Analysis of Biological Potential of Methanol Extract of leaves of *Thladiantha cordifolia*

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The thesis report submitted to the Department of Pharmacy as a requirement for the degree of Bachelors

Department of Pharmacy
Brac University
March 2020

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Declaration

It is hereby declared that-

1. The thesis submitted which consist of my own original works during acquiring the degree at Brac University.
2. All the works are done by myself and with my ideas and implementations.
3. The thesis does not contain any material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
4. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
5. I have acknowledged all main sources of help.

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Approval

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Ethical Statements

1. All the experiments are done by considering required ethical rules and regulations.
2. I have donated blood for the current project.
3. This work will only be published after ethical permission has been taken.

Abstract

The thesis which includes 4 different experimental processes, was performed to evaluate and determine the biological potential of *Thladiantha cordifolia* which is a medicinal plant which is from cucurbitaceae family. All the different experiments were done to evaluate its antioxidant, cytotoxicity, thrombolytic and antimicrobial property. Antioxidant property tests included 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, total flavonoid content (TFC) test and total phenolic content (TPC) test, which showed acceptable results. The plant reflected very mild thrombolytic property and remarkable level of cytotoxic property. However, antimicrobial property was completely absent in the plant extract. From this research it is evident that the plant *Thladiantha cordifolia* is able to provide moderate effect in the development of medicinal world.

Keywords: *Thladiantha cordifolia*; In-vitro evaluation: antioxidant; cytotoxicity; thrombolytic and anti-microbial.

Acknowledgement

The persistent appreciations and abundant supports from many people throughout all the project works are gratefully acknowledged here.

First of all, I would like to express my respect and gratitude to my most esteemed supervisor Shahana Sharmin, Senior Lecturer, Department of Pharmacy, Brac University, in completing this thesis. Her excellent guidance, instructions, insightful discussion, constant encouragement and support throughout the project works are base for the establishment of this research. From core of my heart I deeply acknowledge my guide for picking me up as one of her research students and providing me knowledge and an excellent atmosphere for completing the project work in time.

I take pride in acknowledging the initiative guidance of professor Dr. Eva Rahman Kabir, Chairperson, Department of Pharmacy, Brac University, for sparing her valuable time whenever I needed.

My acknowledgement would be incomplete without taking the biggest source of my strength, my family. I am thankful for the blessings of my parents and the love and care of my siblings.

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Chapter 1

INTRODUCTION

Plants are the most significant part of human race and always played a conspicuous role in the betterment of our health. Medicinal plants are an indispensable component used traditionally in healthcare sector. Since primitive age, plant extracts had been used to treat different kind of diseases. When a plant is necessary as a drug or an active component of a medicinal composite it can be marked as 'medicinal'. Many new drugs and drug therapies are formulated and developed from those primitive agents, so it is still one of the most valuable source of medicine which leads us to identify naturally available medicinal plants for the production of different and new medicines for the betterment of human race as well as animal kingdom. Among about 7.5 lakhs of plant species that has been invented so far, higher plants are 5 lacs" and 2.5 lacs are "lower plants". Natural herbal derivatives contribute about 60% of all clinically used medicines and natural products. Antibiotics, antidiabetic, antioxidant, antitumor agents and anticoagulants- all of these classified medicines come from natural products (about 87%) and their derivatives. One of the safest and harmless way of treating disease or ill conditions is the treatment with medicinal plant because it reflects slightest side effects. The people all ages and sex can consume the herbal medicines which is also convenient for acceptance. According to the World Health Organization (WHO), for primary treatment almost 80% people around the world are directly dependent on medicinal plants.

1.1 Antiquity of medicinal plants

We still could not figure it out exact when or how treating diseases with medicinal plant was started. It is assumed that people used to far in the search of food and they found different types of plants. They collected different parts of the plant to use in various purpose, eventually people found that plants (different parts of the plant) showed healing effects as well as poisonous effects. Human were intelligent enough to adapt those more easily, started researching and shared knowledge throughout the clan. Some great civilizations like, the Assyrians, the Chinese, the Babylonians, the Egyptians, the Persians figured out the medicinal properties of plants. The Ancient Egyptians wrote the book (Ebers Papyrus), which provides medicinal details on over 850 plants. Babylonians had vast knowledge about medicinal plants and modern medicine still uses those speciic plants among those in the same way as Babylonians.

The first typescript on the use of medicinal plants, which is almost 5000 years old was found on a Sumerian clay slab. The clay slabs also include hundreds of medicinal plants where Myrrh, Aloe, Mandrake and Opium were also listed along those, mentioned in 1700, more than 700 methods were discovered. The ancient book titled "Pen Tsao" which holds the information on usage and application of 365 medicinal plants.

Researchers stated that Indian forests had a great quantity of medicinal and aromatic plants since primitive ages. Indian medicine system named AYUSH enlisted around eight thousand herbal remedies and also for native significant medicine system (Tribal, Unani and Ayurveda).

Researchers conducted a lot of researches on medicinal plants, attempted to evaluate with specific findings on the effectiveness of diverse herbs that provide pharmacological and efficient significance against various diseases. The main reason behind the quality treatment with herbal medication all over the world is these formulated medications from different plants have negligible or no side effects or any kind of adverse reactions. In fact, there are some diseases which gets cured with medicinal herbs or plants but are hard to treat with any other medicines. Not only this it also strengthens our immune response naturally. In fact, there are few diseases which are only curable by herbal medicines or products from medicinal plants.

1.2 Medicinal Plants available in Bangladesh

With the geographical benefits as many rives run through Bangladesh (subtropical country), we have rich resources of many medicinal plants which are the main ingredients in the production of medicines. Plant-based traditional medicines for healthcare is still the basis for primary treatment in rural areas in Bangladesh. Bangladesh is enriched with 5000 different types of medicinal plants which is mentioned in the book 'Meteria Medica'. Different areas of Bangladesh have different species of plants. Due to a large amount of plant resource nowadays many pharmaceutical companies are growing up considerably and contributing in the country's economy.

Table 1: List of medicinal plants available in Bangladesh

Plant species	Family	Local name	Traditional Use
Acrostichum aureum	Ptridiaceae	Tiger fern	Rheumatism, treat wounds and boils, to stop bleeding
Adiantum caundatum	Adintaceae	Mayurshikha	Expectorant, antipyretic, diabeters, skin disease, antibacterial, hypoglyceamic
Aegiceras corniculatum	Myrsinaceae	Kholisha	Asthma, diabeters, rheumatism
ammannia baccifera	Lythraceae	Jangli mendi	Rheumatism, skin disease, ring worm and fever
Argemone mexicana	Papaveraceae	Shialkata	antifungal, antiviral, antithelmintic, syphilitic infection, dysentery
Blumea lacera	Compositae	Kukursunga	astringent, stimulant, antithelmintic, antimicrobial, anti-inflammatory and diuretic
Bruguiera gymnorrhiza	Rhizophoraceae	kankra	astringent, diarrhoea, stops bleeding, blood pressure
Clerodendron inerme	Verbenaceae	Bon jui	Rheumatism, hypotensive, fever, cytotoxic
Ficus religiosa	Moraceae	Pan bot	antibacterial, astringent, diarrhoea, dysentery, gonorrhoea, antiprotozoal, antiviral and ulcers, skin disease
Hibiscus tiliaceous	Malvaceae	Bhola	fever, coughs and dry throat, bronchitis, ear infections, dysentery
Hygrophila auriculata	Acanthaceae	Talmakna	Tonic, diarrhoea, dysentery, urinary discharge, gonorrhoea, diuretic, inflammation, antineoplastic, rheumatism
Limnophila indica	Scrophulariaceae	Karpur	Antiseptic, dysentery, elephantiasis, fever
Mollugo pentaphylla	Molluginaceae	Khetpapra	antiseptic, digestion problem, ear ache, spermicidal, antifungal
Pandanus foetidus	Pandanaceae	Kewa kata	leorosy, small pox, syphilis, scabies, brain disease, diabetes
Xylocarpus moleccensis	Meliaceae	Passur	astringent, febrifuge, dysentery, diarrhoea, swelling of breats, itching, elephantasis
Aegiceras corniculatum	Myrsinaceae	Kholisha	asthma, diabetes,inflammation and rheumatism
Argyreia nervosa	Convovulaceae	Bichtarak	hypotension, hallucinogenic, stomach trouble, small pox, syphilis, diarrhoe, dysentery, wound healing, skin disease, diuretic, rheumatism, urinary disease, chronic ulcers

Avicennia alba	Avicenniaceae	Morcha bean	treatment to infertility, skin diesase, tumors, ulcers
Caeaolpinia pulcherrima	Caesalpiniaceae	Khrishnachura	purgative, abortifacient, anticancer, liver disorders, cough, asthma, bronchitis, malaria fever, cholera, infantile convulsion
Clerodendrum viscosum	Verbenaceae	Bhant	hypotensive,anthelmintic, emetic, antiperiodic, malaria treatment, asthma, tumor, snakebite
Clitoria ternatea	Papilionaceae	Aparajita	demulcent, aperients, laxative,diuretic, cathertic, ascites, sore throat, tumors, dropsy, skin diseases, gonorrhoea
Dillenia indica	Dilleniaceae	Chalta	expectorant,laxative, tonic, abdominal pain, astringent, anstifungal
Diospyros peregrina	Ebenaceae	Gab	astringent, dysentery, biliousness, astringent, sore throat, wound, ulcer, cough, dyspnoea
Dipterocarpus turbinatus	Dipterocarpaceae	Garjan	Gonorrhoea, gleet, ulcer, rheumatism, ringworm, skin diseases
Ecbolium viride	Acanthaceae	Nilkanta	antitumor, jaundice, rheumatism, gout, dysuria, cardiovascular disease
Glinus oppositifolius	Molluginaceae	Gima	CNS depressant, diuretic, stomachic, aperients, antiseptic, skin diseases
Glycosmis pentaphylla	Rutaceae	Daton	fever, liver complaints, coughs, rheumatism, anemia, jaundice, eczema
Gnaphalium luteoalbum	Compositae	Boro karma	astringent, diuretic, haemostatic, counter irritant
Hymenodictyon excelsum	Rubiaceae	Bhui kadam	astringent, febrifuge, antiperiodic, hypotensive, antimicrobial, diarrhoea, herpes
Jasminum sambac	Oleaceae	Beli phul	CNS depressant, hypotensive, indolent ulcer, breast tumors
Lannea cormandelica	Anacardiaceae	Jeol	astringent, leprous, obstinate ulcer, mouth sores, swelling and pain
Mussaenda glabrata	Rubiaceae	Patralekha	white leprosy, jaundice, diuretic, asthma, dropsym, chest pain, ulcer
Myrica nagi	Myricaceae	Kaiphal	astringent, carminative, antiseptic, fever, asthma, cholera, dysentery, anemia, piles, ulcer, bronchitis, antitumors
Saraca asoca	Caesalpinaceae	Ashok	antitumor, menorrhagia, haemorrhagic dysentery, colic, ulcer, blood purification, biliousness, syphilis, uterine tonic.

1.3 Importance of medicinal plants in drug finding

Against many diseases therapeutic agents are derived from plant components which can be directly uses as drugs or as semi-synthesized or synthesized drugs. Even in modern age more than 80% of antimicrobial, anticancer, antioxidant, antidiabetic, cardiovascular, cardiovascular, pulmonary, narcotic agents are plant origin. Besides API around 60% of pharmaceuticals are derived or isolated from plants or herbs including animals, insects, organisms as active ingredients. Drug discovery from herbs may be divided into three stages, namely, pre-drug stage, quasi-drug stage, full drug stage.

Medicinal plants provide us with various types of therapeutic agents that are used for treating different diseases. These therapeutic agents are derived from plants and are used directly as drug or as semi-synthesized or synthesized drugs. In the past remedial was used to be produce by mixture, extracts or preparations prepared by herbalists who used to know the therapeutic treatment for most of the diseases. Today approximately 80% of antimicrobial, cardiovascular, immunosuppressive, anticancer drugs are of plant origin. And in fact, around 50% of pharmaceuticals are derived from compounds first identified or isolated from plants or herbs including animals, insects, organisms as active ingredients. Drug discovery from herbs may be divided into three stages, namely, pre-drug stage, quasidrug stage, full drug stage (Pan et al., 2013).

The importance of medicinal plants is immense and it is also increasing day after day. To improve the healthcare system and economic growth medicinal plants and plant-based pharmaceuticals playing an important part.

1.4 Drug as natural product obtained from medicinal plants

The medicinal plants are the main source for APIs. Around 25% of drugs that are currently in use for different treatments are directly isolated from herbal plants. According to World Health Organization (WHO) 252 drugs are registered as essential medicine where 11% drugs are directly from plant extracts. Along with this around 70% percent drugs are semi-synthetic that means which are formulated from the original plant source by adding or removing different functional groups. Moreover, there are many synthetic drugs which is also associated with natural plant products.

Table 2: Medicines from plant source

No.	Name of the drug	API	Plant source
1.	Aspirin	Salicylic Acid	Willow bark
2.	Quinine	Qualaquin	cinchona tree
3.	Opiates	Oxycontin, Morphine, Codeine	Opium poppy (*Papaveraceae somniferum*)
4.	Myriocin		*Mycelia sterilia*
5.	Penicillin		Penicillium mold
6.	Digoxin		foxglove plant (*Digitalis lanata*)
7.	Paclitaxel	Taxol	Pacific Yew (*Taxus brevifolia*)
8.	Vincristine & Vinblastine		Madagascar periwinkle plant (*Catharanthus roseus*)
9.	Lysergic Acid Diethylamide (LSD)		ergot (*Claviceps purpurea*)
10.	Irinotecan		*Camptotheca acuminata*

1.5 Selection of *Thladiantha cordifolia* for this project

The major reason behind selecting this plant for the project is, no significant research had been done on this plant before. After exploring many different journals and publications no sufficient amount of information was found on *Thladiantha cordifolia*. This leads to the identification of various biological properties like- antioxidant activity, antimicrobial activity, thrombolytic activity and cytotoxicity of *Thladiantha cordifolia*.

1.5.1 Introduction to the selected plant *Thladiantha cordifolia*

Thladiantha cordifolia belongs to family Cucurbitaceae which is also known as Himalayan Goldencreeper. This type of plant can only be found in hilly areas. In Bangladesh they are available in Chittagong specially Bandorban, Khagrachari, Rangamati. *Thladiantha cordifolia* is a rare kind of plant and very hard to be found.

Gesneriaceae is also called the pumpkin Family. Cucurbitaceae, the gourd family of flowering plants, belonging to the order Cucurbitales and containing 98 genera and about 975 species of food and ornamental plants. Members of the family are annual or perennial herbs native to temperate and tropical areas and include cucumbers, gourds, melons, squashes, and pumpkins. Most species are extremely sensitive to temperatures near freezing, a factor that limits their geographic distribution and area of cultivation. Cucurbits have a generally low nutrient content, one exception being the winter squashes.

Figure 1: *Thladiantha cordifolia*

1.5.2 Morphology of plant *thladiantha cordifolia*

Thladiantha cordifolia is a beautiful large climber with ovate-heart-shaped leaves and bell-shaped golden yellow flowers. Flowers are bell-shaped, about 2 cm, with petals turned back at the tips. Male flowers are borne in the axils of broad, fringed bracts, in stalked clusters 5-8 cm long. Ovate leaves have long stalks, and the blade is 10-15 cm, deeply heart-shaped at the base, with marginal tooth-like projections. Leaves are round and bristly haired. Fruit is ellipsoidal, about 3.5 cm long, longitudinally 12-25 nerved. Himalayan Goldencreeper is not a common plant, and is found in the Himalayas from Bhutan to North-East India, Nepal, south-east side of Bangladesh at altitudes of 600-2500 m.

1.5.3 Plant Taxonomy (*Thladiantha cordifolia*)

Table 3: Taxonomy hierarchy of *Thladiantha cordifolia*

Rank	Scientific name
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Cucurbitales
Family	Cucurbitaceae
Genus	*Thladiantha*
Species	*Cordofolia*

Figure 2: Flowers from Cucurbitaceae

1.5.4 Pharmacological properties of other genera and species of this family

Thladiantha cordifolia is included in the family Cucurbitaceae and several species of this family also possesses biological activities, such as antioxidant, antidiabetic, antimicrobial, cardiovascular, anti-inflammatory, and antitumor properties. Moreover, some species from Cucurbitaceae family have been used in traditional medicine against fever, cough, colds, skin problems, snakebite, pains, and infectious and inflammatory diseases. Different components from the plants of this family were used to develop medicine to treat many other uncommon diseases.

1.5.5 Related publication on *Thladiantha cordifolia*

*Thladiantha cordifolia*is is a rare plant of hilly areas which still doesn't have any publication on scientific researches on its medicinal properties.

Therefore, many valuable properties and uses of medicinal plants still need to be identified.

1.6 Project justification /rationale

As several species of this family have reported powerful medicinal properties and also no significant research has been conducted on biological potential of *Thladiantha cordifolia,* the plant was chosen for this thesis work. Therefore, through this study we were solely intended to find out different medicinal and pharmacological properties.

1.7 Aim of the project

To identify and analyze potential biological effects of the selected plant, *Thladiantha cordifolia* (Family: Cucurbitaceae) is the ultimate aim of the project.

1.8 Objectives of the project

Based on this project protocol following experiments were conducted which was done with methanol extract of the *Thladiantha cordifolia* leaves-

1. Determination of thrombolytic activity.
2. Evaluation and screening of the plant's antimicrobial activity.
3. Evaluation of cytotoxic activity
4. Evaluation of the antioxidant property which includes-
    a) DPPH free radical scavenging method,
    b) Determination of the total phenolic content (TPC)of the plant,
    c) Determination of the total flavonoid content (TFC) of the plant

1.9 *In-vitro* evaluation of thrombolytic property of *Thladiantha cordifolia* leaves extract

Since primitive age, different diseases and health conditions has been treated with plant derivatives as it the safe source of medicinal agents. Cardiovascular and heart diseases cause the maximum death in the world. Because people are more into oily/ cheesy foods which result obesity as well as heart diseases. Fat deposits on blood vessels narrow down the veins/ arteries. Again blood can clot inside the blood vessels due to many other dysfunctionalities. In these cases, thrombolysis is a crucial required treatment. (CVST) Cerebral Venous Sinus Thrombosis is a serious condition. Heparin, urokinase, clopidogrel, Streptokinase and many other active agents are uses as the first line treatment. Hence, precise goal of this experiment was to introduce the thrombolytic efficacy of methanol extract of Thladiantha cordifolia leaves.

Thrombolytic drugs are the agents which breaks down the blood clots, used more systematically and effectively to solve problems in blood flow, but it is not applicable for every patient (risk of bleeding) and has to be administrated by professionals. Thrombolytic agents reduce activating the enzyme plasminogen, which removes the primary structure that is cross-linked fibrin mesh. These thrombolytic agents might trigger high blood pressure and active bleeding.

1.10 *In-Vitro* Antimicrobial property evaluation of *Thladiantha cordifolia* leaves extract

Infectious diseases by different microorganism are one of the main reasons of death. It has been observed from the ancient time to till now. Sometimes it became pandemic and caused severe damage to human race. Infectious disease death rate, holding the fifth place in 1981 but became the third in 1992. Infectious diseases are more observed in developing country where a large number of population live, because of low quality of life style, lack of hygiene and consciousness, easy to multiply and poor response to the treatments. Other contributing factors are- increased resistance to antibiotics in infections acquired by our nosocomial and communal infections. However, mortality rate from infectious diseases is increasing in advanced countries simultaneously.

With advanced science and technology, we have created a lot of vaccines and antibiotics many of them are directly isolated from plants. To invent new treatments or prevention strategies we also need medicinal plants. Thousands of medicinal plants are available to serve required active agents but we need to carry on to find more effective plant derivatives.

Antimicrobial screening is the first step in the search for antimicrobial drugs to determine the susceptibility of different fungi and bacteria to each agent. This assay measures the ability of each test sample to inhibit the in vitro growth of fungi and bacteria. This capacity can be estimated by one of the following three methods:

- Disc diffusion method
- Bio-autographic method and
- Serial dilution method.

1.11 Brine shrimp lethality assay of *Thladiantha cordifolia* leaves extract

Any drug at higher doses can show toxicity for the living being, that means no drug is allowed to be administrated above the therapeutic index graph. There is a saying, "pharmacology is simply the highest dose toxicology and simply lower dose pharmacology". The brine shrimp test is the convenient and fast biological test to analyze any bioactive substances of synthetic, semi synthetic and natural origin and this is easiest system for monitoring biological activities of the plant derivatives (natural product, fractions of substances as well as pure substances). The in-vivo lethality test is used for examining biological test specifies cytotoxicity and an extensive series of pharmacological or biological properties such as antimicrobials, antivirals, pesticides and antibodies, etc as well as it is inexpensive and does not require special equipment or aseptic techniques

Shrimp eggs are hatched in replicated seawater and added calculated amount of dimethyl sulfoxide (DMSO) with the sample in desired concentration which would be kept for 24 hours. The positive and negative control groups are measured also in required concentration and process. The number of dead/ living naupliis are counted at the end which gives a result.

1.12 *In-vitro* evaluation of antioxidant property of Tetraphyllum bengalense leaves extract

Free radical induced oxidative stress can be the reason of my illness and physical disorders like atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia and degenerative eye disease. Antioxidant agents are taken which can influence unnecessary oxidation reactions in our body by involving with free radicals, metal complexes and chelates. Antioxidant

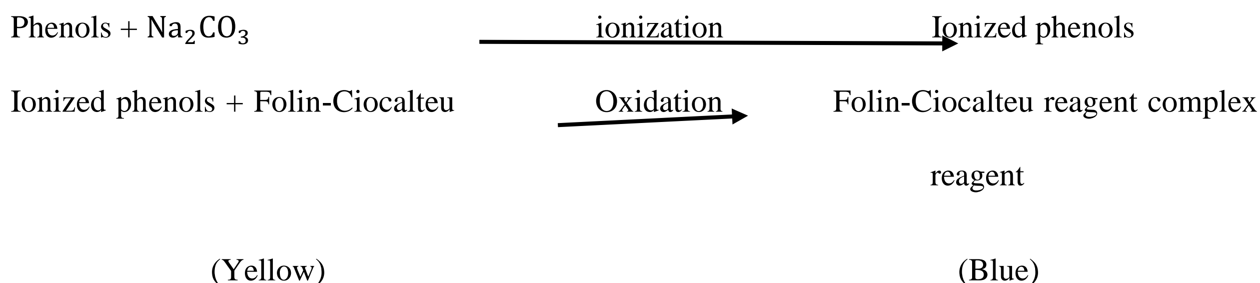
from plant origin is playing remarkable role in this conditions which is also relevant in terms of nutritional impact. In modern medicinal science it is important to examine different plants due to their effective antioxidant activity, minimum amount of side effects and also financial viability

Antioxidant property can be evaluated by:

- Determination of total phenolic content (TPC).
- Determination of total flavonoid content (TFC)
- Determination of antioxidant properties: DPPH assay.

1.12.1 Evaluation of total phenolic content (TPC)

Phenolic compounds from the plant derivatives can neutralize or absorb the free radicals, peroxides and triplet oxygen by redox reaction, thus it shows its antioxidant property. The phenols completely ionize in the stage of alkaline. Folin Ciocalteu considered as standard for these reactions which can easily oxidize the phenols when used in ionic phenolic solution. Here yellow color of Folin Ciocalteu becomes dark blue and this strength of color changes is measure in spectrophotometer with 760 nm which indicates TPC of the compound.



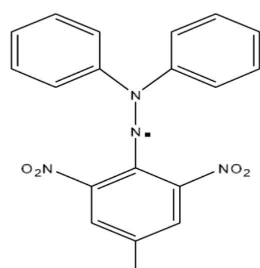
1.12.2 Evaluation of total flavonoid content (TFC)

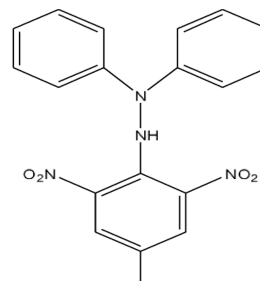
The spectrophotometric assay based on aluminium complex formation is one of the most commonly used procedure to determine the total flavonoid content of the extracts. Quercetin was used as standard where flavonoid content was determined as quercetin equivalent. A calibration curve for quercetin was drawn for this purpose. The absorbance of this reaction mixture was recorded on UV spectrophotometer with 510 nm. The same procedure was repeated with the pure methanol extracts of the plant and total flavonoid content was calculated as quercetin equivalents (mgQE/g).

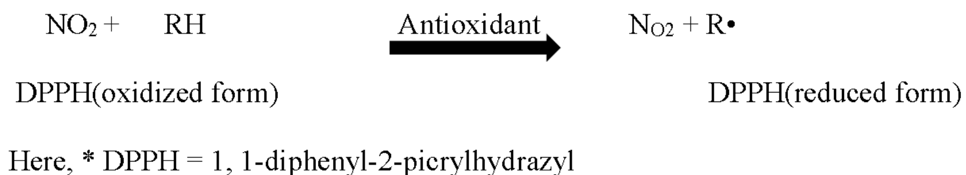
There are 2 distinguishable procedures for TFC calculation. For the 1st one solution of $AlCL_3$ in the required concentration {2-10% (m/v)}is added with the sample with methanol. In some cases, acid/ acetate solution is added. The measurements will be taken after 60 minutes. Different flavonoid can be used here like Quercetin, Rutin, Galanin etc. Besides for the 2nd procedure complexation reaction is carried out in the presence of NaNO2 in an alkaline medium. The method is based on the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked. A yellow colored solution of complex was formed, which turned immediately to red after addition of NaOH and Al(III). The value of absorbance is measured with 510nm. The standard compound is Catechin here.

1.12.3 Evaluation of antioxidant property by DPPH examine

Free radical scavenging capability of the methanol extract od the plant on stable 1,1-diphenyl-2-picrylhydrazyl is analyzed in this experiment. Required concentration is occurred by serial dilatation of the methanol solution of the plant and mixed with 3mL DPPH (2mg/10ml). Ascorbic acid as standard is also assayed here. All the measurements were taken at 517 nm by UV spectrophotometer.







Chapter 2

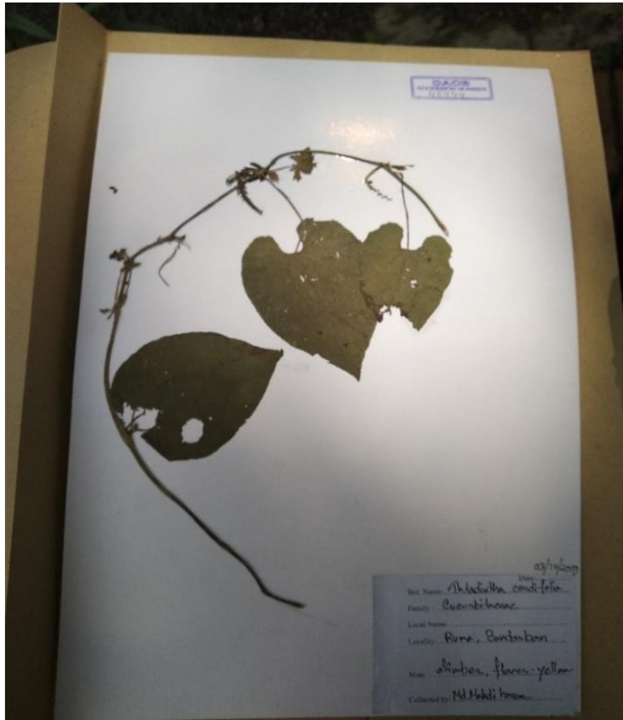
Methodology

2.1. Plant collection

Thladiantha cordifolia is a rare kind of plant and only available in Hilly areas in Bangladesh. Before starting all the project works and experiments an order was placed to the National Herbarium Bangladesh(NHB), Mirpur, Dhaka to provide sufficient amount of plant leaves and barks. They accumulated the plant here from Bandorban and submitted to me.

Figure 3: Bangladesh National Herbarium

2.2. Verification of plant



During receiving the specific plant (*Thladiantha cordifolia)* from the NHB, they provided a token number and the accession number for the given specimen that proves the specimen is identified which was authenticated by National Herbarium Bangladesh (NHB), Mirpur, Dhaka, Bangladesh.

Figure 4: *Thladiantha cordifolia* plant's accession number was collected from National Herbarium Bangladesh (NHB).

2.3 Process of extraction

Extraction of medicinal plant involved various steps.

The entire extraction procedure can be divided into two parts they are firstly, Preparation and drying of plant material, secondly, extraction by solvent (methanol) process.

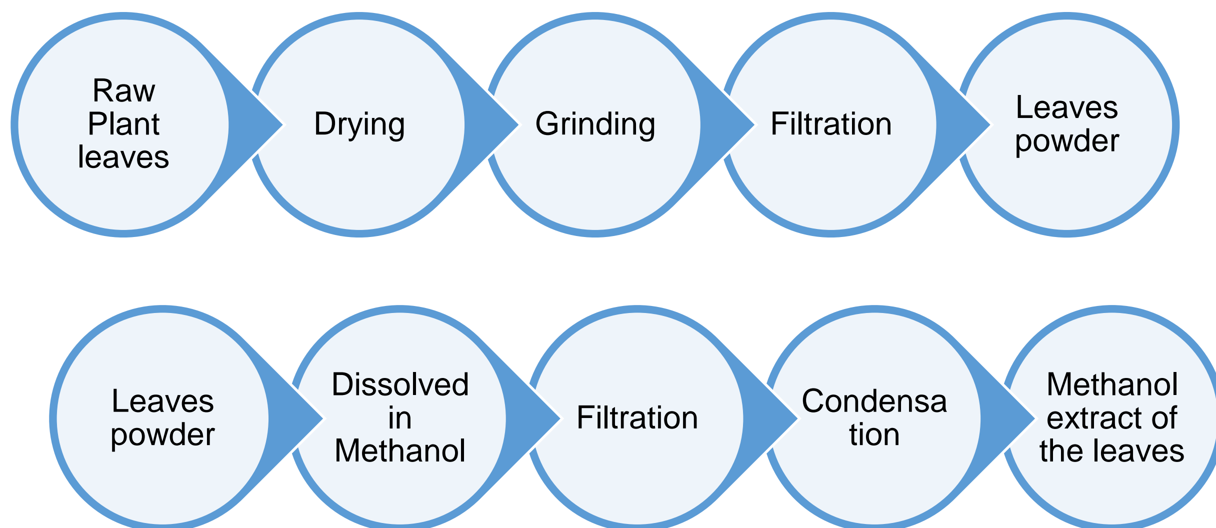


Figure 5: Flow chart of the methanol extraction process of plant materials

2.3.1 Preparation of plant material for crude extract

a) Drying

Initially the leaves of *Thladiantha cordifolia* were snatched off from the plant stem and washed with clean water to remove the dirt, plant scrap and dust particles. Then the fresh leaves were kept on big dishes to dry under sunlight in a clean space where less airflow was allowed for about 6/7 days and then again the leaves were dried for 30 minutes at 30-40°C in hot air oven. Finally, dried leaves were ready for the following experiments.

Figure 6: Raw leaves of the plant (before drying)

b) Grinding and size reduction

The dry and crusty leaves of *Thladiantha cordifolia* were then grinded by using a high capacity grinding machine. Then the grinded powder was filtered using steel mesh screen filters and more fine powder were obtained. To avoid cross-contamination essential steps were taken. Approximately, 300g of powder of leavs of *Thladiantha cordifolia* were collected and packed in an air-tight and sterile glass container which was labeled carefully. The container was finally stored in a cool, dry, and dark place.

Figure 7: Grainded leaves of *Thladiantha cordifolia* after drying

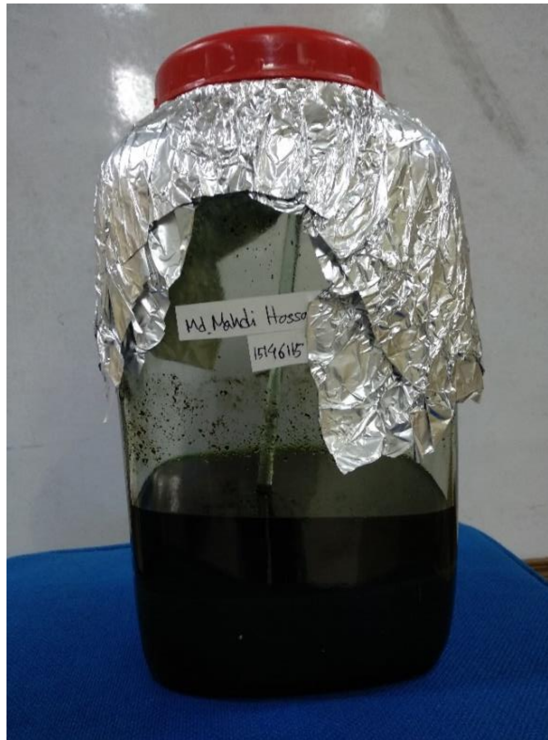


Figure 8: grinded leaves of the plant are dissolved in the methanol inside an airtight Jar

2.3.2 Extraction of plant by solvent

a) dissolution in the solvent

Based on the types of solvents used, the extraction methods can be divided into two parts:

- Extraction with aqueous solvents
- Extraction with organic solvents

Figure 9: Methanol (solvent)

For this experimental study, methanol was used as organic solvent. Beaker containing plant material (leaves powder of

(*Thladiantha cordifolia)* was soaked in 1L of methanol for a period of 7 days at normal ambient temperature (22-25°C) with occasional stirring.

b) Filtration

After seven days of soaking, the mixture in the beaker was filtered using a white thin cloth (Markin cloth) and Whatman filter (pore size: 110 mm).

c) Evaporation and Condensation

The collected deposit of plant extraction was processed through rotary evaporator machine at 100 rpm at 48°C for about 1.5 hours, to get concentrated methanol extract of the plant and recompensed methanol on the other bottle. Then thick concentrated mixture was collected in a petri-dish.

Figure 10: Rotary Evaporation of dissolved plant materials in methanol

d) Drying

Lastly, to vaporize the remaining solvent adhering with the extract the petri-dish was placed inside a Laminar AirFlow (LAF) machine. It also aids in preventing microbial growth in the petri-dish.

Figure 11: Pure methanol extract of leaves of Thladiantha cordifolia

Table 4: The weight of *Thladiantha cordifolia* leaf extract obtained as a result of complete extraction procedure.

Initial weight of petri dish (gm)	73.81
Final weight (gm) (petri-dish+ extract)	85.79
Weight of extract (gm)	11.98

2.4. *In-vitro* Evaluation of Thrombolytic activity

Thrombolytic property analysis is a very easy in-vitro experiment. Though it has to be done very carefully and in aseptic way because blood need to be drawn from a person. Heparin (anti-platelet agent) is used as positive standard and water is used as negative standard.

a. Materials and Reagents

Sl. No.	Name of the Materials
1.	Blood
2.	Distilled water
3.	Heparin
4.	Micro centrifuge tube
5.	Plant extract

b. Test sample preparation

To prepare the test sample at first 10ml distilled water was added with 200mg of plant methanol extract. The mixture was kept overnight in a dry and dark place. On the next day the solution was filtered properly.

c. Standard solution preparation

75mg of Heparin as a standard anti-coagulant agent was dissolved in 10ml of distilled water and mixed properly. The suspension was stored as stock standard solution from which required concentration was made by dilution and used for the thrombolytic test.

d. Blood sample preparation

I collected the blood sample with the help of a nurse under an aseptic condition. A healthy volunteer has to be selected who has no history of bleeding or coagulation problem. 4ml of blood was collected into pre-weighed micro-centrifuge tube (Eppendorf). After that tubes were kept in an incubator to form clots for about 45 minutes at 25-30 °C.

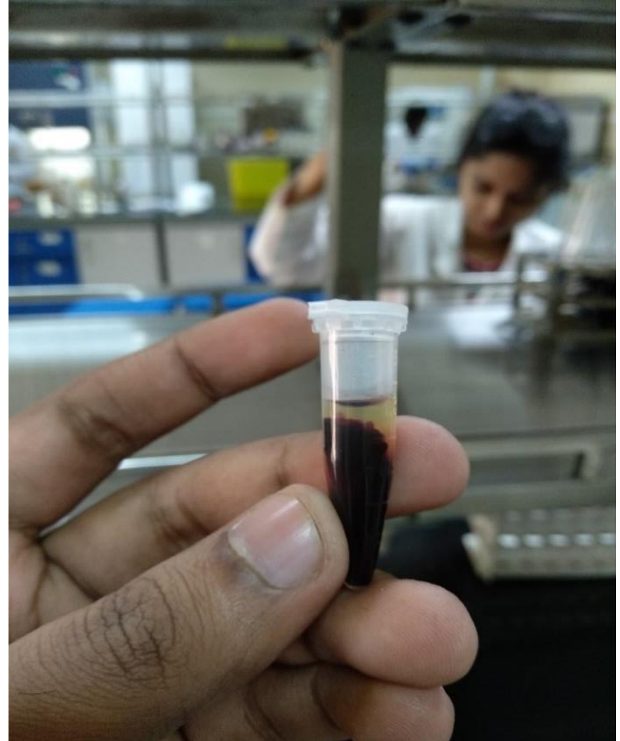


Figure 12: Blood sample collected before Thrombolytic test

e. Thrombolytic property test process

At first after blood collection, blood was transfer from syringe to 3 micro-centrifuge tubes. Then the tubes were allowed to incubate at 37°C for about 45minutes. The serum was completely and carefully ejected from all the tubes. The weight of the clot was determined by excluding the weight of pre-weighed micro centrifuge tubes. 1 ml of heparin which was used as thrombolytic positive control and 1ml of distilled water were used as a negative control. Besides the test sample (*Thladiantha cordifolia* leaves methanol extraction) was also added 1 ml in one micro centrifuge tube. Then immideiatly all three tubes were placed for incubation at 37°C for about 90minutes. After the incubation, the liquid part from the

clot (blood part due to lysis) was removed very carefully and the weight was measured to find the difference.

Finally, percentage (%) of clot lysis was determined by the following equation:

Percentage (%) of clot lysis = (weight of released clot /clot weight) $\times$ 100

2.5 Evaluation of the antimicrobial property of the plant

Here the evaluation has done by only microbial screening test. Methanol extract of *Thladiantha cordifolia* leaves were used as test sample which was applied on 2 gram-positive (+) and 2 gram-negative (-) bacteria. It is three consecutive days' experiment.

a. Materials and Reagents

Sl. No.	Name of the Materials
1.	Nutrient agar medium
2.	Petri dishes
3.	Micropipette
4.	Filter paper discs
5.	Screw cap test tubes
6.	Sterile forceps
7.	M.H agar
8.	Spirit burner
9.	Autoclave
10.	Refrigerator

11.	Incubator
12.	Laminar air flow hood
13.	Nose mask and hand gloves

b. Micro-organisms used in this test

Both gram positive and gram negative bacteria were collected from pure culture for this test.

Table 5: List of the microorganisms used

Bacteria (+ve)	**Bacteria (-ve)**
Bacillus subtilis	*Escherichia coli*
Staphylococcus aureus	*salmonella typhi*

c. Sterilization procedure of test

Sterilization is the most significant procedure in any microbial test. So, all the apparatus which includes beakers, conical flasks, petri-dishes, cottons, forceps and others were sterilized very well before starting the test and also kept in an aseptic condition provided by Laminar Air Flow (LAF) hood. UV light was turned on for 1 hour in the Laminar Air Flow Hood before the experiment. Petri-dishes and other apparatus were sterilized by using

the autoclave machine where the temperature was set at 12 ºC with 15-lbs/sq. pressure for about 1 hour. Micropipette tips, cotton, forceps, blank discs were sterilized properly by UV light. All the equipment was sterilized again to be 100% sure.

d. Procedure of antimicrobial test

At the beginning, 21.4g of nutrient agar powder was dissolved in 50ml distilled water and the culture was prepared. 10ml of broth mixture was taken in each 4 conical flasks for each bacteria. Then bacterial strains were added very carefully kept in shaking incubator for 24 hours at 37 ºC. As the bacterial culture was left for 24 hours it increased above the required concentration, which was confirmed from their absorption in the UV spectrometry at 600nm. In this regard I added required amount of broth solution to dilute all the bacteria concentrations. In the next step, agar medium was prepared using 1.3g of agar and 100ml of distilled water which was placed into petri-dishes and kept for cool down to room temperature. The bacterial strains were then spread into the petri dishes when M.H. agar in the petri dishes became solid. On the other hand, plant extracts of different concentration ranging from 20mg/10ml to 2mg/10ml were prepared. Small filter paper discs were used to soak the extract solution of different solutions. Then the test samples along with 2 standard antibiotics of known concentration were placed into petri-dishes. Then again all the petri-dishes were incubated for 24 hours at 37 ºC which is considered as convenient environment for bacterial growth. On the next day, the inhibition zoned of all samples and antibiotics were measured.

e. Special Precaution

As we sterilized all the required equipment and machines before the test it is also necessary to sterile all the accessories after the test. Microorganism can grow in many other different media as well which cannot be allowed. So, all the glass and steel materials gone through the autoclave process and other disposals were thrown to airtight and isolated containers.

f. Determination of inhibition zone for test

Antimicrobial potency was evaluated by observing the zone of inhibition that is the capability to stop the microbial growth around the discs in the medium of the petri-dishes. Only the clear region of inhibition indicates the antimicrobial property. By calculating the diameter of the inhibition area with a clear scale the strength can be determined.

2.6 Evaluation cytotoxic property

In this experiment, we used Brine Shrimp Lethality assay to find out cytotoxicity of the plant sample. The toxic effects of the sample and positive and negative control are observed and evaluated where Vincristine sulfate is used as positive control and sea water is used as negative control.

a. Materials and Reagents

Sl. No.	Name of the Materials
1.	Brine shrimp (*Artemia salina)* egg
2.	Test tubes
3.	Small tank
4.	NaCl
5.	Pipette, Micropipette
6.	Vincristine sulfate
7.	Plant extract
8.	Glass vials
9.	Dimethyl sulfoxide (DMSO)
10.	Lamp to attract shrimps

b. Preparation of sea water

38g of salt (pure NaCl) and 1 liter distilled water were added to prepare a sea water solution and filtered to get a clear solution.

c. Hatching of Brine shrimp eggs

Brine shrimp (Artemia salina) eggs were collected from the lab and added into the tank filled with seawater along continuous oxygen supply and heat. Required oxygen and heat is crucial for egg hatching. Pasteur pipette was used to collect 10±2 living shrimps from the container and added to each of the test tube containing 5mL of seawater.

d. Preparation of test solution for experiment

At first, the test sample was diluted to get required concentrations ranging from 20mg/10ml to 2mg/10ml and 5ml of dimethyl sulfoxide (DMSO) were added to each test tube. 10±2 nauplii were dispersed into each test tube.

e. Preparation of control group for experiment

To justify the analysis process and results from the experiment control groups are very important which are equivalent to the performance of the test sample. Generally, there are 2 types of control groups- positive control and negative control.

1) Preparation of positive control

Positive control group is a standard compound with known concentration and results are certain for a specific experiment which helps to compare with the result of test sample. Here, Vincristine sulfate which is a cytotoxic compound widely used in this test as standard. At first step vincristine sulfate was mixed with 1 ml DMSO to obtain 1st dose and then the concentration diluted as required ranging from 20mg/10ml-2mg/10ml. Finally, 5ml seawater along with 10±2 nauplii was added with the solution.

2) Preparation of negative control

Negative control group is also a standard compound which will not show any effect to the specific experiment to observe if the death of the subject is for other unwanted reason or not. Here, water is negative control. 1 ml of DMSO was added to the test tube containing 5ml of sea water along with 10±2 nauplii.

f. Nauplii counting

After 24 hours the number of nauplii that survived was counted very carefully and accurately using a magnifying glass. According to each dilution, the percentage of mortality was calculated and a linear regression graph was made. From this graph that is the relationship between the concentration and mortality rate by the sample was used to calculate lethal concentration (LC_{50}). And all the data was compared with the negative and positive control groups.

2.7 In-vitro antioxidant property analysis

2.7.1 Evaluation of free radical scavenging DPPH assay

By using DPPH test we can evaluate the destroying power on the free radicals of the plant extract.

Materials and Reagents

Sl. No.	Name of the Materials
1.	Test tubes
2.	Volumetric flask
3.	Light-proof box
4.	Pipette (1ml and 5 ml)
5.	UV-spectrophotometer
6.	DPPH (2,2-Diphenyl-1-Picrylhydrazyl)
7.	Methanol

8.	L-ascorbic acid
9.	Extract of the experimental plant
10.	Distilled water

a. Control preparation for evaluation

L-ascorbic acid is widely considered as the standard (positive control). Here, L- ascorbic acid was dissolved in methanol to prepare several required concentration ranging from 12mg/10ml to 2mg/10ml.

b. Test sample preparation for evaluation

In this case, 24 mg plant exact was mixed with 20 ml methanol to get 12mg/10ml concentration and then by serial dilution of that stock solution we got required more concentrations of test sample.

c. DPPH solution preparation for evaluation

2mg of DPPH was dissolved in 50ml of distilled water to get 0.004%(2/v) DPPH solution which was covered by aluminium foil paper and kept in a dark and cool place.

d. Procedure of DPPH free radical scavenging activity test

1 ml of both sample and standard (L-ascorbic acid) were taken in two different test tubes and 2ml of 0.004% DPPH solution was added to the tubes. Then both the test tube was incubated for 30minutes at 25 ℃. Finally, the absorbance was taken at 517nm for sample and standard against the blank (methanol).

2.7.2. Evaluation of total phenolic content (TPC)

By following the Folin-Ciocalteu method the TPC of the plant was analyzed and Gallic acid was used as the standard.

a. Materials and Reagents

Sl. No.	Name of the Materials
1.	Test tubes
2.	Volumetric flask
3.	vials
4.	Pipette (1ml and 5 ml)
5.	UV-spectrophotometer
6.	Micropipette
7.	Methanol
8.	Folin-Ciocalteu reagent
9.	Sodium carbonate
10.	Gallic acid

b. Test sample preparation for evaluation

The stock solution was made by dissolving 120mg of the plant extract with 100ml methanol which gives the concentration of 12mg/10ml. Then the stock solution was diluted as required that is 12,8,4,2 mg/10ml.

c. Standard preparation

Similarly, the preparation of the test samples with different concentration, Gallic acid as standard was also prepared. we have four serially diluted concentrations 12,8,4,2 mg/10ml.

d. Preparation of the blank

Here the blank solution only contains FCR, Sodium and methanol added as required to make the volume up to 10ml.

e. Procedure of Total phenolic content (TPC) test

1 ml of both sample and standard (gallic acid) were taken in two different test tubes and 2.5ml Sodium carbonate solution and Folin-Ciocalteu reagent (FCR) was added to each test tube. The solution was vortexed for 15 seconds and then it was kept 30 minutes in a water bath at 40ºC. Finally, at 765 nm the absorbance of sample, standard was taken using a spectrophotometer against the blank.

2.7.3. Evaluation of total flavonoid content (TFC)

By following the Flavonoid-Aluminum chloride ($AlCl3$) complexation method the TFC of the plant was analyzed and Gallic acid was used as the standard.

a. Materials and Reagents

Sl. No.	Name of the Materials
1.	Test tubes
2.	Volumetric flask
3.	Micropipette
4.	Pipette (1ml and 5 ml)
5.	UV-spectrophotometer
6.	Potassium accetate
7.	Methanol
8.	Quercetin
9.	Aluminium chloride

b. Reagents preparation

Aluminium chloride solution: 100ml of 10% aluminium chloride solution was made by dissolving 10g of aluminium chloride in 100ml distilled water.

Potassium acetate solution: 100ml of 1M potassium acetate solution was prepared by dissolving 9.8g of potassium acetate in 100ml distilled water.

c. Test sample preparation for evaluation

200mg of *Thladiantha cordifolia* leaves extract was mixed with 100ml of methanol in a test tube to have 20mg/10ml concentration. Then required concentrations were prepared by dilution process- 20, 12, 8, 6, 2 mg/10ml**.**

d. Standard preparation

Quercetin was used as standard here. This was prepared in the similarly as the stock solution and diluted in five concentrations, 20- 2 mg/10ml.

e. Preparation of the blank

The blank contained 2ml of 10% aluminium chloride solution, 2ml of 1M potassium acetate, 10ml methanol and 10 ml distilled water.

f. Procedure of Total flavonoid content (TFC) test

1ml of each 5 concentrations were taken from sample and standard (Quercetin) in each test tubes. Adding 3ml methanol to each test tubes. Then 2ml of 10% aluminium chloride and 2 ml of 1M potassium acetate were added to the mixture. Finally, to make volume of 10ml,

4 ml distilled water was added to each tubes. The test tubes were incubated at room temperature for 30minutes. Lastly the absorbance of each concentration from the sample and standard solutions were taken against blank using spectrophotometer at 415 nm. As Quercetin equivalents

the total flavonoid content of each concentration were expressed using this equation:

C= (c x V)/m

where,

C= Total content of flavonoid compounds, mg of quercetin per gram plant extract expressed as quercetin equivalent (QE).

c= concentration of quercetin obtained from calibration curve (mg/10ml).

V= volume of the sample solution(ml).

m= weight of the sample(g).

Chapter 3

Observations and results of all the experiment

3.1 Analysis of Thrombolytic property

Completing all the experiment procedure correctly I collected several data and calculated required results. The whole experiment was done for 3 times and took an average % of clot lysis.

Table 6: Calculation and result of Thrombolytic activity

NO.	Weight of the empty tube (A) gm	Weight of the tube with clot (B) gm	Weight of clot (C) C=B-A	Weight of tube with clot after lysis (D) gm	Weight of lysis E (B-D)	% of clot lysis (E/C)x100
1.	0.82	1.45	0.63	1.24	0.21	33.33
2.	0.83	1.46	0.63	1.23	0.23	36.51
3.	0.83	1.45	0.62	1.25	0.2	32.25

From this test it can be said that, the leaves of Tetraphyllum bengalense have shown less effect on clot lysis, on average 34.03 %.

3.2 Analysis of Anti-microbial property

After completing all the procedure successfully and carefully we got the results to evaluate anti-microbial activity of methanol extract of *Thladiantha cordifolia* leaves. Concentrations ranging from 20mg/10ml to 2mg/10ml were applied on the bacterial growth. But no significant result was found. As we can see on the following figures no inhibition zone is noticeable that means this leaves extract has no effect of bacterial growth. On the other hand, the antibiotics used here as standard that is streptomycin and amoxicillin showed inhibition zone very clearly on bacterial strains. This means there is minimum chance of experimental error

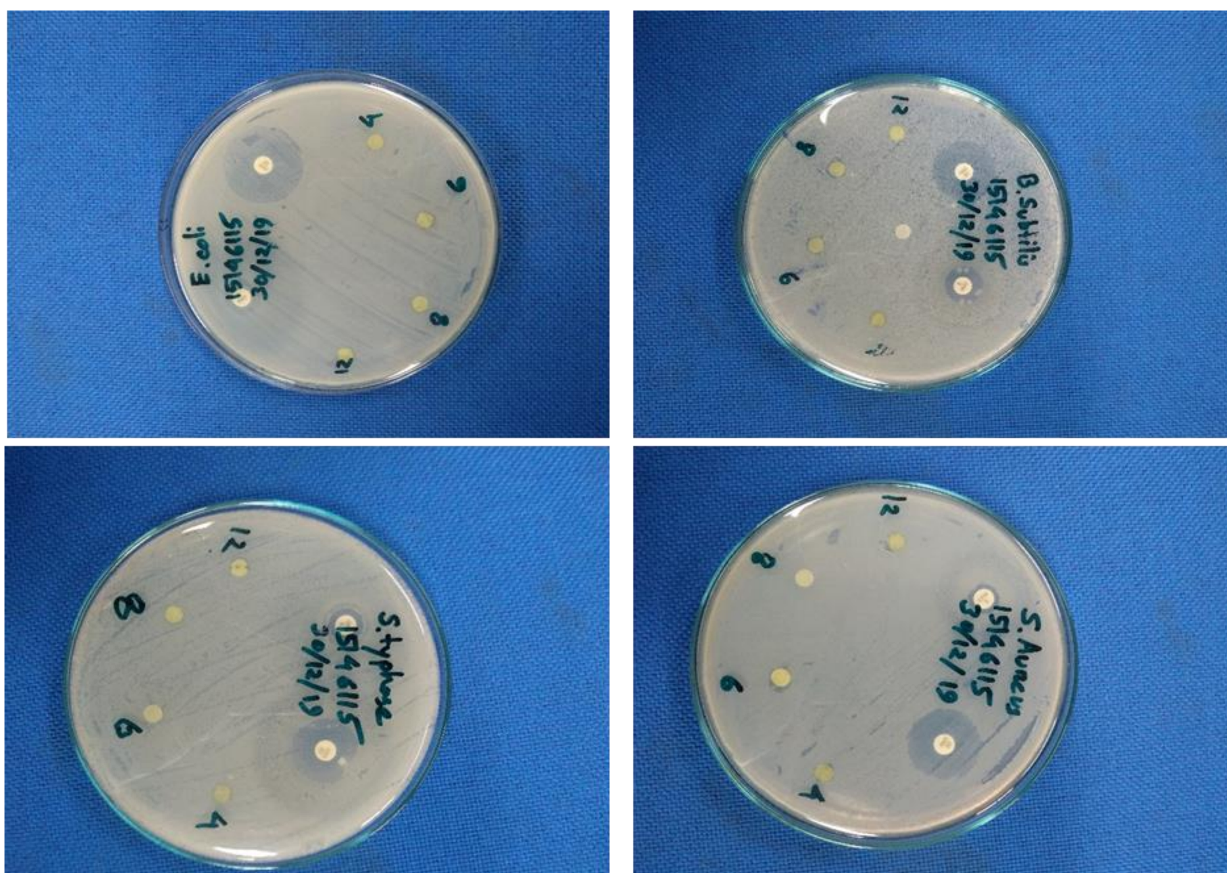


Figure 13: Petri-dishs showing the microbial growth against antibiotics and plant samples

3.3 Analysis of In-vitro Cytotoxic property

After completion of all the procedure required data was collected and analyzed to find out the lethal concentration (LC_{50}) and a graph was made.

Table 7: Calculation and result of cytotoxic activity of the standard

Concentration mg/10 ml	Log_{10}	Number of Naupli taken	Number of dead Naupli	Naupli alive	Mortality %	LC_{50} mg/10ml
20	1.301	15	15	0	100	
12	1.0792	15	13	2	86.6667	
8	0.9031	15	12	3	80	2.199
6	0.7782	15	12	3	80	
4	0.6021	15	10	5	66.6667	
2	0.301	15	9	6	60	

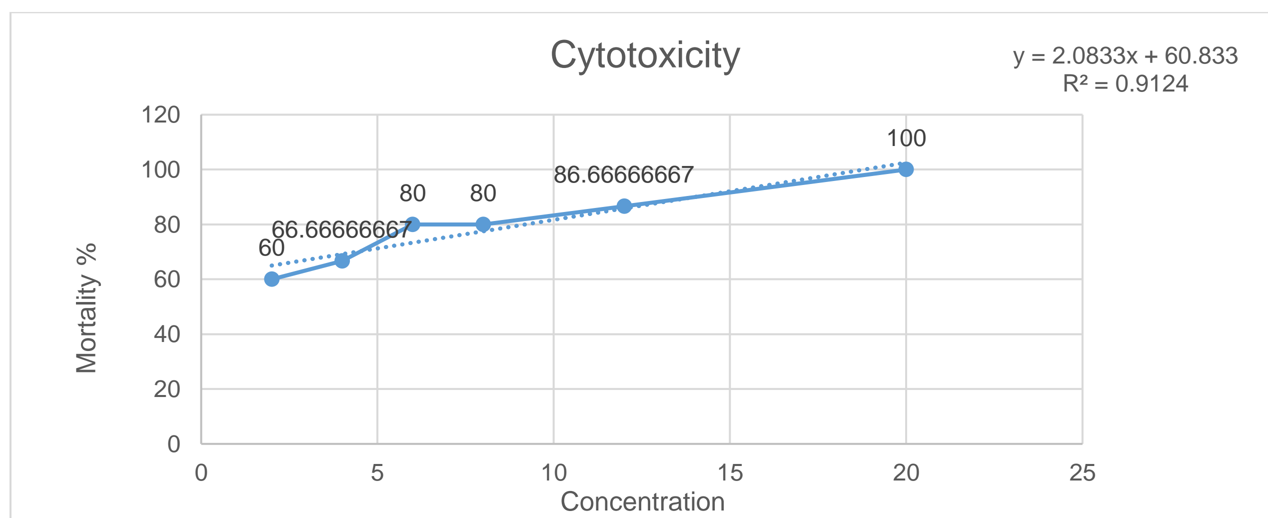


Figure 14: Graph of cytotoxic potency of the standard

Table 8: Calculation and result of cytotoxic activity of the sample

Concentration mg/10 ml	Log10	Number of Naupli taken	Number of dead Naupli	Naupli alive	Mortality %	LC50 mg/10ml
20	1.301	15	11	4	73.333	
12	1.079	15	8	7	53.333	
8	0.903	15	8	7	53.333	10.96
6	0.778	15	7	8	46.667	
4	0.602	15	4	11	26.667	
2	0.301	15	0	15	0	

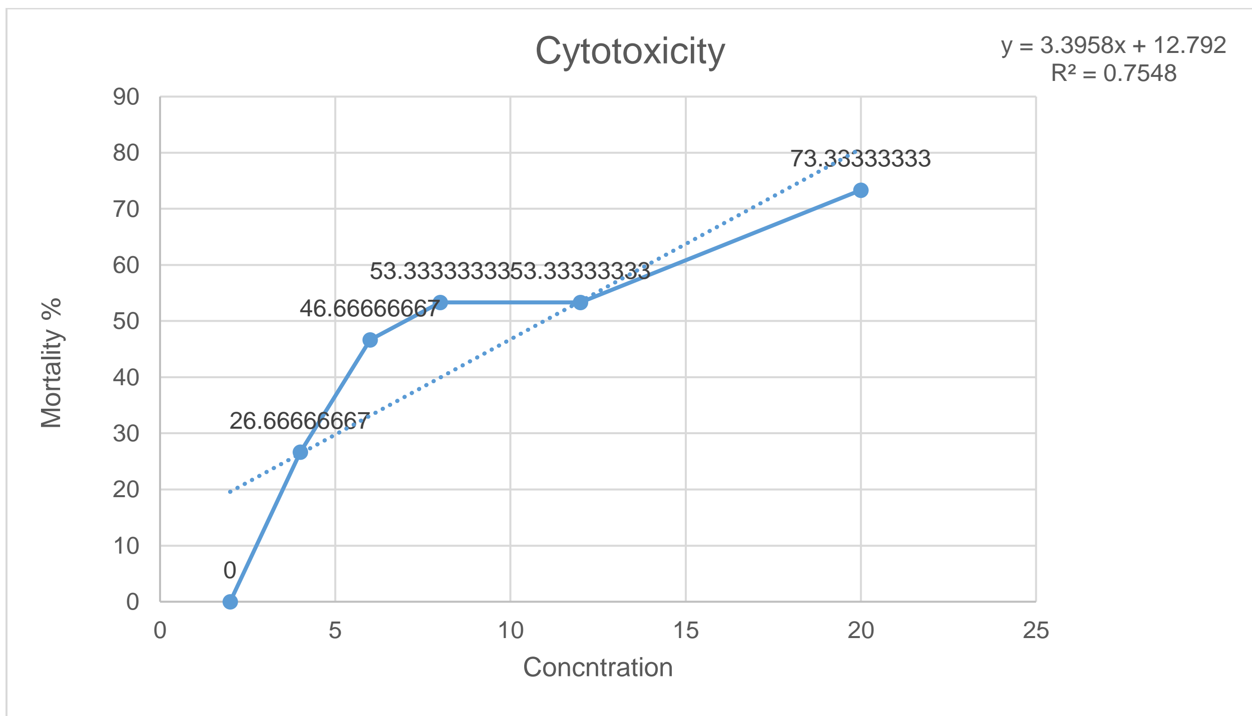


Figure 15: Graph of cytotoxic potency of the sample

From the above graphs and tables, it is evident that this plant sample shows a significant result on cytotoxicity. Although according to the standard it has higher value for LC_{50} but it can be used in further research or scientific experiments.

3.4. Antioxidant property analysis

To find out antioxidant property we did 3 different experiment and all of them showed antioxidant property after all the acquired data were analyzed. However, they did not show expected results against the standard.

3.4.1. Results for DPPH free radical scavenging assay of *Thladiantha cordifolia*

Table 9: Calculation and result of DPPH test of the standard

Absorbance of the blank	**Concentration (mg/10ml)**	**Absorbance of ascorbic acid**	**Inhibition %**	**IC50 (mg/10ml)**
	5	0.0049	98.49	
	4	0.0075	97.86	
0.35	3	0.0101	94.54	17.99
	2	0.0288	91.78	
	1	0.0459	86.89	

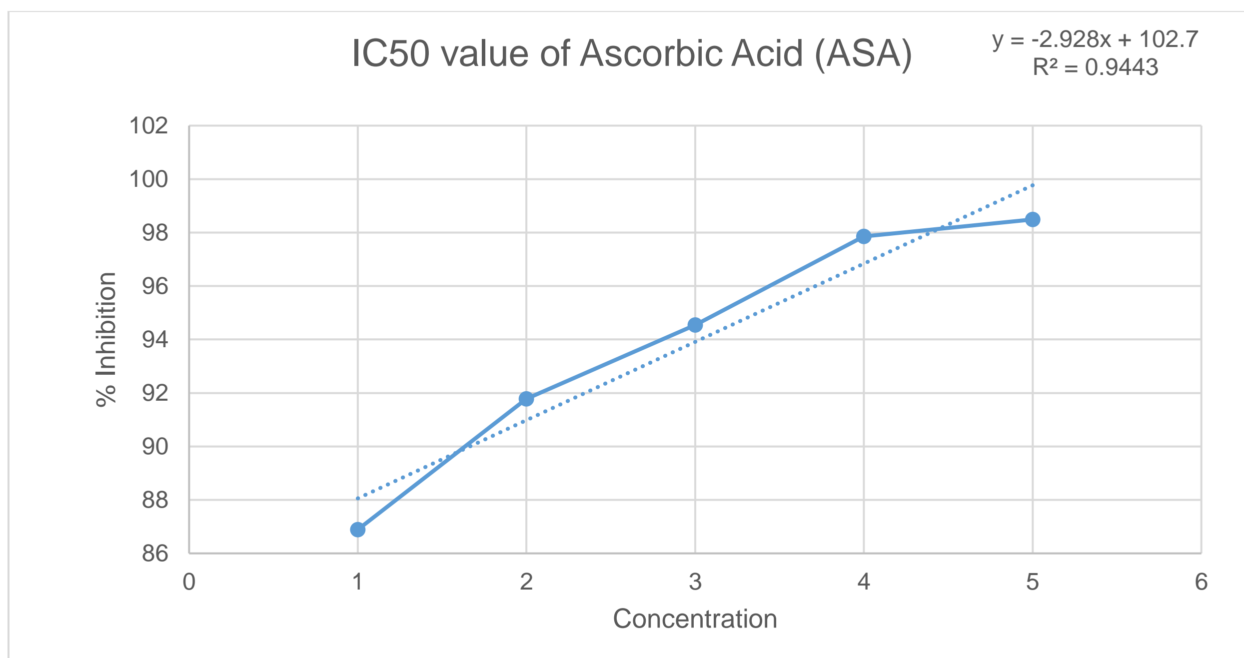


Figure 16: Graph of IC_{50} potency of the sample

Table 10: Calculation and result of DDPH test of the sample

Absorbance of the blank	Concentration (mg/10ml)	Absorbance of ascorbic acid	Inhibition %	IC50 (mg/10ml)
	5	0.058	83.43	
	4	0.088	74.86	
0.35	3	0.104	70.29	165.40
	2	0.127	63.72	
	1	0.152	56.69	

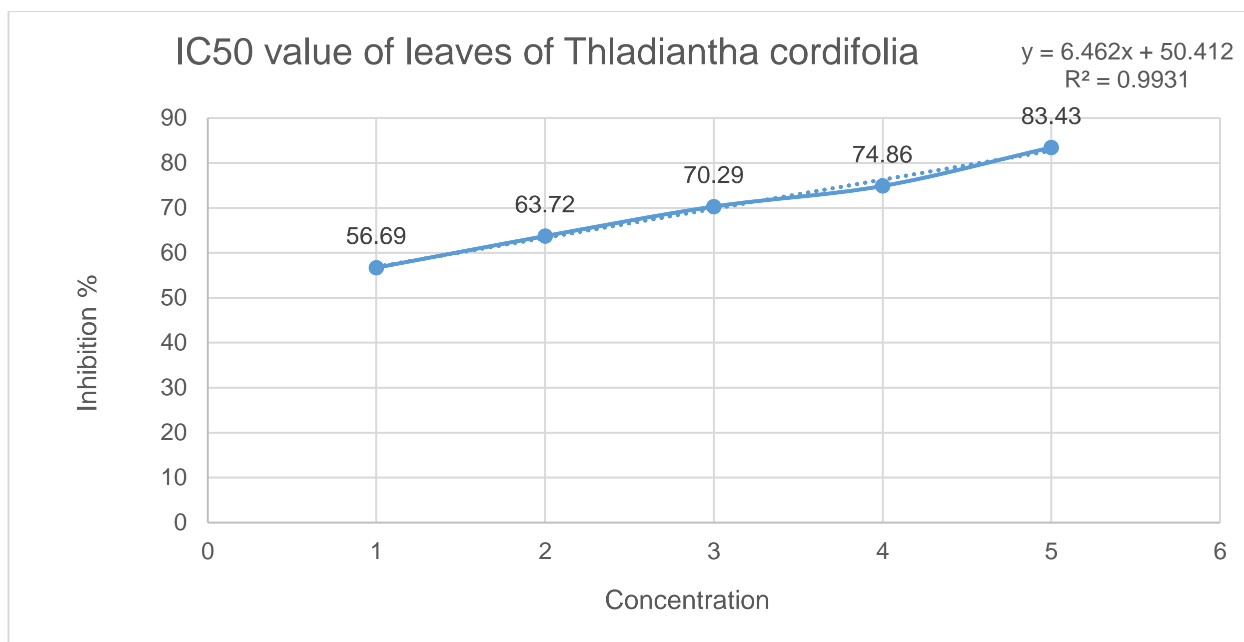


Figure 17: Graph of IC_{50} potency of the sample

3.4.2. Results for Total Phenolic Content (TPC) *Thladiantha cordifolia*

Table 11: Acquired absorbance for different concentration

Concentration mg/10 ml	**Absorbance**	**Regression line**	**R^2**
20	1.7	y = 0.0624x + 0.441	0.9956
12	1.2		
8	0.9		
6	0.8		
2	0.6		

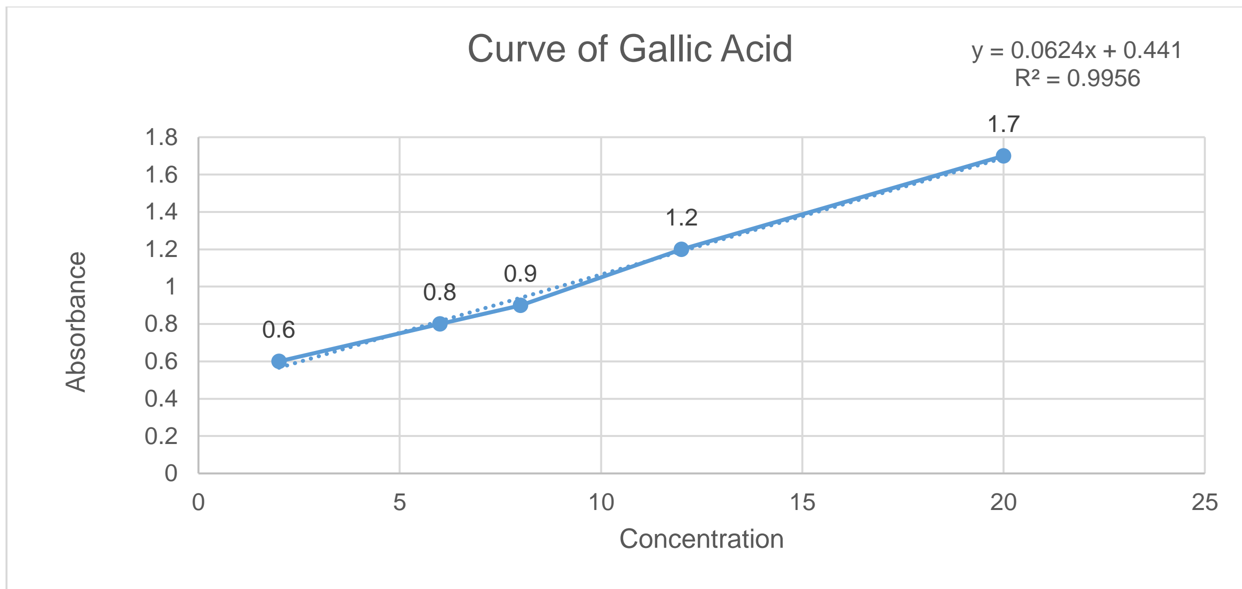


Figure 18: Graph of absorbance of Gallic acid

Table 12: Calculation and result of TPC test

Sample solution mg/10ml	**Weight of the dry extract/ml (gram)**	**Absorbance**	**GAE concentration mg/10ml**	**TPC as GAE $A = \frac{CV}{m}$ mg/g**
2	0.002	0.562	1.94	9.7
2	0.002	0.56	1.91	9.5
2	0.002	0.565	1.95	9.7

Therefore, the total phenolic content was obtained average 9.6 (mg of GAE/gm of extract) of the methanol extract of *Thladiantha cordifolia* leaves. When the concentration of methanol crude extract of *Thladiantha cordifolia* increased total phenolic content also increased.

3.4.3. Results for Total Flavonoid Content (TFC) *Thladiantha cordifolia*

Table 13: Acquired absorbance for different concentration

Concentration mg/10ml	**Absorbance (420nm)**	**Regression line**	**R^2**
20	1.9	y = 0.0224x + 1.4467	0.9788
12	1.7		
10	1.7		
8	1.6		
2	1.5		

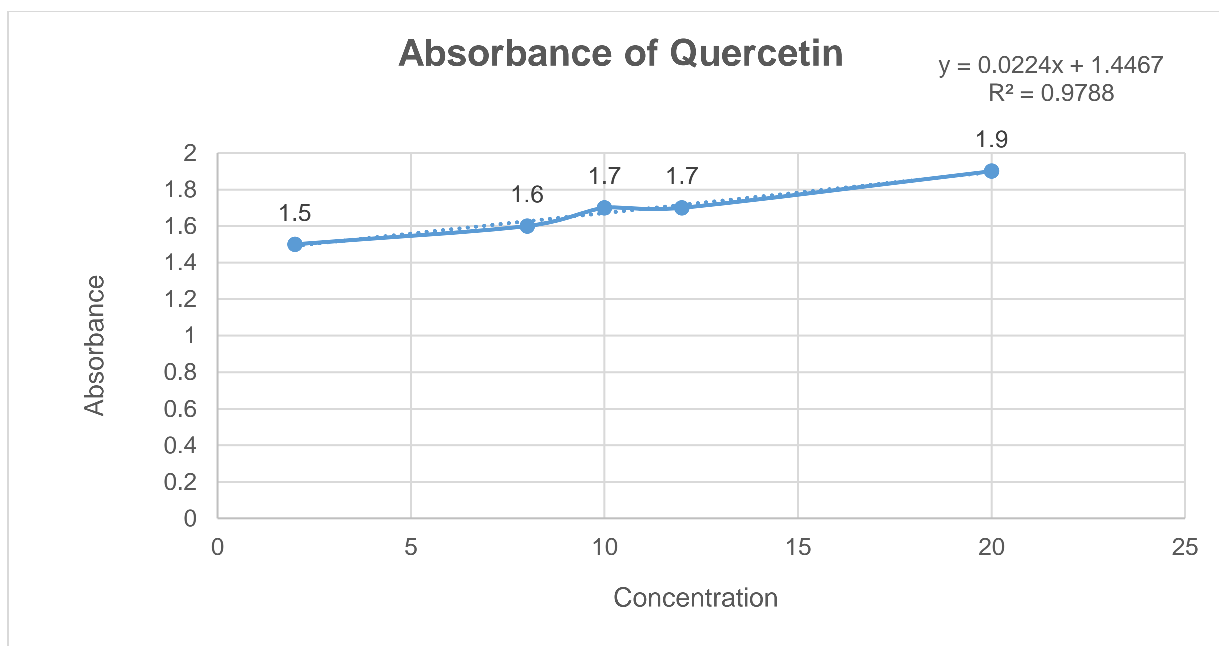


Figure 19: Graph of absorbance of Quercetin

Table 14: Calculation and result of TFC test

Sample solution mg/10ml	**Weight of the dry extract/ml (gram)**	**Absorbance**	**QE concentration mg/10ml**	**TFC as QE,** $\mathbf{A = \frac{CV}{m}}$ **mg/g**
2	0.002	1.98	2.38	11.9
2	0.002	1.96	2.29	11.5
2	0.002	1.99	2.24	12.1

Therefore, the total flavonoid content was obtained average 11.5 (mg of GAE/gm of extract) of the methanol extract of *Thladiantha cordifolia* leaves. Flavonoid content is proportional with the concentration of methanol extract of the plant.

Chapter 4

Discussion

After completing all the experiments perfectly and maintaining all the precautions, we found some particular information about the test sample that is methanol extract of *Thladiantha cordifolia* leaves.

Thrombolytic activity of *Thladiantha cordifolia* leaves showed 34.03% clot lysis capability whereas, heparin as positive control showed 71.21% and negative control shoed 10.58%. According to these data it can be proclaimed that the sample did not show thrombolytic effects up to the mark. Though leaves of *Thladiantha cordifolia* showed very mild effect but further investigation on other parts of the plant might show adequate results.

Similarly, the methanol extract of *Thladiantha cordifolia* leaves did not show any perticular result against microbial growth. Conspicuously, there was very minor chance of experimental error. In fact, no sign of inhibition zone was found. So, it can be declared that the plant sample the does not possess any antimicrobial property.

We found LC_{50} value of the methanol extract of *Thladiantha cordifolia* leaves to determine its cytotoxicity. In this case, it showed a remarkable result against positive control vincristine sulfate that is LC_{50} value of the plant sample was 10.96 and vincristine sulfate had 2.19. It is necessary to

test this extract in low concentration to determine its potency. So further experiments are required to clarify whether *Thladiantha cordifolia* possess cytotoxic activity at lower concentration. And as a cytotoxic agent it can be used in anti-cancer drug production as well.

We followed three experimental procedures to find out antioxidant capability of *Thladiantha cordifolia* leaves, those are DPPH assay total flavonoid content (TFC) and total phenolic content (TPC). For DPPH test we found out IC_{50} values for the sample and standard. Ascorbic acid was used as standard which gave 17.99 IC_{50} value. On the other hand, IC50 value of Thladiantha cordifolia leaves was 165.4. In comparison, the sample showed very less potency in free radical scavenging capacity. In the 2nd experiment, it is evident that TPC increases if the sample amount increases. As Gallic Acid Equivalence (GAE) it showed average 9.6 mg/g. Similarly, in total flavanoid test (TFC) it was noticeable flavonoid content is proportional to the concentration of methanol extract of the plant. As Quercetin Equivalence(QE) it showed average 11.5 mg/g. However, it can be said that dried extract of *Thladiantha cordifolia* did not show acceptable amount of antioxidant potentiality.

Chapter 5

Conclusion

Through all these experiments the methanol extract of *Thladiantha cordifolia* leaves has been studied to evaluate four particular biological properties. From the results and evaluation, it is confirmed that the plant possesses few biological properties in very low amount which is not acceptable in new medicine development. Although the cytotoxic property was remarkable but antimicrobial activity is completely negligible. However, the plant might have other medicinal properties like anti-diabetic, anti-cancer, cardiovascular and others. Additional experiments on other extractions like aqueous, petroleum and so on can be analyzed in near future. Not only the leaves of the plant but also roots, barks and flowers can be evaluated through many other experiments. Further investigation is required which might give more effective and useful results for the health care sector.

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