Preliminary Phytochemical Screening and Biological Investigation of Methanol and Hexane Extracts of *Borreria Pusilla*

A Project
By

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A thesis submitted to the Department of Pharmacy
in partial fulfillment of the requirements for the degree of
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This work is dedicated to my parents to whom I owe my achievements.

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.

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Approval

The project titled "Preliminary Phytochemical Screening and Biological Investigation of Methanol and Hexane extracts of *Borreria pusilla*" submitted by Shakera Islam Keya, ID-15346035 of Summer, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor on 10.10.2019.

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Ethics Statement

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

Abstract

Borreria pusilla is a traditional medicinal plant in Bangladesh that traditionally used to treat bone fracture. The aim of this study was to identify the phytochemical components and to investigate *in vitro* antioxidant and antibacterial activity of its methanol and hexane extracts. It's phytochemical screening revealed the existence of carbohydrates, flavonoids, alkaloids, phenolic compounds and tannins in methanol extract whereas, hexane extract contained alkaloids, phenolic compounds, glycosides, carbohydrates and steroids. The plant extracts, along with its total phenolic content and flavonoid content, showed notable antioxidant activities through DPPH free radical scavenging assay. Moreover, Disc diffusion method evaluated the antimicrobial activity in which the both plant extracts mostly showed activity against the *Staphylococcus aureus, Bacillus subtilis*, and *Enterococcus faecalis* bacteria. The present data that investigated may be valuable for conducting research and developing new drugs to treat various types of diseases in our country as well as elsewhere in the world.

Keywords: *Borreria pusilla*; phytochemical screening; methanol; hexane; antioxidant activity; antimicrobial activity.

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List of Acronyms

WHO	World Health Organization
OTC	Over the Counter
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic Acid
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
MDR	Multidrug Resistant
GC	Gas Chromatography
NHB	National Herbarium of Bangladesh
LAF	Laminar Air Flow
BHT	Butylated hydroxytoluene
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent
NB	Nutrient Broth
ASA	Ascorbic acid
IC	Inhibition Concentration

Chapter 1

Introduction

1. Introduction

1.1 General information

Medicinal plants are used for medicinal purposes throughout the world, including Bangladesh from the prehistoric period. There are many plants which are known as medicinal plants possess many medicinal activities. Almost more than 3.3 billion people of less developed countries used medicinal plants frequently. Thereby medicinal plants are called “backbone” of traditional medicine (“Medicinal Plants: A Review,” 2015). The demand and acceptance of medicinal plants are expanding day by day. Moreover, they play a vital role in ecosystem in many ways (Jamshidi-Kia, Lorigooini, & Amini-Khoei, 2017). These medicinal plants are growing volume and as well in trade also. One study found that the global trade of medicinal plants is US$800 million per year (Hoareau & DaSilva, 1999). According to World Health Organization (WHO), almost 80% of residents of earth are relied on traditional medicines to satisfy their primary health care need. Most of the therapy they are used include plants extract and their bioactive components (Krishnaiah, Sarbatly, & Nithyanandam, 2011).

A study by World Health Organization (WHO), defined traditional medicines as based on theories, beliefs and experiences indigenous to different cultures how peoples’ knowledge, skills and practices treat medicinal plants to maintain health, also to prevent diagnose, improve, treat physical and mental illness. Depending on different regions and cultures, the practices of traditional plants can be varied including plant, animal, mineral based medicines, massage, spiritual therapies and other techniques as well. These traditional medicines are different from conventional medicines including allopathic, modern, orthodox or western medicines that are mainly depend on biochemical theories of illness (Tesfahuneygn & Gebreegziabher, 2019).

It is found that, over the past 50 years, many important drugs that have been turned into modern medicinal practice were obtained from plants. The chemical components of plants exert different therapeutic properties. Recently, WHO initiates and promotes different national health care programs based on herbal drugs as they can be accessed easily with minimal price by common people. Moreover, the herbal drugs are found much safer than modern synthetic drugs (Dar, Shahnawaz, Qazi, & Qazi, 2017).

Morphine was the first pharmacological active compound that was isolated from plant in a raw form in 1805. In 19th century, many alkaloids were isolated from different plants like *Atropa belladonna, Coffea Arabica, Erythroxylum coca, Ephedra species, Papaver somniferum, Pilocarpus cordifolia, Physostigma venenosum, Cinchona cordifolia, Salis species, Theobroma cacao,* and *Camellia sinensis* etc. plants extracts from these plants were being used as drugs as well. After these discoveries, people started to use secondary metabolites as drugs in both original and modified form as they give greater pharmacological effects on mammalian systems (Kumar et al., 2015).

Though herbal medicines play an important role in health issues of individuals and communities but their quality need to assured before use. In the last few decades, people are more using herbal medicines as a treatment tool, proper use of these medicines are great matter of concern. Therefore, a proper process is necessary to identify them (Dey, Srikanth, Wanjari, Ota, & Jamal, 2012). According to Pharmacopoeia, some steps need to follow for ensuring the quality of herbals drugs like identify the desire plants specie, purity confirmation and identify the presence of active compounds at minimum concentrations which are matter of challenging as they are being used to manufacture herbal medicines.

1.2 Medicinal plants as alternative of synthetic drugs

In 18th century, all kinds of people used herbal medicines to treat many diseases. With the time, synthetic drugs took the places of natural medicines due to advancement in medicine fields by indicating the fact of possess side effects. Though synthetic drugs are being used mostly, but many medicines show the severe side effects as well. For example, Aspirin, Diclofenac, Ibuprofen, Warfarin and other over the counter (OTC) drugs can possess minor to severe side effects including breathing difficulties, excessive bleeding, hemorrhage and many more. If herbal or handmade medicines are taken in consideration, they seem less costly rather than synthetic drugs. Among the rural people the tendency of using herbal medicines is high as plants do not have any lethal side effects. Moreover, some other species like cloves, chilies, turmeric, curcumin, cinnamon etc. have pharmacological effects on human body.

On the contrary, synthetic drugs are manufactured in laboratory with the help of different methodology. They do not exist in natural sources. Synthetic drugs might able to cure many diseases but causes severe side effects also. For instance, Paracetamol might cause liver poisoning, Naproxen might show gastrointestinal side effects, Ibuprofen may cause nephrotoxicity (Nisar, Sultan, & Rubab, 2018).

1.3 Significance of medicinal plants

With the evolution of human civilization, people were dependable to different plants to combat with various diseases. Moreover, along with pharmacological interest, they are also popular in pharmaceutical, cosmetic and nutritional fields also in both developed and developing countries. The reasons behind growing their recognition are less side effects, easy accessibility and affordable cost. Essential oils also can be obtained from medicinal and aromatic plants by distillation method and they have high demand in cosmetology, food and

perfumes industry. In Europe, approximately 2000 aromatic and medicinal plants are traded commercially. Currently, the future perspective of medicinal plants are on demand as there are half million of plants around the world from where all the pharmacological activities of plants are not investigated yet (Ghorbanpour & Varma, 2017).

Recently, researchers are becoming more concerned to isolate pharmacologically active compound from the natural resources that are effective than available synthetic drugs. Now a days people are giving more concern towards herbal systems than modern drug systems so that there is greater opportunity for rejuvenation of medicinal science that are known as alternative medicine (Aslam & Ahmad, 2016).

Table 1 List of some common medicinal plants and their usages

Botanical Name	**Plants**	**Family**	**Part used**	**Therapeutic use**
Adina sessilifolia L.		Rubiaceae	Leaf	Used in treatment of various infectious diseases impetigo, folliculitis, minor cellulitis, fungal infections.
Adhatoda vasica		Acanthaceae,	leaf	Expectorant, coughs asthma

Asparagus racemosus L.		Liliaceae	Roots	upset stomach (dyspepsia), constipation, stomach spasms, and stomach ulcers
Brassica nigra (L.) Koch.		Brassicaceae	Seeds	respiratory infections
Boerhaavia diffusa		Nyctaginaceae	leaves	Asthma, Diuretic, skin diseases
Caesalpinia bonduc L.		Fabaceae	leaves and roots,	urinary tract infections, skin infections, helminthiasis
Ixora arborea roxb		Rubiaceae	Root, Bark, Flower	Gynaecological disorder, Anaemia
Ocimum sanctum		Labiatae	leaf	Cough, fever, dysentery, stomach diseases, mosquito repellent

Nodiflora (l.) Greene phyla.		Verbenaceae	Leaves	Rheumatic fever
Terminalia arjuna.		Combretaceae	Bark. Seed, Fruit	Cardiovascular disorder, Hepatic disorder, Sexual transmitted diseases
Terminalia bellirica		Combretaceae	Fruits	Stomach diseases
Saraca indica		legume	Leaves	female diseases, dysentery

From the table 1, it has been showed that many plants are being used to treat variety of diseases. There are a total of 422,000 plant species on earth, 52,885–72,000 of which are used worldwide as medicinal plants. Their different parts like root, bark, leave, stem fruits, seed flowers are being used for different purposes as they possess many chemical constituents that essentials for therapeutic uses (Bardhan, Ashrafi, & Saha, 2018; Haque, Bari, Hasan, Sultana, & Reza, 2015).

1.4 Chemical constituents of different medicinal plants

There are many commonly occurring chemical substances that are responsible for possessing variety of medicinal properties including toxic effects as well Source (Pengelly, 2004). Some of these are as follow:

1. Organic acids (not strictly metabolites): acetic acid, oxalic acid, fumaric acid, tartaric acid, benzoic acid, acyclic, cyclic and heterocyclic (furan, pyrrole, pyran, pyridine) compunds.
2. Phenols: (pyrogallol, hydroquinone, acetophenones, salicylic acid)
3. Glycosides: (anthraquinone glycoside, cardiac glycoside, saponin glycoside, thiocyanate glycoside)
4. Tannins
5. Polysaccarides
6. Terpenoids and saponins
7. Essential oils
8. Resins
9. Alkaloids
10. Sterols
11. Vitamins and minerals

1.5 Current status of medicinal plants in Bangladesh

Bangladesh being arranged in a sub-tropical zone establishes a bigger piece of South-Asian focal point of plant hereditary decent variety, imparting to India. It is wealthy in field crops, natural products, nuts and woods plants covering a wide exhibit of animal varieties, genera and families with colossal hereditary decent variety. Around 5700 types of higher plants have been recorded up until this point, and of these, somewhere in the range of 160 species are

utilized as harvests. The remainders of the species are for all intents and purposes left on developing in regular vegetation in woodlands and in town bushes, which have been a significant wellspring of various non-wood backwoods items, similar to foods grown from the ground, drug and aromatics, fuel, grain, bamboo, rattans, palms, and fancy (M M Rahman & Fakir, 2015).

In our nation, 75% of our populace utilizes natural medication for essential social insurance. More than 4 billion of total populace utilizes the home grown medication. Both in home and abroad, home grown drugs are presently the most well-known type of customary prescriptions, free of symptoms and are acclaimed profoundly. What's more, ranchers are developing various kinds of therapeutic plants in Mymensingh, Tangail, Sylhet, Modhupur, Kushtia and Chittagong Hill Tracts (By, 2018).

A long custom of indigenous home grown therapeutic frameworks, in light of the rich neighborhood plant assorted variety is viewed as a significant segment of the essential human services framework. Among the different frameworks of conventional medication existing together inside the nation are the homeopathic, ayurvedic, unani and the customary restorative framework. The last framework is drilled by people or customary medicinal specialists, also called Kavirajes. There are more than 87,000 towns in Bangladesh and most towns have a couple rehearsing Kavirajes. Learning of the therapeutic plants utilized by the Kavirajes of Bangladesh can be a decent hotspot for further logical investigations in the journey for better medications from the restorative plants utilized and with lesser symptoms (M. Mizanur Rahman, Masum, Sharkar, & Sima, 2013).

1.6 Literature review

1.6.1 The plant family Rubiaceae

Rubiaceae is the fourth biggest family inside angiosperm (= Magnoliophyta) plants. With an expected all out number of around 13,000 species inside 650 genera they involve about 4% of every blooming plant, making them one of the biggest plant families among the eudicotyledons.

1.6.2 Characters of Rubiaceae

- ✓ Trees or herbs.
- ✓ Leaves interchange or inverse.
- ✓ Stipules interpetiolar or intrapetiolar, inflorescence cymose.
- ✓ Blossoms tetra or pentamerous, bisexual, actinomorphic, epigynous, corolla, gamopetalous.
- ✓ Stamens 4-5.
- ✓ Epipetalous, introrse, dithecous.
- ✓ Ovary second rate, bilocular with one or numerous ovules in every loculus.
- ✓ Organic product container or berry.

1.6.3 Habit

- ✓ For the most part bushes (Gardenia, Ixora, Mussaenda, Hamelia)
- ✓ Trees (Morinda, Adina) and a couple of herbs (Galium, Rubia).

1.6.4 Distribution of Rubiaceae

It is usually known as Madder or Coffee family. It incorporates 6000 species and 500 genera. In India it is spoken to by 551 species. The individuals from this family are conveyed in tropics, sub-tropics and mild areas.

1.6.5 Phytochemicals of Rubiaceae family

Rubiaceae of different blossoming plants called the madder family, bedstraw family or espresso family. Rubiaceae family is a huge group of 630 genera and around 1300 species found around the world, particularly in tropical and warm districts. Numerous Rubiaceae family plants showed antimalarial, antimicrobial, anti-hypertension, anti-diabetic, cancer prevention agent, and mitigating exercises. Bioactive mixes including indole alkaloids, terpenoids and anthraquinones have been separated from these plants. Different normal items happen in Rubiaceae plants. Broad phytochemical examination has been acknowledged with respect to the common event of terpenoids, anthraquinones and indole alkaloids in the family. The event of alkaloids in some Rubiaceae individuals is all around reported. Phytochemical examination of *Canthium horridum* was accounted for. *Canthium multiflorum* concentrates uncovered the nearness of a few substance mixes, for example, alkaloids, terpens and tannins. Compound constituents of the stems of Canthium comparison was accounted for.

Canthium mannii plant bark concentrates involve nematicidal action and important auxiliary metabolites, for example, alkaloids and saponins. In Rubiaceae family species, phytochemical screening has demonstrated that in Rytigynia nigerica some bioactive mixes, for example, tannins, saponins, diminishing mixes, steroids, and flavonoids are available. In *Rytigynia umbellulata*, alkaloids, tannins, saponnins, diminishing mixes, and flavonoids were available. Borreria and Spermacoce species has alkaloids, iridoids, flavonoids and terpenoids. Canthium multiflorum leaves displays high esteem phytochemicals like Saponins, Tannins, Flavonids, Alkaloids, Proanthocyanidins, Anthracenosides, Coumarins, Terpenoids, Sterols and Carotenoids (Chandra Kala, 2015).

1.6.6 Pharmacological properties of Rubiaceae family

The study incorporated some nearby plants speaking to the family Rubiaceae, used to fix different diseases. Distinctive 8 plants of family are utilized for the therapeutic reason.

Diverse plant part, for example, roots leaves blossoms, gums and at times entire plant is utilized with the end goal of medication. From the table 2 it has been showed that greater part of the plants of family Rubiaceae are utilized to fix looseness of the bowels and diarrhea, fever, wounds, bronchitis, dyspepsia, joint maladies and so on (Samudra, 2016).

Table 2 Outlines the information in regards to certain individuals from Rubiaceae, including its plant name, nearby name; parts utilized and medicinal properties

Botanical name	**Local Name**	**Plant part used**	**Traditional medicinal use**
Canthium coromandelicum (Burm.F.) Alston.	Karbit	Fruits	Diarrhea and urinary problem
Gardenia lucida Roxb.	Dikamali	Resin	Bronchitis, indigestion, dysentery
Hedyotis corymbosa (L) lamk.	Pittapapda	Whole plant	Jaundice, liver diseases, fever, giddiness
Lxora coccinea linn.	Lal lokhandi	Leaves, flower	Diarrhea and dysentery
Mitragyna parvifolia (Roxb) Korth.	Kalam	Leaves, flower	Cuts, wounds, and tumor
Morinda pubescens J.E.Sm.	Bartondi	Leaves	Diarrhea, wounds and ulcer
Spermacoce articularis Lf.	Madanghanti	Roots	Joint diseases

1.6.7 The plant genus: *Borreria*

The Rubiaceae family involves one of the biggest angiosperm families, with 650 genera and around 13,000 species, distributed predominantly not just in tropical and subtropical districts. The genera *Borreria* and *Spermacoce*, the biggest of the clan *Spermacoceae*, contains around 280 species dispersed in tropical and subtropical America, Africa, Asia, and Europe. In Brazil, 36 *Borreria* species were recorded, of which 22 are endemics. The across the board

employments of *Borreria* and *Spermacoce* species in conventional medication have brought about significant synthetic examination of the plants and their dynamic standards. Today, more than 60 mixes dispersed in various classes have been confined. Alkaloids, iridoids, flavonoids, and terpenoids are the primary gatherings of constituents. Among them, alkaloids and iridoids showed *in vivo* or *in vitro* some organic exercises.

Borreria and *Spermacoce* species have a wide assortment of therapeutic properties. Up until now, a couple of species have been screened for affirmation of their natural exercises. Test results have demonstrated a few animal categories as antimicrobial, antitumor, cancer prevention agent, mitigating, hepato-protective, larvicidal, and so on. The different organic exercises detailed from various concentrates of *Borreria* (Conserva, Ferreira, & Júnior, 2012).

1.6.8 Other botanical information

Borreria is the biggest variety in the clan *Spermacoceae*, and is hazardous as to both conventional and infrageneric grouping. Numerous creators incorporate *Borreria* (frequently likewise Dioida) in *Spermacoce s. l.*, however as the status of certain species treated here isn't clear, they are kept in *Borreria*. Different creators keep in any event the American *Borreria* species discrete; however, incorporate Dioida at the subgeneric level as it were. *B. articularis (L.f.)* F.N. Williams is considered here as an equivalent word of *B. hispida*. As indicated by certain creators they have a place with various animal categories, speaking to the boundaries of unpredictable animal categories. *B. hispida* is for this situation an erect herb, with terete stem, long spreading hairs, flexuous leaves, campanulate blooms, huge foods grown from the ground, while *B. articularis* is prostrate, with pointedly 4-calculated stem, scabrid hairs, ordinarily level leaves, and channel like blooms and little foods grown from the ground.

1.6.9 Ecology

Borreria develops for the most part in areas with a short or long dry period, on many soil types, frequently in consistently aggravated regions, yet additionally in meadows and in auxiliary backwoods.

1.6.10 Propagation and planting

Borreria is proliferated by seed. The seeds show little lethargy. A solitary plant of *B. laevis* may create around 22 000 seeds under ideal developing conditions, and an actual existence cycle can be finished in 3 months. Seed weight is around 18 mg/100 seeds.

1.6.11 Diseases and pests

Borreria is a host for a few nematode animal varieties. Hatchlings of certain hawk moths use *B. verticillata* as essential feed plant. In India, *B. hispida* is an off-season have for the hatchlings of the groundnut leaf excavator (Aproaerema modicella). Numerous *Borreria* species are weeds in yearly and enduring yields all through the world.

1.6.12 Harvesting

Borreria is reaped all through the season, when required. Typically, entire plants are pulled up.

1.6.13 Handling after harvest

Borreria is utilized new, or dried in the shade for later use.

1.6.14 The Plant: *Borreria pusilla*

The description of the whole plant of *Borreria pusilla* (*Borreria pusilla* in Global Plants on JSTOR, n.d.) as follows-

- ✓ Erect or once in a while prostrate yearly typically extended herb (2–) 7.5–60 cm. tall, with notched ± glabrous or marginally papillate-puberulous frequently rosy stems.
- ✓ Leaf-edges straight lanceolate to barely lanceolate, 1–5.3 cm. long, 2–5.5 mm. wide, intense at the summit, limited to the base, minutely scabrid above, glabrous underneath; genuine petiole missing; stipule-sheath 1.5–2 mm. long, glabrous or pubescent, 1.5–2 mm. long, bearing 7 setae 2–4 mm.
- ✓ Blooms in thick conservative circular bunches all things considered hubs, 0.6–1(– 1.5) cm. in distance across; bracteoles filiform, various, 2 mm. long.
- ✓ Calyx-tube ovoid, 1 mm. long, pubescent; teeth 4, equivalent or somewhat inconsistent, subulate, 0.6–1.2 mm. long, scabrid.
- ✓ Corolla white or pink; tube barely pipe molded, 1.3 mm. long; projections 0.8–1.1 mm. long, 0.4 mm. wide, with some since quite a while ago smoothed hairs at the pinnacle.
- ✓ Fibers exserted ± 1 mm. Style exserted 0.5 mm. Container ellipsoid, 1.5 mm. long, glabrous or ± inadequately pubescent.
- ✓ Seeds chestnut-dark colored, sparkling, elongated ellipsoid or barely oval, 1.3 mm. long, 0.55 mm. wide, 0.4 mm. thick, with a wide ventral furrow.

1.6.15 Distribution

Tropical Africa, tropical Asia, Pakistan to Japan and Philippines.

India: Assam, Bihar, Maharastra; Sri Lanka, Bhutan.

1.6.16 Taxonomic classification

Kingdom: plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Gentianales

Family: Rubiaceae

Subfamily: Rubioideae

Tribe: Spermacoceae

Genus: *Borreria*

1.7 Preliminary screening of biological activities of plants

1.7.1 Antioxidant property by DPPH

Phenolic mixes are broadly circulated in plants and as of late they have increased much consideration, because of their cancer prevention agent movement and free radical-rummaging capacity with potential useful ramifications in human wellbeing. At the point when added to sustenance, cancer prevention agents limit rancidity, hinder the arrangement of poisonous oxidation items, keep up wholesome quality, and increment time span of usability. These cancer prevention agents may soothe oxidative pressure, for example

keeping free radicals from harming biomolecules, for example, proteins, DNA, and lipids. The cancer prevention agent action of concentrates of a few plants, including their leaves, bark, roots, organic products, and seeds has been broadly considered. In any case, numerous scientists revealed the unfavorable impacts of engineered cell reinforcements, for example, poisonous quality and cancer-causing nature. Common cancer prevention agents are in extreme interest for application as nutraceuticals, bio-pharmaceuticals, just as sustenance added substance as a result of customer inclination (Sowndhararajan & Kang, 2013).

The DPPH examine technique depends on the decrease of DPPH, a steady free radical. The free radical DPPH with an odd electron gives a most extreme ingestion at 517 nm. This test has been the most acknowledged model for assessing the free radical rummaging action of any new medicate. At the point when an answer of DPPH is blended with that of a substance that can give a hydrogen particle, at that point this offers ascend to the diminished structure (Diphenyl picryl hydrazine; non radical) with the loss of this violet shading (Van Goethem, Zurita, Martin Bermejo, Lemaî, & Bischoff, 2001).

1.7.2 Antioxidant property by TPC

Plant phenolic are regularly found in both eatable and non-palatable plants and their cancer prevention agent exercises primarily because of their redox properties which enable them to go about as lessening specialists, hydrogen donators and singlet oxygen quencher. Phenolic mixes have hostile to bacterial, against viral and enemies of parasites activities in the plant itself and by prudence of being hydrogen-giving cancer prevention agents, they ensure against oxidative pressure which can unfavorably influence the soundness of plants just as people. In people, cells react to polyphenols for the most part through direct connections with receptors or compounds engaged with sign transduction which may bring about alteration of the redox status of the phone and may trigger a progression of redox-subordinate responses.

Phenolic mixes are great electron benefactors on the grounds that their hydroxyl gatherings can legitimately add to cancer prevention agent activity. Moreover, some of them animate the blend of endogenous cancer prevention agent particles in the cell. As indicated by different reports in the writing, phenolic mixes show free extreme restraint, peroxide decay, metal inactivation or oxygen searching in natural frameworks and counteract oxidative sickness load (Aryal et al., 2019).

1.7.3 Antioxidant property by TFC

Flavonoids are auxiliary metabolites with cancer prevention agent movement, the intensity of which relies upon the number and position of free OH gatherings. In a review of past writing reports it was discovered that Kumar et al. revealed a TFC of 21.53 mg QE/g dry load in the methanol concentrate of *C. tora*, Adebooye et al. decided a TFC of 0.64 mg catechin counterparts per gram crisp load in the water concentrate of *S. nigrum*, and Uddin et al. discovered the TFC of 49.2 ± 3.4 mg rutin reciprocals per gram dry load in the methanol concentrate of *P. oleracea*. As announced in the writing, hereditary decent variety and Plants 2019, 8, 96 4 of 12 biological, ecological, occasional and year-to-year varieties essentially influenced the flavonoid substance of vegetables (Aryal et al., 2019).

1.7.4 Antimicrobial activity by Disc Diffusion Method

Antimicrobial operators are basically significant in decreasing the worldwide weight of irresistible maladies. Be that as it may, development and spread of multidrug safe (MDR) strain in pathogenic microorganisms have turned into a noteworthy general wellbeing risk as there are less, or even now and again no, viable antimicrobial operators accessible for the contamination brought about by pathogenic microbes (Manandhar & Manandhar, 2002). In the course of recent decades, these medical advantages are under risk the same number of normally utilized anti-infection agents have turned out to be less and less successful against specific diseases not, simply because a large number of them produce dangerous responses,

yet in addition because of development of medication safe microscopic organisms. It is fundamental to explore more up to date tranquilizes with lesser opposition (Bhalodia & Shukla, 2011).

The utilization of unrefined concentrates of plants parts and phytochemicals, of known antimicrobial properties, can be of incredible noteworthiness in the restorative medications. Lately, various examinations have led in different nations to demonstrate such productivity. Numerous plants have utilized in light of their antimicrobial attributes, which are because of the auxiliary metabolites incorporated by the plants. These items are known by their dynamic substances like, phenolic mixes which are a piece of the basic oils, just as in tanning (Khan et al., 2013). Plants are wealthy in a wide assortment of optional metabolites, for example, tannins, terpenoids, alkaloids, flavonoids, glycosides, and so on, which have been found in vitro to have antimicrobial properties (Bhalodia & Shukla, 2011). Higher plants have been demonstrated to be a potential hotspot for new enemy of microbial operators (Garner, 1996).

1.8 Significance of solvents

1.8.1 Methanol

Bioactive mixes from Plants have a place with different compound gatherings, for example, tannins, alkaloids, glycosides, lignans, terpinoids, and so forth. Methanol has an extremity file of 5.1. For the most part methanol is utilized for extraction different polar mixes however certain gathering of non-polar mixes are genuinely solvent in methanol if not promptly dissolvable. Methanol is an amphiphilic compound. It separates all the different concoction gatherings, from the plant material. Thusly, methanol is normally utilized for extraction of bioactive mixes. In addition, methanol among every one of the alcohols has low breaking point of only 65 degree Celsius. So extraction and centralization of bioactive mixes is simple.

1.8.2 Hexane

Hexane in its unadulterated structure is a dull fluid, and its breaking point is between 50℃ – 70℃ all of which work in support for oil extraction. The dissolvable is decently non-lethal and does not create any harmful exhaust. With its low breaking point, hexane can hold its fluid states even in virus atmospheres. Hexane can be helpful for extraction on the off chance to separate non-polar segments and very little else. Some different mixes which are not dissolvable in hexane won't be removed, and this will give a cleaner GC follow. Obviously this strategy is material just in the event that is keen on examining non-polar hexanes-dissolvable part.

Different points of interest of hexane could be low harmfulness, simple expulsion and littler opportunities to frame emulsions during the extraction procedure. It is additionally earth more benevolent and more affordable than chlorinated solvents. Hexane as the best decision of the non-polar solvents for some buys.

1.9 Related publications on *Borreria pusilla*

There are no past reports of distribution on *Borreria pusilla*. No investigations have yet been completed on its cancer prevention agent property, antimicrobial property, and antioxidant property. Subsequently, extraordinary significant properties and therapeutic estimations of the plant still fundamental to be perceived.

1.10 Rationale of the study

Historically pharmacological screening of natural or synthetic compounds was the source of countless therapeutic agents. In different countries, plants are used and are a source of many powerful and powerful drugs. A broad variety of medicinal plant components are used as raw

drugs for extract and have diverse medicinal properties. Seeds, root, stem, flower, fruit, twigs exudate and altered plant organs are the various components used (Submitted et al., 2012).

Here, the literature review on *Borreria pusilla* reveals that no pharmacological investigation has been studied. Considering the vast area of plant potential as drug sources and taking into account traditional local uses, a systematic investigation was undertaken to screen *Borreria pusilla's* phytochemical and pharmacological activity. Traditionally this plant is used to treat bone fracture (Khisha, Karim, Chowdhury, & Banoo, 2012). The main rationale of the research is therefore to define and explore the various pharmacological characteristics of the plant's methanol and hexane extracts.

1.11 Aim of the project

The aim of this project is to recognize the significant therapeutic estimations of the chosen plant for the more prominent welfare of the human culture and set up various obscure natural properties.

1.12 Objective of the project

The main objectives of this project are as follows:

1. Assessment of the cancer prevention agent properties of the methanol and hexane concentrate of the whole plant *Borreria pusilla* by deciding DPPH free radical rummaging movement, all out phenolic substance and absolute flavonoid content.
2. To perform phytochemical screening trial of the methanol and hexane concentrate of the whole plant *Borreria pusilla*.
3. To find out antimicrobial activity of methanol and hexane concentrate of the whole plant *Borreria pusilla*.

Chapter 2

Methodology

2. Methodology

The accompanying plan was pursued for the whole examination:

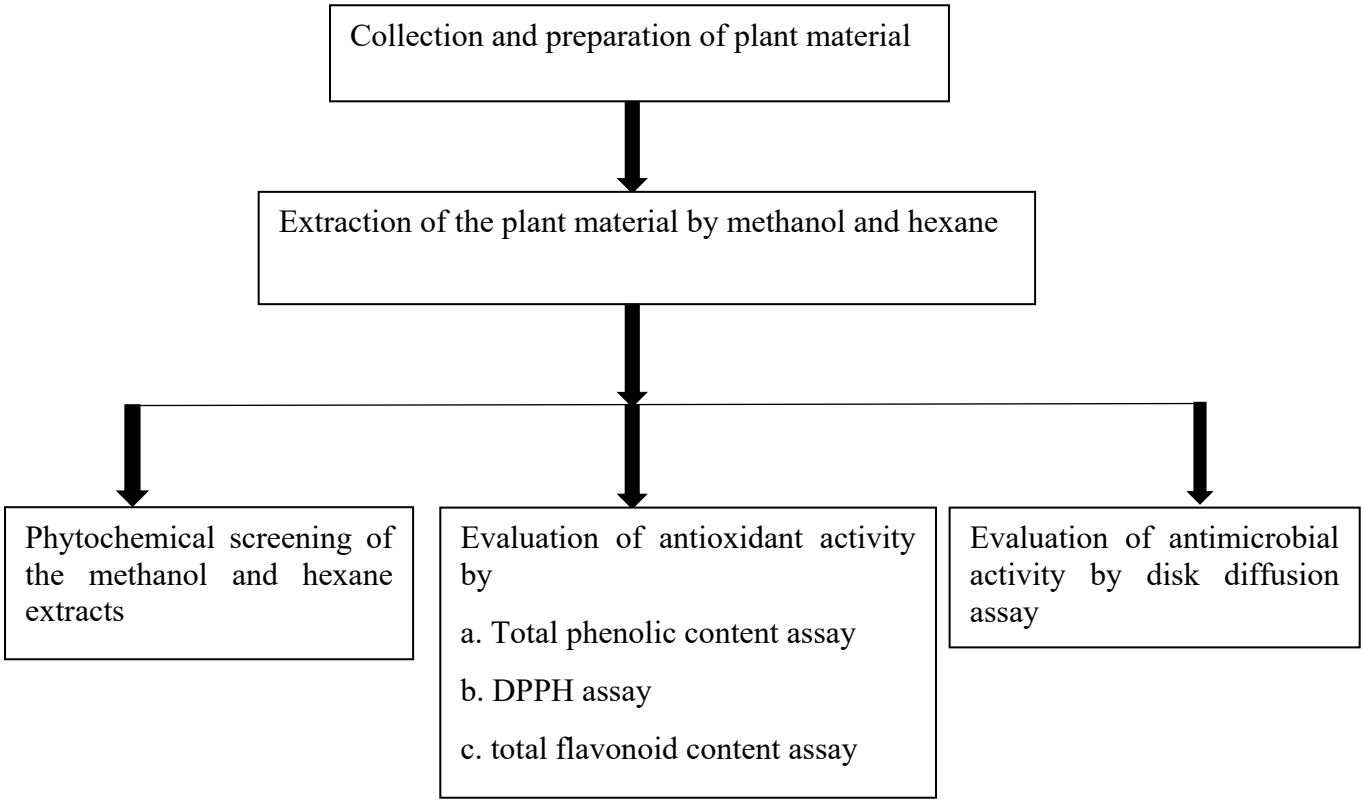

Figure 1: Protocol for pursuing the whole examination

2.1 Collection and authentication of plant

Borreria pusilla plant was chosen during this examination work for screening profitable synthetic parts that are available in plants and exploring distinctive pharmacological exercises. The new leaves of the plant were gathered from Sylhet during May 2019. The plant test was then sent to National Herbarium of Bangladesh (NHB), Mirpur for distinguishing proof where they recognized the plant and gave a check number 48418.

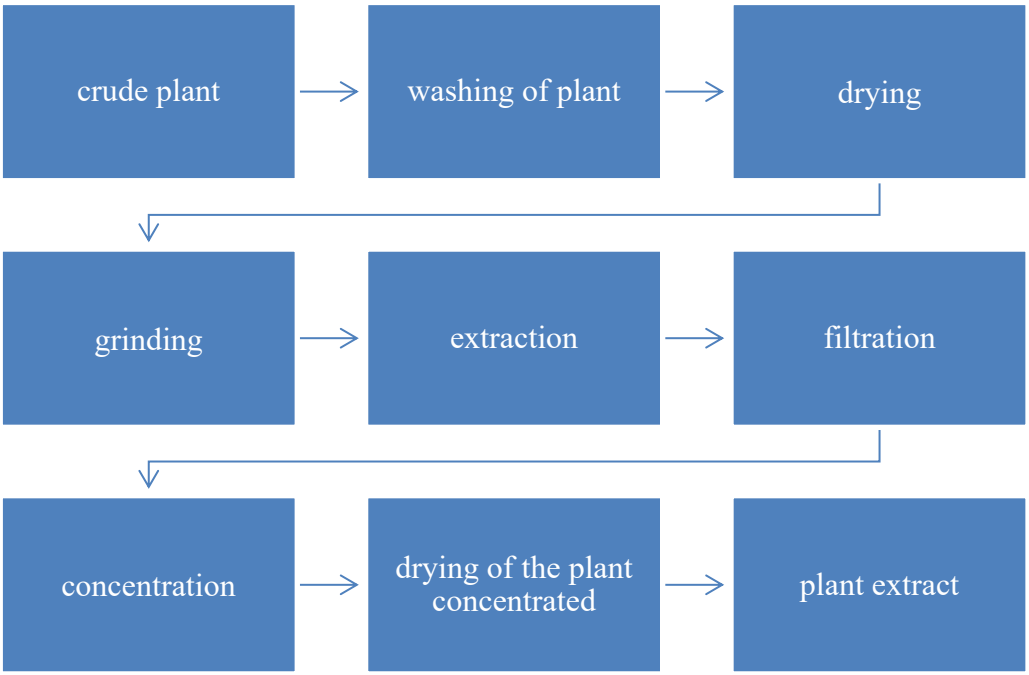

Figure 2 Flow chart of obtaining plant extract

2.2 Plant leaves preparation and drying

In the wake of gathering the leaves of *Borreria pusilla*, the leaves were oppressed for water wash to evacuate plant garbage just as residue. Afterward, the leaves were isolated from the branch and left for shed drying for around about fourteen days. The leaves were shed dried to anticipate any sort of denaturation of compound constituents of *Borreria pusilla*.

2.3 Extraction process

2.3.1 Drying of plant material

When the plant material has been gathered, a typical practice includes drying the examples on plate at surrounding room temperature with satisfactory ventilation (Chikezie, Ibegbulem, & Mbagwu, 2015). Laves of *Borreria pusilla* were culled off from plant stem and cleaned appropriately with water to expel pointless residue particles. After that leaves were shade dried for a few days and dried leaves were then arranged for next technique.

2.3.2 Grinding and weighing of plant material

After fruition of drying, a high limit pounding machine was utilized to squash the leaves into fine powder. From that point onward, it was pressed into a hermetically sealed plastic compartment and appropriate marking on the holder was finished. From that point forward, marked holder was kept in a dull, cool and dry spot till it required for next procedure. The last weight of the powdered plant material was 650 gm.

2.3.3 Extraction

To remove the substance constituents from plant parts by maceration process, methanol and hexane were utilized as the natural dissolvable. Here 2L beaker was utilized for this procedure. About 350 gm. and 300 of grounded leave powder of *Borreria pusilla* which was soaked in 1L of methanol 1L of Hexane for 7 days' time span in a room temperature (22-25°C) with irregular fomentation. The after effect of maceration procedure was a 2-layer stage. The lower-most stage is the dregs and the upper-most is a methanol and hexane arrangement of the concentrate which was isolated by a filthy suspension of plant parts.

2.3.4 Filtration

Following 7 days of drenching, the substances of the container were purged out first to channel them by utilizing Firstly, through material and secondly, through cotton.

2.3.5 Concentration

By utilizing turning evaporator (Heidolph) at 45°C, and at 100rpm the gathered filtrate was moved into concentrated methanol and hexane separate. At that point, the concentrates were moved onto petri-dishes for drying under laminar wind stream.

2.3.6 Drying

In the wake of concentrating the unrefined concentrate, the thick focused arrangements were moved into containers. The containers were held under Laminar Air Flow (LAF) to dissipate

the dissolvable from the concentrates. Subsequent to drying for five days, the concentrates turned out to be completely dried. The dried concentrates were then put away in vials to keep away from any sort of cross defilement, microbial development and furthermore for further examination. At long last, aluminum foil was utilized to cover petri-dishes and refrigerated.

2.4 Phytochemical screening of whole plant extracts of *Borreria pusilla*

The phytochemical screening of *Borreria pusilla* extracts were evaluated by standard strategy. Phytochemical screening was completed all in all plant concentrates utilizing various solvents to distinguish the significant characteristic concoction gatherings, for example, tannins, saponins, flavonoids, phenols, alkaloids, glycosides, carbohydrates, phytosterols, resins and steroids. General responses in these examinations uncovered the nearness or nonattendance of these mixes (Biotech, 2016).

2.4.1 Chemicals and reagents

The reagents and synthetic compounds utilized for phytochemical screening were of investigative evaluation and were acquired from the Department of Pharmacy, Brac University.

2.4.2 Detection of alkaloid

Three tests were performed for the subjective assurance of the alkaloids. 0.5 gm. of methanol and hexane concentrates of *Borreria pusilla* were broken down in 5 mL of 1% Hydrochloric corrosive bubbled in a water shower pursued by filtration respectively. Utilizing the filtrate got the accompanying tests were performed for both extracts.

2.4.2.1 Mayer's test

To a couple of mL of plant test extricate, two drops of Mayer's reagent (potassium mercuric iodide arrangement) are included at the edges of test tube. In the event that ruddy darker

shading precipitation structures, at that point it will point to the nearness of alkaloids (Bandiola, 2018).

2.4.2.2 Hager's test

The concentrates were treated with Hager's reagent (soaked picric corrosive arrangement). Nearness of alkaloids is affirmed by the nearness of yellow hasten (Bandiola, 2018).

2.4.2.3 Wagner test

In 1mL of each plant concentrate was included with a similar measure of Wagner's reagent (Iodine in potassium iodide). In the event that ruddy darker shading precipitation structures, at that point it will point to the nearness of alkaloids.

2.4.3 Detection of carbohydrates

2.4.3.1 Molish test

To 2 mL of each concentrate, two drops of alcoholic arrangement of α-naphthol are included. The blends are shaken well and a couple of drops of concentrated sulphuric corrosive are included gradually at the edges of the test tube. The presence of violet ring at the intersection means sugars are distinguished (Bandiola, 2018).

2.4.3.2 Fehling's test

To 1 mL of the each concentrate, 1 mL of Fehling's A and 1 mL of Fehling's B arrangements were included a test tubes and warmed in a water shower for 10 minutes. Arrangement of red hasten demonstrates the nearness of a diminishing sugar. The filtrates were treated with 1 mL of Fehling's A and B, and warmed in a bubbling water shower for 5-10 minutes. Appearance of ruddy orange encourage demonstrates the nearness of starches.

2.4.3.3 Benedict's test

Filtrates were treated with Benedict's reagent and warmed delicately. Orange red accelerate shows the nearness of lessening sugars (Prashant, Bimlesh, Mandeep, Gurpreet, & Harleen, 2011).

2.4.4 Detection of Flavonoids

2.4.4.1 Lead-acetic acid derivation test

Extracts were treated with few drops of lead acetic acid derivation arrangement. Arrangement of yellow shading hasten demonstrates the nearness of flavonoids (Prashant et al., 2011).

2.4.4.2 Shinoda's test

One mL of each concentrate is included with 0.5 mL of hydrochloric corrosive and magnesium metal. The nearness of flavonoids is affirmed by rosy hue (Bandiola, 2018).

2.4.4.3 $FeCl_3$ test

A couple of drops of nonpartisan ferric chloride arrangement were included 1-2 mL of each concentrate. On the off chance that the testimony of blackish red shading encourages structure, at that point it will point to the nearness of flavonoids.

2.4.5 Detection of phenols or phenolic compounds

2.4.5.1 Lead acetate test

The concentrates (50 mg) were broken down in refined water. At that point 3 mL of 10% lead acetic acid derivation arrangement is included in each of concentrate. A massive white hasten shows the nearness of phenolic mixes (Bandiola, 2018).

2.4.5.2 $FeCl_3$ test

0.2 gm of each concentrate was broken down in Ferric chloride arrangement. A green or grimy green encourage demonstrated the nearness of phenolic compound (Odeja, Ogwuche, Elemike, & Obi, 2017).

2.4.5.3 Dilute iodine solution test

To 2-3 mL of each concentrate, a couple of drops of weaken iodine arrangements were included. Development of transient red shading demonstrates the nearness of phenolic mixes.

2.4.6 Detection of glycosides

2.4.6.1 Keller Killiani test

From the outset 1mL of cold acidic corrosive was stirred up with 1 mL of each concentrate and cooled. After that 2-3 drops of ferric chloride was blended and 2mL of concentrated H_2SO_4 was included cautiously in sideways of test cylinder dividers. On the off chance that rosy dark colored hued ring at the intersection of two layers' structure, at that point it will point to the nearness of glycoside.

2.4.6.2 Concentrated H_2SO_4 test

1mLof Concentrated H_2SO_4 was included 1mL of each concentrate kept still for 2 minutes. On the off chance that rosy shading accelerates structure, at that point it will point to the nearness of glycosides.

2.4.6.3 Borntrager's test

5mL of each concentrate was taken; 5mL of 5% $FeCl_3$ and 5mL dilute HCl were included. Substances were warmed for 5 minutes in bubbling water shower pursued by cooling. To this blend, 5 mL of benzene was included and shaken well. Natural layer was isolated and equivalent volume of weaken smelling salts arrangement was included. Appearance of pinkish red shading in the ammonical layer demonstrated the nearness of glycosides (Kamalakar, Prabhakar, & Shailaja, 2014).

2.4.7 Detection of tannins

2.4.7.1 Ferric chloride test

A couple of drops of $FeCl_3$ were included in each of concentrate. On the off chance that blackish shading hastens structure, at that point it will point to the nearness of tannins.

2.4.7.2 Lead acetate test

A couple of drops of fundamental lead acetic acid derivation were included 1-2 mL of each concentrate. In the event that massive red shading hastens structure, at that point it will point to the nearness of tannins.

2.4.7.3 Alkaline reagent test

A couple of drops of sodium hydroxide arrangement were included in each of concentrate. In the event that red shading structures, at that point it will point to the nearness of tannins.

2.4.8 Detection of phytosterol

2.4.8.1 Libermann-Burchard's test

Each of concentrate is included with chloroform. After filtration, they treated with a couple of drops of acidic anhydride, bubbled, and cooled. At that point conc. sulphuric corrosive is included. Arrangement of dark colored ring at the intersection demonstrates the nearness of phytosterols (Bandiola, 2018).

2.4.9 Detection of steroids

2.4.9.1 Acetic anhydride test

2 mL of acidic anhydride was added to 0.5 mL unrefined of each concentrate of plant extract with 2 mL H_2S0_4. The adjustment in shading from violet to blue or green in tests shows the nearness of steroids.

2.4.9.2 Liebermann-Burchard Test

The concentrates were broken down in 2 mL of chloroform to which 10 drops of acidic corrosive and five drops of concentrated sulphuric corrosive were included and blended. The difference in red shading from blue to green shows the nearness of steroids.

2.4.9.3 Salkowski's test

Concentrates were treated with chloroform and sifted. The filtrates were treated with few drops of conc. sulphuric corrosive, shaken well and permitted to stand. Appearance of red shading in the lower layer demonstrated the nearness of steroids.

2.4.10 Detection of saponins

2.4.10.1 Foam test

Fifty (50) mg of each concentrate is weakened with refined water and made up to 20 mL. The suspension is shaken in a graduated chamber for 15 minutes. Saponins are recognized by the development of two-cm layer of froth (Bandiola, 2018).

2.4.10.2 Froth test

In the test tube 5mL of each concentrate was taken and shake enthusiastically to get steady foam. 5-6 drops of olive oil were included into foaming arrangement. In the event that the emulsion is shaped, at that point it will point to the nearness of saponins.

2.4.11 Detection of resins

2.4.11.1 Copper acetate solution test

5 mL of copper acetate solution were mixed with 5 mL of extract. The solution that resulted was strongly shaken and permitted to separate. A green solution is a proof of the existence of resin (Odeja et al., 2017).

2.4.11.2 Copper sulphate solution test

The $CuSO_4$ solution was mixed in 3-4 mL with an extract that was shaken strongly for 1-2 minutes and permitted to be discreet. If green color precipitate happens, the presence of resins will be indicated.

2.5 Evaluation of Antioxidant activity

Some herbal plant extracts have been evaluated with different in vitro assays (Sathisha, Lingaraju, & Prasad, 2011). In the following ways, this research explored the antioxidant characteristics-

1. Determination of antioxidant properties: DPPH assay
2. Determination of Total Phenolic Content (TPC)
3. Determination of Total Flavonoid Content (TFC)

2.5.1 DPPH Free Radical Scavenging Assay

2.5.1.1 Principle

It is possible to measure the free radical scavenging activity (antioxidant ability) of plant extract (*Borreria pusilla*) on stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). In this case, 2 mL of a sample extract (methanol and hexane) solutions at distinct levels (500 µg/mL to 0.97 µg/mL) were mixed with 3 mL of a DPPH methanol and hexane solutions (20 µg/mL). The plant extracts were used to compare the bleaching of purple colored DPPH methanol solution with two norms called ascorbic acid and tert-butyl-1-hydroxytoluene (BHT) (Brand-Williams, Cuvelier, & Berset, 1995).

Table 3: Materials and Reagents for DPPH assay

Materials	Reagents
UV-spectrophotometer	2,2-Diphenyl-1-Picrylhydrazyl (DPPH)
Test tubes	Ascorbic Acid
Volumetric flask	Methanol
Light proof box	Hexane
Pipette (1ml and 5ml)	Distilled water
Glass rod	Extracts of the selected plant

2.5.1.2 Procedure

2.5.1.2.1 Standard preparation

Ascorbic acid (ASA) was chosen for positive control and the complete amount of ascorbic acid needed was calculated. The required quantity was dissolved to obtain a prime mixture maintaining a concentration of 500 µg/mL. On the prime mixture, sequential dilution was performed to achieve multiple concentrations ranging from 500.0 to 0.977 µg/mL.

2.5.1.2.2 Test sample preparation

In a test tube, 2 mg of each extract from the chosen *Borreria pusilla* plant and 4mL of methanol and hexane were drawn and blended for sample preparing. The sample concentrations were 500 µg/mL. On these tester mixtures, sequential dilution of both extracts has now been performed to achieve several concentrations ranging from 500.0 to 0.977 µg/mL.

2.5.1.2.3 DPPH solution preparation

Weighed and dissolved 20 mg DPPH powder in methanol to obtain a 20 µg/mL DPPH solution.

2.5.1.2.4 Assay of free radical scavenging activity

2 mL of each sample extract solution at various concentrations (500 µg/mL to 0.977 µg/mL) was mixed with 3 mL of the DPPH methanol solution (20 µg/mL). The absorbance was measured by UV spectrophotometer at 517 nm against blank after 30 minutes reaction period at room temperature in dark place. Methanol has been used as a blank solution here.

2.5.1.2.5 Calculation

From the equation, the proportion of free radical scavenging was calculated as follows:

Free radical scavenging activity = $(A_0\text{-}A_1) \times 100/ A_1$

Where, A_0 = the absorbance of the control

A_1 = the absorbance of the sample/standard

Extract concentration of 50 percent inhibition (IC_{50}) was calculated against extract concentration from the graph plotted inhibition percentage.

2.5.2 Determination of total phenolic content (TPC)

A method was used to evaluate the complete phenolic content of *Borreria pusilla* leave extractives. Folin-Ciocalteu reagent was used in this method as an oxidant in addition to gallic acid as a standard (Majhenič, Škerget, & Knez, 2007).

2.5.2.1 Principle

Folin-Ciocalteu chemical readily oxidizes phenols when these chemicals are added to this ionic phenolic solution. Folin-Ciocalteu is a chemical that transformed into dark blue when the oxidation operation was finished in the solution yellow color. This color altered the

combination in a UV spectrophotometer measured at 760 nm. The absorbance value plotted in the calibration curve of Gallic acid and data were evaluated as equivalent Gallic acid (GAE) (Sharmin et al., 2018).

Table 4: Materials and reagents for TPC

Materials	**Reagents**
UV-spectrophotometer	Folin-Ciocalteu reagent
Test tubes	Na_2CO_3 solution (7.5 %)
Volumetric flask	Gallic acid
Micropipette (50-200 µl)	Extracts of the selected plant
Conical flask	Distilled water

Table 5: Composition of Folin-Ciocalteu reagent

Serial No.	**Component**	**Percentage**
1.	Water	57.5
2.	Lithium Sulfate	15.0
3.	Sodium Tungstate Dihydrate	10.0
4.	Hydrochloric acid ≥ 25%	10.0
5.	Phosphoric Acid 85% solution in water	5.0
6.	Molybdic Acid Sodium Dihydrate	2.5

2.5.2.2 Procedure

2.5.2.2.1 Gallic acid solution preparation for standard curve

By default, Gallic acid is used worldwide to determine complete phenolic content. Different Gallic acid solution levels ranged from 0.5 mg/mL to 0.1 mg/mL were created for this

experiment. The solution was prepared by blending Folin-Ciocalteu chemical 2.5 mL, sodium carbonate solution 2 mL (7.5 percent w/v) and Gallic acid 0.5 mL. Folin-Ciocalteu chemicals have been diluted with water 10 times. The blend was then held for 20 minutes in the dark place. Using UV spectrometer, absorbance was taken at 760 nm after 20 minutes. By plotting sample concentration against absorbance, a linear equation was achieved.

2.5.2.2.2 Preparation of test sample for evaluation

20 mg of extract was dissolved in 1mL distilled water to produce 20 mg/mL of sample concentration.

2.5.2.2.3 Assay of total phenolic content test

Half mL of the sample, 2.5 mL of Folin-Ciocalteu 10 times diluted, and 2.0 mL of Na_2CO_3 (7.5 percent w/v) were blended. Then the mixture was kept for 20 minutes in the dark. UV spectrophotometer was noted at 760 nm after passing the necessary time absorbance. For the complete sample, the assessment of the normal Gallic acid estimation curve was produced.

2.5.2.2.4 Calculation:

Total phenolic content of the sample was confirmed as mg of GAE (equivalent Gallic acid)/gm extract using the following equation:

$C = c \times V / m$

Where,

C = Total content of phenolic compounds

c = Concentration of Gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (gm)

2.5.3 Determination of total flavonoid content (TFC)

2.5.3.1 Principle

The complete flavonoid content of *Borreria pusilla* was determined by the technique by which the aluminum chloride colorimetric technique determined the complete flavonoid content of *Borreria pusilla* extracts.

2.5.3.2 Chemicals and reagents required

1. Methanol
2. Hexane
3. Quercetin (standard)
4. Aluminium Chloride
5. Sodium nitrite
6. Sodium hydroxide

2.5.3.3 Reagent preparation

1. For 10 mL of 5% (w/v) sodium nitrite, 0.5 gm of sodium nitrite dissolve in 10 mL of H_20 distilled.

2. To prepare 10 mL of 10% (w/v) aluminum oxide ($AlCl_3$), dissolve 1 gm of $AlCl_3$ in 10 mL of H_20 distilled.

3. To prepare 4% (w/v) sodium hydroxide, 4 gm of NaOH dissolve in 80 mL of distilled water. After dissolving the strong NaOH into distilled water then is adding more 20 mL of distilled water to make up volume up to 100 mL.

2.5.3.4 Procedure

The two different extract TFC were determined by colorimetric assaying aluminum chloride. In short, 5 mg/mL of each extract and standard solution of quercetin (0.1-5 mg/mL) were introduced with 2 mL of distilled water and 0.15 mL of sodium nitrite (5 percent -$NaNO_2$, w/v) was added and mixed. 0.15 mL (10 percent $AlCl_3$, w/v) solution was added after 6 minutes. The solutions were allowed to stand for another 6 minutes and after the mixtures were added 2 mL of sodium hydroxide (4% NaOH, w/v) solution. With instant addition of distilled water, the final amount was adapted to 5 mL, carefully blended and permitted to stand for another 15 minutes. The absorption of each blend against the same blend was determined at 510 nm but without extract as a blank. Using the calibration curve of quercetin, TFC was determined as mg quercetin equal per gram of sample.

2.5.3.5 Calculation

The complete flavonoid content, C, was displayed as the equivalent of Quercetin (QE) for each fraction using the following equation:

$C = c \times V/m$

Where,

C = Total content of flavonoid compounds

c = Concentration of Quercetin obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (gm)

2.6 Antimicrobial assay by disc diffusion method

Plants have been a precious source of natural products to maintain human health for a long time, with more extensive natural therapy research, particularly in the last century. The use of plant extracts and phytochemicals can be of excellent importance in therapeutic medicines, both with known antimicrobial characteristics. A number of researches have been carried out in various nations over the past few years to demonstrate such effectiveness. Many plants were used owing to their antimicrobial characteristics, which are due to compounds produced in the plant's secondary metabolism. These goods are renowned for their active substances, such as the phenolic compounds that are component of vital oils and tannins (Nascimento, Locatelli, Freitas, & Silva, 2000).

2.6.1 Principle

A range of laboratory techniques can be used to assess or screen an extract or pure compound's in vitro antimicrobial activity. Disc-diffusion and broth or agar dilution techniques are the most recognized and fundamental techniques. Other techniques, such as poisoned food techniques, are used particularly for antifungal testing (Balouiri, Sadiki, & Ibnsouda, 2016).

In this well-known process, agar plates are inoculated with the sample microorganism's standardized inoculum. Filter paper disks (about 6 mm in diameter), comprising the test compound at the required concentration, are then put on the surface of the agar. Under appropriate conditions, the Petri dishes are incubated. In general, the antimicrobial agent diffuses into the agar and inhibits the germination and development of the test microorganism (Balouiri et al., 2016).

2.6.2 Apparatus and reagents

1. Filter paper discs
2. Nutrient agar medium
3. Petri dishes
4. Sterile cotton
5. Micropipette
6. Inoculating loop
7. Sterile forceps
8. Screw cap test tubes
9. Autoclave
10. Laminar Air Flow (LAF) hood
11. Spirit burner
12. Refrigerator
13. Incubator
14. Dichloromethane
15. Ethanol
16. Nose mask and hand gloves

2.6.3 Test microorganisms

In antimicrobial sensitivity trials, five sample microorganisms were used. They were the gram-positive *Staphylococcus aureus* bacteria, *Enterococcus faecalis* and *Bacillus subtilis*, and included *Escherichia coli* and *Pseudomonas aeruginosa* gram-negative bacteria. These were acquired from the Brac University, Department of Pharmacy.

2.6.4 Disk diffusion assay

2.6.4.1 Preparation of medium

Medium was prepared in 100 mL distilled water by adding 3.8 mg Mueller Hinton agar. Then it was carefully blended until the agar was dissolved entirely. The blend was cooled up to 45-50°C after autoclave at 121°C for 20 minutes and poured into 10 sterile Petri dishes to create a uniform depth in each plate. Every 100 mm Petri dish has roughly 25 mL of agar solution. Then at room temperature it was permitted to cool and solidify.

2.6.4.2 Preparation of the inoculum

Nutrient Broth (NB) was used in the preparation of bacterial broth cultures. The broth media was prepared on the orders of the producers. The autoclaved media was transmitted aseptically to approximately 6ml sterile capped test tubes. Sub-cultured bacterial isolates were transmitted aseptically to the corresponding broth media. These were incubated for 37°C overnight in the incubator and for bacteria for 48 hours at 30°C (Broadbent ∗, 2004).

2.6.4.3 Preparation of crude extract sample

The sample was prepared by dissolving Borreria pusilla's 500 mg methanol and hexane extract of 1mL of methanol and hexane at 500 mg/mL respectively. Then it was diluted in series for both extracts to get 250 mg/mL.

2.6.4.5 Preparation of disc

The disk was made of 6 mm diameter Whatman paper and was autoclaved in a test tube. Then plant extract solutions were introduced to the disk. Then all plant extracts were permitted to be soaked for 10-15 minutes.

2.6.4.6 Standard

The norm here was kanamycin and streptomycin.

2.6.4.7 Disc diffusion evaluation

First, a cotton swab was immersed in bacteria's suspension. The swab was gently pressed against the tube to get rid of surplus fluid. The swab was then used to streak the bacterial suspension in one direction to the nutrient agar plate and then diagonally streaked in. The agar plates were permitted to get dry for five minutes after that. Then the disks containing plant extract with two different concentrations were placed on the surface of the place individually using forceps. As a reference standard, Kanamycin 30 and Streptomycin 10 were used and also put on the plate surface. After using each time, the forceps were sterilized by

flame and softly pushed the disk onto the tray. Finally, at 37°C, the Petri dishes were incubated 24 hours a day to obtain the bacteria's lawn development.

Chapter 3

Results

3. Results

3.1 Phytochemical analysis

Preliminary phytochemical analysis was performed for methanol and hexane extracts of *Borreria pusilla* successively. The results are presented in the following table:

Table 6: Preliminary phytochemical screening of methanol and hexane extracts of Borreria pusilla (whole plant)

Phytochemical tests		**Methanol extract**	**Hexane extract**
Alkaloids	Wagner's test	-	-
	Mayer's test	+	-
	Hager's test	+	+
Flavonoids	Lead acetate test	+	-
	Ferric chloride test	+	-
	Zinc ribbon test	-	-
Phenols or phenolic compounds	Lead acetate test	+	-
	Ferric chloride test	+	-
	Dilute Iodine solution test	+	+
Glycosides	Keller killiani test	-	+
	Conc. H_2SO_4 test	-	-
	Borntrager's test	-	+
Tannins	Ferric chloride test	+	-
	Lead acetate test	+	-
	Alkaline reagent test	-	-
Carbohydrates	Molish test	-	+
	Fehling's test	-	+
	Benedict's test	+	+

Phytosterol	Libermann-burchard's test	-	-
Steroids	Acetic anhydride test	-	+
	Libermann-burchard test	-	-
	Salkowski's test	-	-
Saponins	Foam test	-	-
	Forth test	-	-
Resins	Copper acetate solution test	-	-
	$CuSO_4$ solution test	-	-

Note: Here, (+) means present of compounds in plant extract and (-) means absence of compounds in plant extract.

3.2 Antioxidant properties of *Borreria pusilla* extract

The presence of flavonoids, phenols, phenolic compounds and tannins in the phytochemical screening of *B. pusilla* contribute to the presence antioxidant properties in these experimental studies.

3.2.1 DPPH free radical scavenging activity of *B. pusilla*

The *Borreria pusilla* (whole plant) methanol and hexane extracts have been screened for free radical scavenging activity. Ascorbic acid (ASA) was used. Table 7, 8 and 9 include the % of inhibition values ASA and samples (methanol and hexane), respectively. IC_{50} value for ASA was 89.202 µg/mL.

Table 7: IC_{50} value of Ascorbic acid (ASA)

Conc. (µg/mL)	Absorbance of Standard (ASA)	% of inhibition	IC_{50} µg/mL
500	0.032	94.58544839	89.202
250	0.041	93.06260575	
125	0.067	88.66328257	
62.5	0.181	69.37394247	
31.25	0.283	52.11505922	
15.625	0.379	35.8714044	
7.813	0.421	28.76480541	
3.906	0.47	20.47377327	
1.953	0.483	18.2741117	
0.977	0.498	15.7360406	
Blank	0.591	Not applicable	

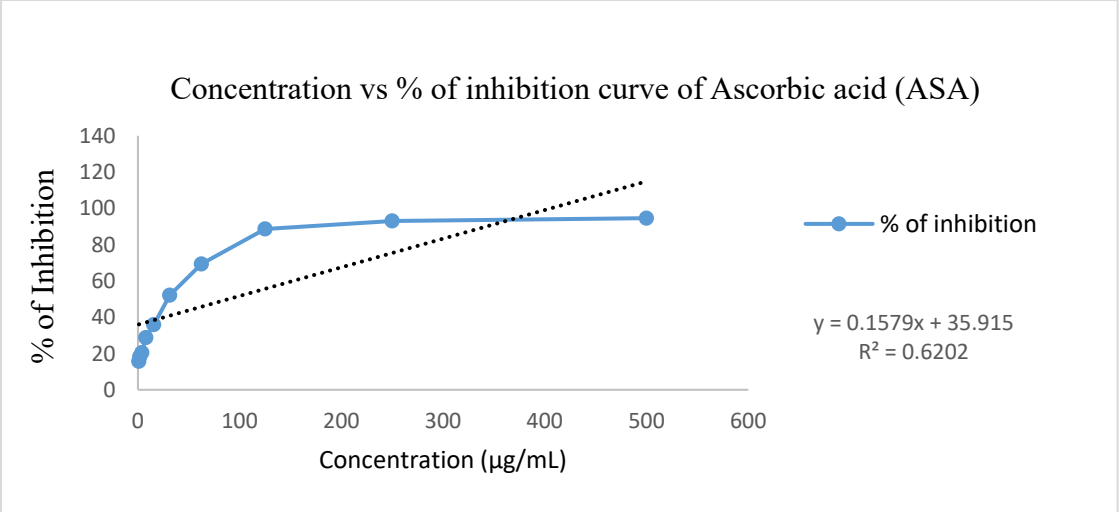

Figure 3: Concentration vs % of inhibition curve of ASA

Table 8: IC_{50} value (µg/mL) of methanol extract of Borreria pusilla

Conc. (µg/mL)	Absorbance of Standard (ASA)	% of inhibition	IC_{50} µg/mL
500	0.091	85.39325843	37.941
250	0.121	80.57784912	
125	0.154	75.28089888	
62.5	0.189	69.66292135	
31.25	0.215	65.48956661	
15.625	0.269	56.82182986	
7.813	0.293	52.96950241	
3.906	0.324	47.99357945	
1.953	0.361	42.0545746	
0.977	0.397	36.2760835	
Blank	0.623		

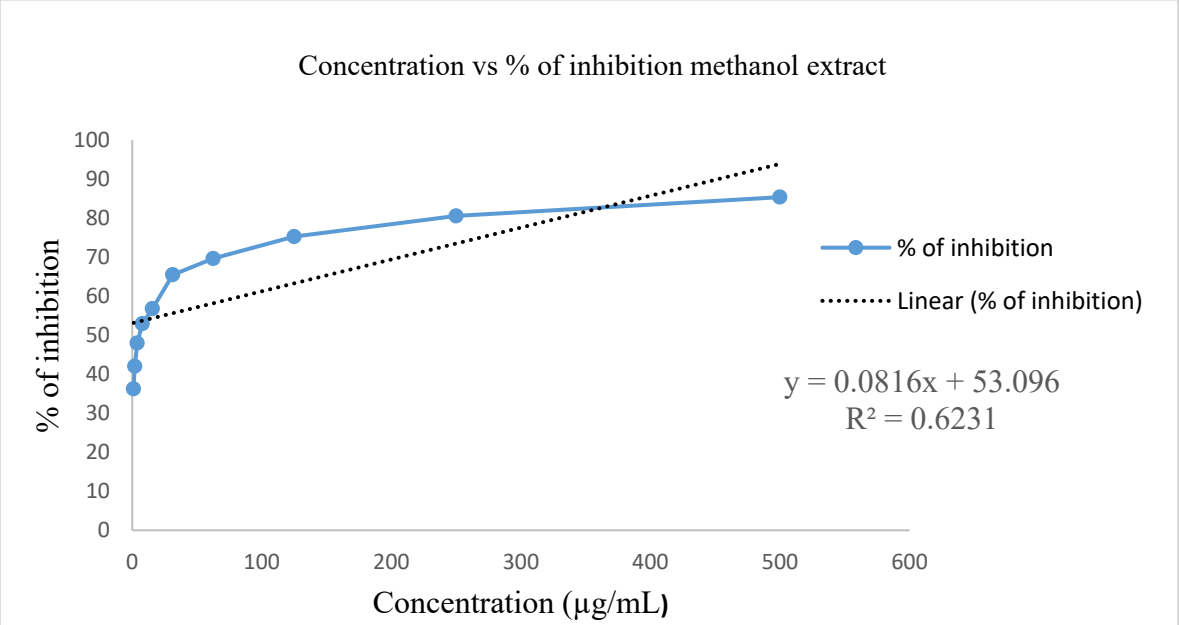

Figure 4: Concentration vs % inhibition of Methanol extract of Borreria pusilla

Table 9: IC_{50} value (µg/mL) of Hexane extract of Borreria pusilla

Conc. (µg/mL)	Absorbance of sample (hexane)	% of inhibition	IC_{50} µg/mL
500	0.049	88.83827	23.867
250	0.061	86.10478	
125	0.092	79.04328	
62.5	0.107	75.62642	
31.25	0.181	58.76993	
15.625	0.207	52.84738	
7.813	0.244	44.41913	
3.906	0.279	36.44647	
1.953	0.294	33.0296	
0.977	0.316	28.0182	
Blank	0.439	Not applicable	

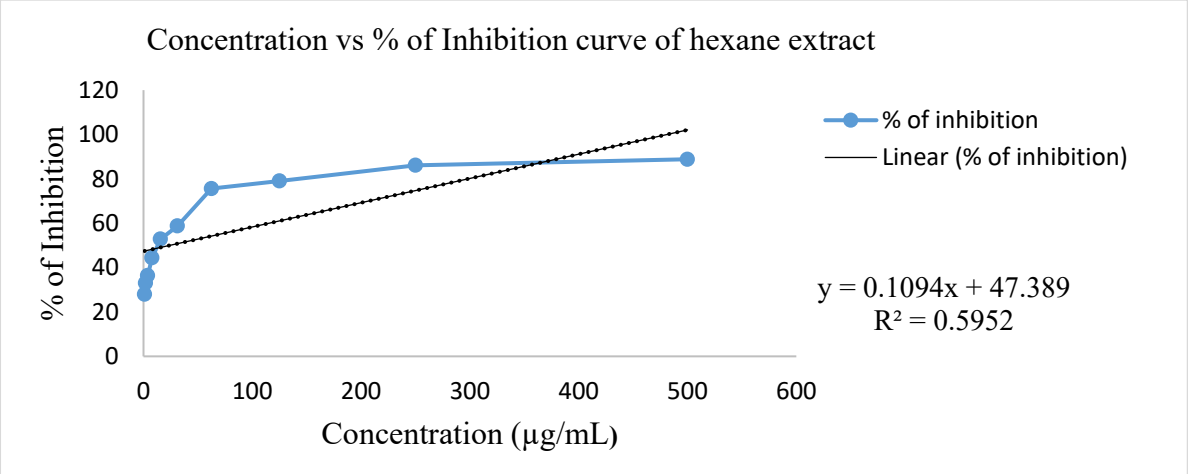

Figure 5: Concentration vs % inhibition of Hexane extract of Borreria pusilla

3.2.2 Determination of Total Phenolic Content (TPC)

The extracts of methanol and hexane were subjected for determination of complete phenolic content. The value of the complete phenolic content of the two different extracts were determined and distinguished with the normal solutions of Gallic acid equivalents, depending on the absorbance values of the extracts solution. The sample's complete phenolic content is displayed as mg of extract GAE (Gallic acid equivalent)/gm.

Table 10: Standard curve preparation by using Gallic acid

Conc. (µg/mL)	**Absorbance**	**Regression Line**	**Regression coefficient (R^2)**
0	0	y=0.0002x+0.1315	0.88
100	0.094		
500	0.221		
1000	0.449		
2500	0.979		
5000	1.113		

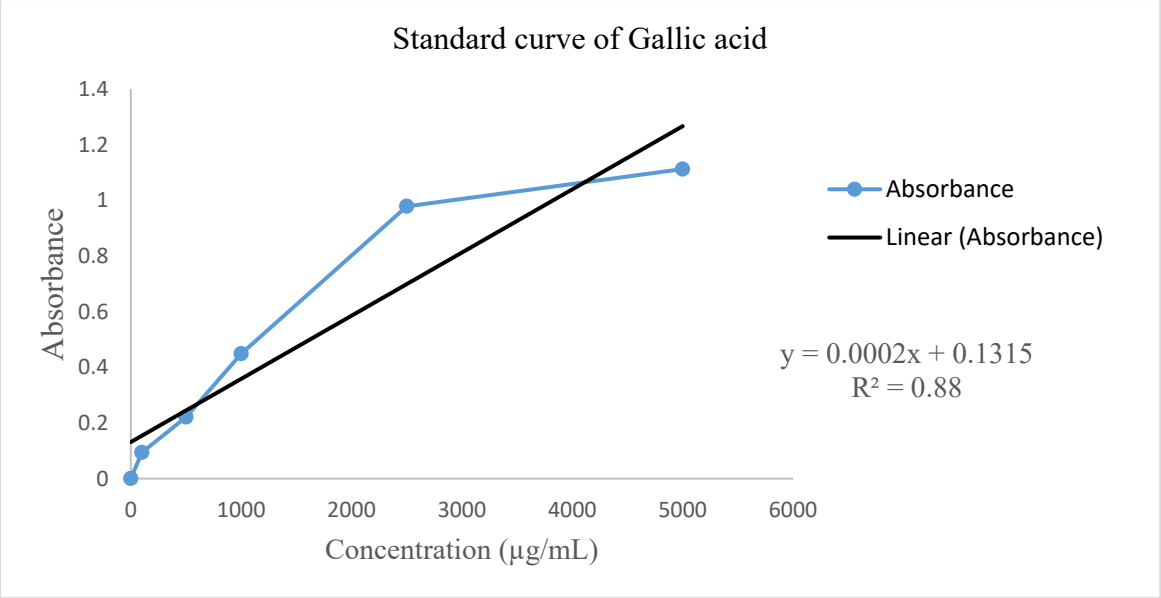

Figure 6: Standard curve of Gallic acid for total phenolic content determination

Table 11: Total phenolic content of methanol extract of B. pusilla

Sample Conc. (µg/mL)	**Absorbance**	**Total phenolic content (mg of GAE/gm of extract)**
20000	1.7	7842.5

Table 12: Total phenolic content of hexane extract of B. pusilla

Sample Conc. (µg/ml)	**Absorbance**	**Total phenolic content (mg of GAE/gm of extract)**
20000	1.7	9492.5

3.2.3 Determination of total flavonoid content (TFC)

Total flavonoid content was determined for the methanol extract and hexane extract of the *Borreria pusilla*. Quercetin has been used for this test as a standard. The value of the total flavonoid content of the extract was investigated and differentiated with standard quercetin equivalent solutions, depending on the absorbance values of the extract solution. The sample's complete flavonoid content is displayed as mg of extract QE (Quercetin Equivalent). The complete flavonoid content was discovered to be 3872.5 mg of QE/gm of mercury in the methanol extract of plant of concern whereas 747.5 mg of mercury in the hexane extract of plant of concern is found.

Table 13: Standard curve preparation by using Quercetin

Conc. (µg/mL)	**Absorbance**	**Regression Line**	**Regression coefficient (R^2)**
0	0	y=0.0002x+0.1715	0.9628
100	0.261		
500	0.354		
1000	0.471		
2500	0.779		
5000	1.349		

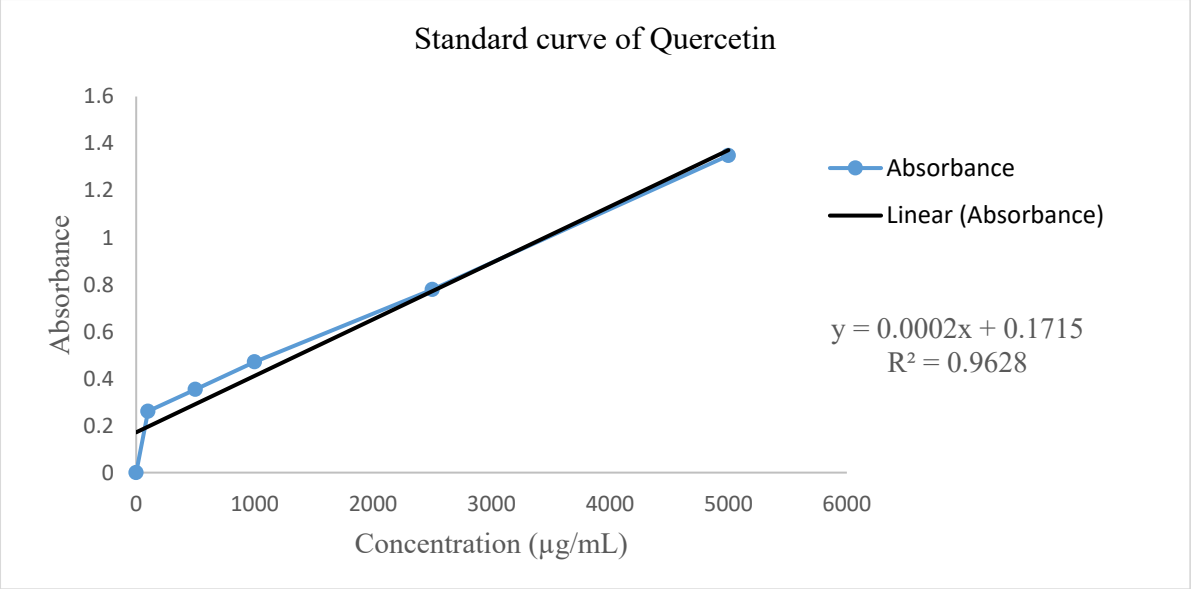

Figure 7: Standard curve of Quercetin for total flavonoid content determination

Table 14: Total flavonoid content of methanol extract of B. pusilla

Sample Conc. (µg/mL)	**Absorbance**	**Total flavonoid content (mg of GAE/gm of extract)**
20000	0.946	3872.5

Table 15: Total flavonoid content of hexane extract of B. pusilla

Sample Conc. (µg/mL)	**Absorbance**	**Total flavonoid content (mg of GAE/gm of extract)**
20000	0.321	747.5

3.3 Antimicrobial activity:

Table 16: Antimicrobial activity of extracts of Borreria pusilla

Test organism	**Diameter of zone of inhibition (mm)**					
	Standard		Plant extracts			
			Methanol extract		Hexane extract	
	Kanamycin	Streptomycin	250 mg/mL	500 mg/mL	250 mg/mL	500 mg/mL
Gram positive						
Staphylococcus aureus	19	20	0	18	0	17
Bacillus subtilis	25	18	0	18	0	13
Enterococcus faecalis	20	21	0	16	0	0
Gram negative						
E.coli	23	19	0	0	0	0
Pseudomonas aeruginosa	18	21	0	0	0	0

Chapter 4

Discussion

4. Discussion

The phytochemical screening of *Borreria pusilla* showed the presence of carbohydrates, flavonoids, alkaloids, phenolic compounds and tannins in the methanol extract. However, glycosides, phytosterol, resins, steroids and saponins were absent in methanol extract. On the other hand, alkaloids, phenolic compounds, glycosides, carbohydrates and steroids were present in the hexane extract of *Borreria pusilla*. It was also found that some of the compounds were also absent in hexane extract and they were flavonoids, tannins, phytosterols, saponins and resins.

The IC_{50} value of *Borreria pusilla* dried extract (methanol) indicates that a slightly greater concentration of 37.941 µg/mL was needed to inhibit 50% of all DPPH-free radical scavengers, whereas a concentration of 89.202 µg/mL was needed to attain the same inhibitory impact for Ascorbic acid. The IC_{50} value of dried extract (hexane) from *Borreria pusilla* showed that a very small concentration of 23.867 µg/mL was required to inhibit 50 percent of all DPPH-free radical scavengers, whereas concentration of 89.202 µg/mL was required to achieve the same inhibitory effect for ascorbic acid.

The overall phenolic content of methanol and hexane extracts were determined by Folin-Ciocalteu technique used Gallic acid as the reference standard. The complete phenolic content of the product was equal to Gallic acid. From the table 10, as the *B. pusilla* concentration rised from 0-5.0 mg/mL, the absorbance of Gallic acid was also increasing. It therefore showed that the antioxidant activity of plant extract also increased with a rise in complete phenolic content. Gallic acid's calibration curve equation was discovered to be $y = 0.0002x + 0.1315$, used to determine the complete phenolic content of our plant's methanol and hexane extracts. Also there acquired was a 0.88 regression coefficient (R^2) showing a moderate connection between concentration and absorbance. The total phenolic content in

methanol and hexane were found to be 7842.5 mg of GAE/gm and 9492.5 mg of GAE/gm of extracts respectively after investigation.

From the table 13, as the *B. pusilla* concentration rised from 0-5.0 mg/mL, the absorbance of quercetin was also increasing. It therefore showed that the antioxidant activity of plant extract also increased with a rise in complete flavonoid content. Quercetin's calibration curve equation was discovered to be y=0.0002x+0.1715, used to determine the complete flavonoid content of our plant's methanol and hexane extracts. Also there acquired was a 0.9628 regression coefficient (R^2) showing a moderate connection between concentration and absorbance. The total phenolic content in methanol and hexane were found to be 3872.5 of GAE/gm and 747.5 mg of GAE/gm of extracts respectively after investigation.

This research concentrated on antimicrobial activity determination of *Borreria pusilla* methanol and hexane extracts by using gram-positive bacterial strains and gram-negative bacterial strains. The methanol and hexane extracts were explored with a concentration of 250 mg/mL and 500 mg/mL in per disk for antimicrobial activity. In case of methanol extract, the maximum zones of inhibition exhibited by standards were found to be moderate against the microbes. With the use of 250 mg/mL concentration of extracts, no activities were showed whereas 500 mg/mL methanol extracts showed positive results against all gram positive organisms: *Staphylococcus aureus, Bacillus subtilis, and Enterococcus faecalis*. Moreover, 500 mg/mL concentration of hexane extract also showed positive results against all gram positive bacteria except *Enterococcus faecalis.* On the other side, against all the gram negative organisms, all the test samples showed adverse reaction. It can therefore be suggested from the observation that the plant does not have antibacterial activity against gram negative bacteria and hexane extract of the plant with 500 mg/mL concentration does not show antibacterial activity.

Chapter 5

Conclusion and Future Direction

5. Conclusion and Future Direction

5.1 Conclusion

Medicinal plants are often used as raw materials to extract from the active components that are then used in the synthesis of various significant drugs. Thus *Borreria pusilla's* crude extracts of leaves can be a source of herbal medicine to heal a broad range of human illnesses. The two distinct *Borrria pusilla* extracts have been subjected to phytochemical screening as well as anti-oxidant activity and anti-microbial activity in this current inquiry. This plant contains flavonoids, alkaloids, glycosides, phenolic compounds, carbohydrates and steroids which were assured by phytochemical screening. The three different methods showed the presence of antioxidant activity in the plant. In addition, the antibacterial research was conducted on the two different extracts of plant by disc diffusion method against five bacterial strains. This study showed that methanol extract of *Borreria pusilla* with concentration of 500 mg/mL can be used against gram positive bacteria.

5.2 Future direction

In future, extensive *in-vivo* studies can be conducted with a view to validating current herbal and traditional uses and exploring any other therapeutic activity along with possible side effects of this plant. Ultimately, this would lead to an awareness of the safety and effectiveness of *Borreria pusilla*. Also, in characterizing and isolating its active compounds, further quantitative phytochemical analysis of separate extracts would be useful.

Further tests of this plant's bioactivity could be conducted that are not yet investigated. Such study projects could contribute to drug discovery, drug isolation, and could serve this plant as a natural source for the growth of new drug compounds.

Chapter 6

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6. References

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