangladesh was used to conduct the study.

2.5 Experimental animal

Swiss albino mice were used for therapeutic investigation and collected from Jahangirnagar University animal lab and ICDDRB, Bangladesh. It was noted that the the mice were approximately 29-34 gm. in body weight. Sufficient amount of food and water was supplied to them for nourishment. Besides, a standard environmental condition was maintained to keep the mice. They were kept in 55-65% relative humidity, 12 hours light/dark cycle and 24.0±0°C temperature.

2.6 Ethical approval

Ethical permission has been achieved from the Department of Pharmacy, Jahangirnagar University and the guidelines of institutional animal ethical committee were maintained. (Zimmermann, 1983).

2.7 Therapeutic inspection of plant extract

To identify the curative effects of the experimental extracts following therapeutic investigation was performed-

- Anti-nociception or analgesic activity

2.8 Anti-nociception of *H. fomes* Plant Extracts

Tests to evaluate the anti-nociception activity of *H. fomes* extracts can be done by using two techniques which are-

- acetic acid induced writhing technique
- formalin induced pain technique.

2.9 Structure of the Analgesic Experiments

First of all, 48 healthy mice were chosen and split into eight groups and were named by the following manner. Every group contained six mice and the body weight of the mice was taken accurately. Then, each mouse were marked and got a certain treatment. The dosages of the test sample and control materials were calculated by the the body weight of the mice for both the control material and test sample.

Table 7: Groups and Treatments

Groups	Treatments
Group I	Control (1% Tween 80 in water)
Group II	Indomethacin (standard)
Group IIIA	EHFB 250 mg/kg
Group IIIB	EHFB 500 mg/kg
Group VA	EHFR 250 mg/kg
Group VB	EHFR 500 mg/kg
Group VIA	EHFL 250 mg/kg
Group VIB	EHFL 500 mg/kg

2.10 Acetic Acid-induced Writhing Method

It is a chemical process used to prompt pain by administrating irritant substances like acetic acid in mice and the analgesic activity of the test compound is estimated from the decrease in the number of writhing (Gawade, 2012). Intraperitoneal administration of acetic acid was

done on the mice to cause sensation of pain. At first, oral administration of the 1% tween 80 in water, extracts at a dose of 250 mg/kg and 500 mg/kg as well as indomethacin (standard) drug were done. Then, 0.7% v/v of acetic acid solution was administered intraperitoneally after 30 minutes.

After 5 minutes of the administration of acetic acid solution, amount of writhing was calculated for 30 minutes. The mice were kept on an observing table to count the number of writhing. Sometimes, partial writhing was observed and so, 2 partial writhing were considered as one full writhing.

2.10.1 List of Chemicals, Reagents and Equipment

Table 8: Chemicals, equipment & reagent used for acetic acid induced test

Name of chemicals, equipment & reagents	**Sources**
Indomethacin	Square Pharmaceuticals Ltd
Acetic acid	Merck, Germany
Tween-80 (as suspending agent)	Sigma Aldrich
Normal saline solution (0.9% NaCl)	Opso Saline
Sterile syringe (disposable, 1ml, 100 divisions)	CHPL, India
Tuberculin syringe having an end of ball shaped	Merck, Germany
Digital electronic balance	Denver Instruments M-220/USA

2.10.2 Preparation of Drug and Chemical Solution

Standard Solution: To prepare standard indomethacin drug solution at a dose of 10 mg/kg, specific quantity of this drug was taken and it was dissolved in 0.9% saline water. Then, each mouse was administered 0.5 ml of standard orally.

Extract Solution: At first, preparation of crude extract was done at a dose of 250 mg/kg and 500 mg/kg as per body weight of mice. So, the doses were calculated and weighed. After that, suspending agent (tween 80) was used in each preparation and mixed accurately. Final solutions were prepared by adding saline water. Finally, oral administration of 0.5 ml of the prepared solution was done in each mouse.

Table 9: List of the test samples used in acetic acid induced writhing test.

Groups	Treatment	Dose	Route of Administration
Group-I (Control)	1% Tween 80 in water	0.1 ml/10gm body weight	Orally
Group-II (Standard)	Indomethacin	10mg/kg	Orally
Group-IIIA (Extract)	EHFB	250mg/kg	Orally
Group-IIIB (Extract)	EHFB	500mg/kg	Orally
Group-VA (Extract)	EHFR	250mg/kg	Orally
Group-VB (Extract)	EHFR	500mg/kg	Orally
Group-VIA (Extract)	EHFL	250mg/kg	Orally
Group-VIB (Extract)	EHFL	500mg/kg	Orally

2.10.3 Step by step process of Anti-nociception Activity of *H. fomes* Extract by Acetic Acid-induced Writhing Method.

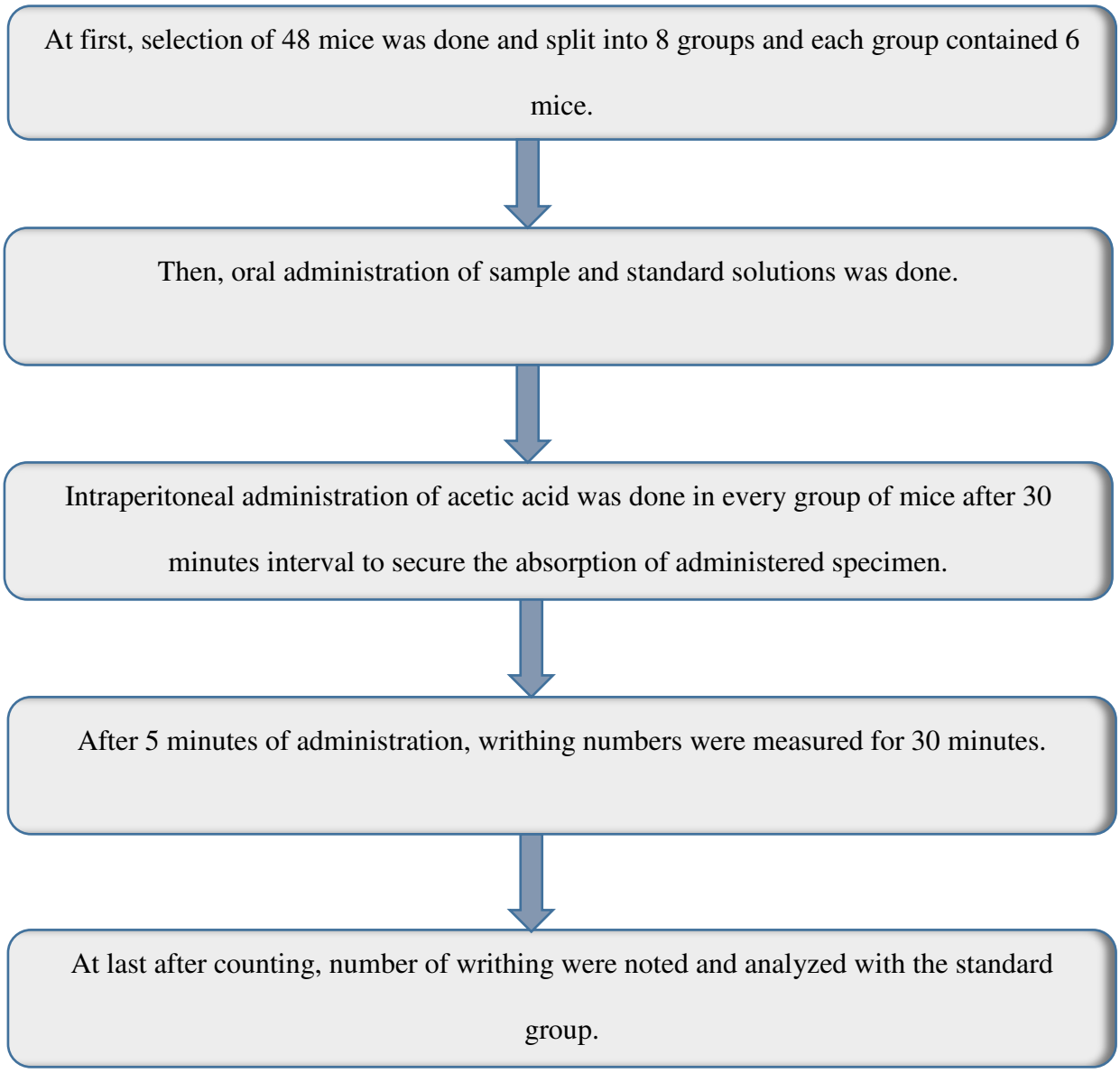

Figure 6: Procedure of acetic acid induced anti-nociception test (Ahmed et al., 2001)

2.11 Process of Formalin Induced Nociception Technique

Formalin test is another method of determining analgesic activity where inoculation of formalin is performed in the right hand paw of the mice to induce a biphasic pain (Sharma et al., 2010). Since this test contains two phases, it is called biphasic. The pain begins in the early phase because of the direct action of formalin in the sensory nerve (neurologic pain). In later phase different inflammatory mediators induce pain (inflammatory pain). Here, indomethacin was chosen as standard since it has pain sensation inhibition control. Then, the comparison of standard with test samples and control were done.

2.11.1 List of Chemicals, Reagents and Equipment

Table 10: Chemicals, Reagents & equipment used for formalin induced test

Name of chemicals, equipment & reagents	**Sources**
Indomethacin	Square Pharmaceuticals Ltd
Formalin	Sigma Aldrich
Tween-80 (as suspending agent)	BDH Chemicals Ltd
Normal saline solution (0.9% NaCl)	Opso Saline
Sterile syringe (disposable, 1ml, 100 divisions)	CHPL, India
Tuberculin syringe having an end of ball shaped	Merck, Germany
Digital electronic balance	Denver Instruments M-220/USA

2.11.2 Preparation of Drug and Chemical Solution

Standard Solution: The solution was prepared by taking required amount indomethacin at a dose of 10 mg/kg and was dissolved in 0.9% saline water. Then, 0.5 ml amount of solution was administered to each mouse orally.

Extract Solution: To prepare crude extract at a dose of 250 mg/kg and 500 mg/kg according to body weight of mice were measured and mixed with few drops of tween 80. After proper mixing, normal saline was also added slowly to make the final solution. 0.5 ml of preparation was administered to each mouse orally.

Table 11: Test samples to evaluate analgesic activity by Formalin test method of H. fomes.

Groups	Treatment	Dose	Route of Administration
Group-I (Control)	1% Tween 80 in water	0.1 ml/10gm body weight	Orally
Group-II (Standard)	Indomethacin	10mg/kg	Orally
Group-IIIA (Extract)	EHFB	250mg/kg	Orally
Group-IIIB (Extract)	EHFB	500mg/kg	Orally
Group-VA (Extract)	EHFR	250mg/kg	Orally
Group-VB (Extract)	EHFR	500mg/kg	Orally
Group-VIA (Extract)	EHFL	250mg/kg	Orally
Group-VIB (Extract)	EHFL	500mg/kg	Orally

2.11.3 Process of analgesic activity of *H. fomes* extracts by formalin test

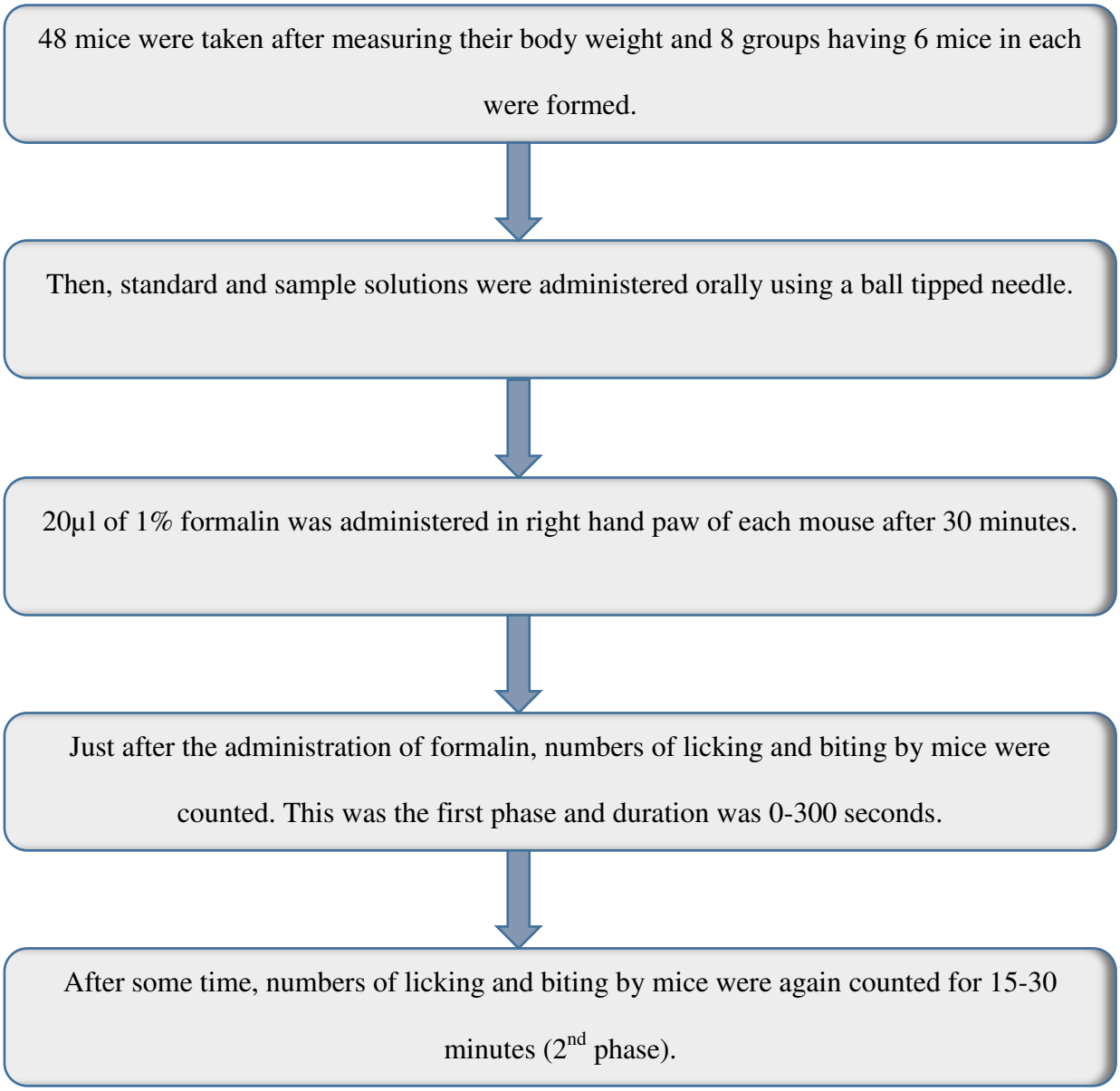

Figure 7: Procedure of formalin induced analgesic test on mice (Sharma et al., 2010)

2.11.4 Counting of Licking and Biting of paws

Induced formalin caused the mice to bite or lick their wounded paws which were determined by using a stopwatch.

2.11.5 Statistical Analysis

All of the values of the experiments are demonstrated as as mean ± standard error of the mean (SEM). One-way Analysis of Variance (ANOVA) was used to evaluate the statistically obtained data. Dunnett's test was also performed using SPSS program (SPSS 16.0, USA).

Chapter 3

Results

3.1 Results of Analgesic activity of plant extracts on mice

Two methods were followed for the determination of analgesic activity- one is the acetic acid induced writhing method and the other one is formalin induced pain method.

3.1.2 Acetic acid induced writhing test

Here, the anti-nociception activity of *H. fomes* was estimated by conducting 250 mg/kg and 500 mg/kg dose to mice. From the test result, it can be observed that there are notable therapeutic effects for the experimented sample compared to standard Indomethacin solution. Out of the crude extract samples, 500 mg/kg of ethanolic leaf extract of *H. fomes* showed effective outcomes in comparison with the standard in VIB group of mice.

Table 12: Anti-nociception activity test data of H. fomes by the writhing method of acetic acid induced test

Groups	**Treatment**	**Dose**	**No. of writhing**	**Percent inhibition**
Group-I (Control)	1% Tween 80 in water	0.1 ml/10gm body weight	25.5 ± 1.20	-----
Group-II (Standard)	Indomethacin	10 mg/kg	11.17 ± 0.79*	56.19
Group-IIIA (Extract)	EHFB	250 mg/kg	5.66 ± 0.25 *	77.80

Group-IIIB (Extract)	EHFB	500 mg/kg	4.16 ± 0.21 *	83.69
Group-VA (Extract)	EHFR	250 mg/kg	6.33 ± 0.42 *	75.18
Group-VB (Extract)	EHFR	500 mg/kg	5.5 ± 0.15 *	78.43
Group-VIA (Extract)	EHFL	250 mg/kg	4.5 ± 0.51640 *	82.35
Group-VIB (Extract)	EHFL	500 mg/kg	3.91 ± 0.37454 *	84.67

The values are demonstrated as mean ± STD (n=6); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.01 significant compared to control.

3.1.3 Formalin induced pain method

The analgesic activity of this test was estimated by counting the numbers of licking and biting of the paws of the mice. Here, ethanolic leaves, stem bark and root extracts of the experimental plant at a dose of 250mg/kg and 500 mg/kg showed significant result to inhibit the licking and biting activity of the mice.

Following table contains the detail outcome of the formalin induced nociception test:

Table 13: Anti-nociception activity of EHFB, EHFR and EHFL extracts in mice by formalin induced nociception test

Groups	Treatment	Dose, route	Early phase (Sec)	Late phase (Sec)	% of inhibition
Group-I (Control)	Distilled water	10 ml/kg	26.17 ± 0.70	43.5 ± 0.76	--
Group-II (Standard)	Indomethacin	10 mg/kg	13.83 ± 0.31*	5.16 ± 1.14*	88.14
Group-III A(Extract)	EHFB	250 mg/kg	5.33 ± 0.42*	11.66 ± 0.33*	73.19
Group-III B(Extract)	EHFB	500mg/kg	5.83 ± 0.30732	8.16 ± 0.60	81.24
Group-VA(Extract)	EHFR	250 mg/kg	10.160 ± 0.48*	8.58 ± 0.49	80.27
Group-VB(Extract)	EHFR	500mg/kg	9.33 ± .33333*	7.16 ± 0.48*	83.54
Group-VIA(Extract)	EHFL	250 mg/kg	6.5 ± 0.4317*	11.83 ± 0.70*	72.80
Group-VIA(Extract)	EHFL	500mg/kg	4.66 ± .33333*	8.33 ± 1.33	80.85

The values are demonstrated as mean ± STD (n=6); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.01 significant compared to control.

Chapter 4

Discussion

Medicinal plants are considered as a great reservoir of medicinal properties since the old times. Many drugs developed from medicinal plants are proven to be less toxic, show good absorption and have lesser side effects than synthetic drugs (Li et al., 2003). In this study, such a medicinal plant, *H. fomes* was chosen for its renowned use in folk medicine and to evaluate its analgesic activity using two test methods on mice. Different crude extracts of different parts of the plant (stem bark, root and leaves) were prepared and administered at two doses (250 and 500 mg/kg) and compared with the standard Indomethacin solution. The test results gave significant outcome which proved the pharmacological efficacy of the plant. Acetic acid induced writhing test and formalin induced pain method were used which showed a considerable decrease in the number of writhing ensuring the anti-nociceptive action of the crude extracts of *H. fomes*.

Acetic acid induced writhing test: From this test, a dose-dependent reduction in writhing was estimated upon the administration of various extracts of the plant. This test was done to demonstrate the central and peripheral analgesic action (Ali et al., 2011) of the crude preparations. At a lower dose (250 mg/kg) of the ethanolic root, bark and leave extracts, the percent inhibition (%) were around 75, 77 and 84 respectively. Besides, at a higher dose (500 mg/kg) the results of percent inhibition (%) was more effective (77%, 78% & 84% for EHFB, EHFR & EHFL respectively) than the lower dose. Moreover, compared to the result of standard Indomethacin solution (56.19 % inhibition) the crude extracts proved themselves to hold effective analgesic potential.

Formalin induced pain method: The second test which was the formalin test method also ensured the central or peripheral action of the crude extracts. Although the percent inhibition of the sample plant extracts did not show a significant result compared to the standard Indomethacin drug (88.14 % inhibition), a remarkable reduction (dose-dependent) in licking and biting activity could be observed. Among the crude extracts, Group-VB (ethanolic root extract at 500 mg/kg) showed the most nearest value (83.54 % inhibition) with the standard preparation. Therefore, the root part of the *H. fomes* plant can be considered as a potential natural source of analgesic drug compounds.

Chapter 5

Conclusion

This study exemplified the anti-nociceptive activity of ethanol extract of leaves, stem barks and roots of *H. fomes* using two test methods (acetic acid induced writhing test and formalin induced pain test) of nociception in mice showing that the species contains central and peripheral mediated analgesic effects. The ethanolic leaves and roots extract at a higher dose demonstrated the most dominant and significant ($P<0.01$) result of analgesic activity on mice compared to the control group.

In conclusion, it can be comprehended that the different parts of the *H. fomes* extracts contain a rich variety of phytochemical constituents of analgesic potential validating the fact of traditional use of this plant for controlling pain. Therefore, it necessitates the isolation of the responsible bioactive compounds for this activity. For this, systematic approaches like bioassay-guided fractionation can be a good option (Mahmud et. al., 2014)

Future Prospects

The long term use of classical analgesic drugs like opiates, non-steroidal anti-inflammatory drugs (NSAID) can generate unavoidable side effects like gastric irritation, renal damage, asthma, cardiac abnormalities etc. Therefore, in spite of the current development in pain management treatments, evolution of effective painkillers with lesser side effects is still essential. In the recent years, the natural substances have established themselves as the richest source of molecular diversity leading the way to discover analgesic drug compounds used in modern medicine (Regalado et. al., 2017). The selected plant sample of this study states that *H. fomes* can also be such a natural resource for developing effective analgesic drug molecules. Since the plant is rich in phytochemical constituents, it requires proper investigation. Utilization of latest scientific techniques can help effectively to discover the

medicinal value of this plant at molecular level. In the future, this study can provide efficient grounds to the scientist and researchers to discover more data about the bioactive compounds through detailed phytochemical screening. Besides, it can pave the way to perform biological assays to create new perspectives for newer analgesic drug discovery.

Chapter 6

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