EVALUATION OF CHEMICAL AND BIOLOGICAL PROPERTIES OF *Tinospora cordifolia* (THUNB.) MIERS STEM EXTRACT FROM KAVREPALANCHOWK DISTRICT OF NEPAL

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By

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DECLARATION

The dissertation entitled **"EVALUATION OF CHEMICAL AND BIOLOGICAL PROPERTIES OF *Tinospora cordifolia* (THUNB.) MIERS STEM EXTRACT FROM KAVREPALANCHOWK DISTRICT OF NEPAL"**, which is being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of Master degree of Science in Chemistry, is carried out by Suresh Bista under the supervision of Assoc. Prof. Dr. Bhushan Shakya, Department of Chemistry, Amrit Campus, Kathmandu and Co-supervision of Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha, Department of Chemistry, Amrit Campus, Kathmandu. I hereby, declare that this work is originally done by me and has not been previously formed anywhere else for other degree. Any literature, data or work done by others or cited in this dissertation has been given due acknowledgement and listed in reference section.

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LETTER OF RECOMMENDATION

This is recommended that the dissertation work entitled "**EVALUATION OF CHEMICAL AND BIOLOGICAL PROPERTIES OF *Tinospora cordifolia* (THUNB.) MIERS STEM EXTRACT FROM KAVREPALANCHOWK DISTRICT OF NEPAL**" has been carried out by **Mr. Suresh Bista** as a partial fulfilment for the Master Degree in Chemistry. To the best of my knowledge, this work has not been previously performed for any other degree. He has fulfilled all the requirement laid down by Institute of Science and Technology (IoST), Tribhuvan University, Kirtipur for the submission of dissertation work for the award of Master Degree in Chemistry.

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DEDICATION TO MY PARENTS

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ABSTRACT

The Menispermaceae family includes *Tinospora cordifolia*, also known as "Gurjo" in Nepali, has various medicinal applications. The current study examined phytochemical screening, total phenolic content and total flavonoid content. Extracts of *T. cordifolia* were also analyzed for antibacterial, antioxidant, antidiabetic and cytotoxicity activities. Extracts were also subjected to Thin Layer Chromatography and Fourier Transform Infra-red Spectroscopy. The air dried powder of *T. cordifolia* were extracted using four different solvents: hexane, chloroform, acetone and methanol. The methanol extract outperformed the other three, yielding 10.07 g, whereas hexane extract produced only 0.50 g. Phytochemical screening discovered the presence of alkaloids, polyphenols, flavonoids, tannins, terpenoids, saponins, resins, steroids and proteins. The quantitative analysis of phytochemicals revealed that the total phenolic content and total flavonoid content of the hexane extract is higher as comparison to the other extracts. Antibacterial susceptibility tests were tested against *Bacillus subtilis and Escherichia coli*. Among the four, three extracts showed better antimicrobial properties. The antioxidant activity was assessed by DPPH free radical scavenging method. The 3,5-dinitro salicylic acid technique revealed a range of α-amylase inhibition properties. Among four extracts, methanol extract was found to have the lowest IC_{50} value 0.06 mg/mL in the DPPH assay whereas acetone extract was found to have the lowest IC_{50} value 0.50 mg/mL in the α-amylase inhibition assay. Similarly, the lowest LC_{50} value of acetone extract as 62.50 µg/mL show its higher cytotoxic potential. The best TLC was shown by all four extracts in three solvent systems. The presence of O-H, C-H, C=O, $-CH_2$, $-NO_2$, C-O, N-H, C=C etc. were verified by FT-IR findings.

Keywords: *Tinospora cordifolia*, extraction, phytochemicals, biological activities.

शोधसार

Menispermaceae परिवार अन्तर्गत पर्ने *Tinospora cordifolia* औषधीय बिरुवा, जसलाई नेपालीमा "गुर्जो" पनि भनिन्छ। यसका विभिन्न औषधीय प्रयोगहरू हुन्छन्। हालको अध्ययनबाट फाइटोकेमिकल अन्वेषण, कुल फेनोलिक सामग्री र कुल फ्लेभोनोइड सामग्रीको जाँच गरियो। *T. cordifolia* एक्स्ट्र्याक्टहरुको एन्टिब्याक्टेरियल, एन्टिअक्सिडेन्ट, एन्टिडायबेटिक र साइटोटोक्सिसिटी गतिविधिहरूको पनि विश्लेषण गरियो। एक्स्ट्र्याक्टहरुको पातलो तह क्रोमेटोग्राफी र फोरियर ट्रान्सफर्म इन्फ्रा–रेड स्पेक्ट्रोस्कोपीबाट पनि विश्लेषण गरियो। *T. cordifolia* को हावा सुख्खा पाउडरलाई हेक्सेन, क्लोरोफर्म, एसीटोन र मेथेनोल चार फरक विलायकहरू प्रयोग गरेर निकालिएको थियो। मिथेनोल एक्स्ट्र्याक्टले अन्य तीन भन्दा 10.07 ग्रामको उच्चतम एक्स्ट्र्याक्ट उत्पादन गर्यो जबकि हेक्सेन एक्स्ट्र्याक्टले 0.50 ग्राम मात्र उत्पादन गर्यो। फाइटोकेमिकल अन्वेषणले अल्कालोइड, पोलिफेनोल, फ्लाभोनोइड, ट्यानिन, टेरपेनोइड, सेपोनिन, रेसिन, स्टेरोइड र प्रोटिनको उपस्थिति पत्ता लगायो। फाइटोकेमिकलको मात्रात्मक विश्लेषणले हेक्सन निकासीको कुल फिनोलिक सामग्री र कुल फ्लाभोनोइड सामग्रीअन्यको तुलनामा उच्च रहेको देखियो। ब्यासिलस सब्टिलिस र एस्चेरिचिया कोलाई विरुद्ध जीवाणुरोधी संवेदनशीलता परीक्षण गरिएको थियो। चार मध्ये तीनवटा एक्स्ट्र्याक्टले राम्रो जीवाणुरोधी गुणहरू देखाए। एन्टिअक्सिडेन्ट गतिविधि DPPH फ्री रेडिकल स्क्याभेन्जिङ विधिद्वारा मूल्याङ्कन गरिएको थियो। 3,5-dinitro salicylic एसिड प्रविधिले α-amylase निषेध गुणहरूको दायरा प्रकट गर्यो। चारवटा एक्स्ट्र्याक्टमध्ये, DPPH परखमा मिथानोल एक्स्ट्र्याक्टको सबैभन्दा कम IC_{50} मान 0.06 mg/mL भएको पाइयो जबकि एसीटोन एक्स्ट्र्याक्टमा α– amylase निषेध परख सबैभन्दा कम IC_{50} मान 0.50 mg/mL भएको पाइयो। त्यस्तै, एसीटोन एक्स्ट्र्याक्टको सबैभन्दा कमी LC_{50} मान 62.50µg/mL ले यसको उच्च साइटोटोक्सिक क्षमता देखाउँछ। सबै भन्दा राम्रो TLC तीन विलायक प्रणालीमा सबै चार एक्स्ट्र्याक्रटले देखाएको थियो। O-H, C-H, C=O, -CH_2, -NO_2, C-O, N-H, C=C इत्यादिको उपस्थिति FT–IR द्वारा प्रमाणित गरिएको थियो।

Keywords: *Tinospora cordifolia*, extraction, phytochemicals, biological activities.

LIST OF ACRONYMS AND ABBREVIATIONS

AOA	:	Antioxidant Activity
AST	:	Antimicrobial Susceptibility Test
DPPH	:	2, 2- Diphenyl-1-picrylhydrazyl
DMSO	:	Dimethyl sulfoxide
FCR	:	Folin-Ciocalteu Reagent
FTIR	:	Fourier-transform infrared spectroscopy
GAE	:	Gallic Acid Equivalent
IC_{50}	:	Inhibitory Concentration 50% Inhibition
LC_{50}	:	Lethal Concentration for 50% Mortality
MHA	:	Muller Hinton Agar
MIC	:	Minimum Inhibitory Concentration
MTT	:	Methyl Tetrazolium Bromide Test
NaEDTA	:	Disodium Ethylene Diamine Tetraacetate
QE	:	Quercetin Equivalent
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
TLC	:	Thin Layer Chromatography
UV	:	Ultraviolet
ZOI	:	Zone of Inhibition

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CHAPTER I: INTRODUCTION

1.1 Background

Nepal is surrounded by mountains and the Himalayan axis is approximately 800 kilometers long and 150–250 kilometers wide. The nation's total land area is roughly 147,516 square kilometers. On three sides, it borders India; on the other side, it borders China. As a border dividing Nepal from Bangladesh, the narrow Siliguri Corridor in West Bengal is limited in width (Nepal Tourism Board, 2018). Nepal is located between latitudes 26º 20ˈ-30º 10ˈ North and longitudes 80º 15ˈ-88º 19ˈEast. The country's unique geographic location combined with changes in altitude and climate, is the cause of the country's rich biodiversity (Bogs *et al*., 2006).

Located in the heart of the Himalayas, Nepal makes up around one-third (800 km) of the full Himalayan range. It is home to eight of the highest peaks in the whole universe including the well-known Mount Everest (8,848m). Beyond the mountains, Nepal is home to flat terrain, river valleys and deep gorges that combine to provide a unique variety of habitats and a notable amount of biodiversity in a comparatively small region. The nation's 1,47,516 km^2 constitute less than 0.1% of the world's land mass but despite this, it is home to an exceptionally diverse range of flora and fauna. More than 2% of the world's flowering plants, 3% of pteridophytes and 6% of bryophytes are found (Paudel *et al*., 2011).

Nepal ranks ninth in Asia for floral richness, home to almost 9,000 different types of blooming plants. Of these, 6,653 species of flowering plants have been identified; catalogues have reported 1,792 to 2,331 excellent medicinal and aromatic plants among them. The biodiversity of the nation is a good economic resource (Parajuli *et al*., 2015).

Since ancient times, people have been searching for natural resources to prepare different medicines. Various medicinal herbs have been utilized to treat a variety of illnesses throughout human history. The customary understanding of these plants has been transmitted across successive generations and is currently employed in contemporary plant research (Šantić *et al.*, 2017).

Natural product is a chemical compound produced by a living organism (Dahanukar *et al*., 2000). Natural products and traditional therapies have greater significance in the treatment of diseases (Yuan *et al*., 2016). Plant-derived substances known as

phytochemicals are produced by plant cells and serve purposes other than the cellular necessities. All parts of the plant organism depend on these compounds for their survival and general functioning (Johnson, 2013). Phytochemicals are a broad category of bioactive substances derived from plants. These substances show potential in treating various medical conditions (Zia *et al*., 2024).

Natural products are the basic sources of bioactive molecules and they used to aid in the creation of lead compounds for the treatment of various diseases (Saraf & Saraf, 2014). Biosynthesis, extraction, identification, quantification, structural elucidation and physical and chemical properties are all part of the natural product chemistry. They are generated by the primary metabolism or secondary metabolism. These natural products are frequently utilized as starting points for drug discovery in chemical synthesis (Chintoju *et al.*, 2015).

Plants create a wide range of organic chemicals which can be divided into two categories: primary metabolites, which are vital for basic life processes like growth and reproduction, and secondary metabolites, which have a variety of activities. Secondary metabolites, also known as secondary products or natural products, have specific purposes. Although they have different distributions and structures from primary metabolites, they have similar metabolic routes. Since the 19th century, secondary metabolites have been known for their importance in defensive mechanisms including phytoalexins, allelopathy, UV protection, and other applications in medicine (Bhatla & Lal, 2023).

Alkaloids are the important class of structurally diverse chemicals that have a nitrogen atom inside their heterocyclic ring. These substances are derived from amino acids. Alkaloids had a significant impact on human history due to their pharmacological qualities which include antibacterial and anticancer actions as well as their varied physiological impacts on animals. Their significance is further increased by their possible usage as stimulants, poisons and narcotics. Approximately 12,000 alkaloids from different plant genera have been found and isolated so far (Kaur & Arora, 2015).

Thin Layer Chromatography is a simple technique of separating the components in a mixture. The low cost, quick development, sensitivity and reliable repeatability make it a versatile and straightforward method in organic chemistry. This technique involves the applying of silica gel (SiO_2) on glass plates. After applying the sample in solution on the plate, it is submerged in a closed container containing the filter paper and

developing solvent. When the solvent almost reaches the top of the plate, it is removed, dried and visualized under a UV lamp. The different colored spot on the plate differentiates the compound one from other (Santiago & Strobel, 2013).

Fourier Transform Infrared Spectroscopy (FTIR) is a quick and non-destructive analytical technique which can reveal information on the different functional groups that are present in various chemical compounds as well as provides the detail about their conformation and molecular structure (Mohamed *et al*., 2017).

1.2 Introduction of the Plant *T. cordifolia*

T. cordifolia is a large extensively spreading glabrous, perennial deciduous twine with succulent stems and papery bark which is widely found in India, Myanmar, Nepal, Bangladesh, China and Sri Lanka. It is native to the tropical region of India ascending to an altitude of 1200 metres in the temperature range of 25 to 45 ºC. The leaves are simple, heart shaped and dark bright green in colour. Further, it is alternate, estipulate, entire and the lamina is broadly ovate 10-12 cm long and 8-15 cm broad (Tiwari *et al*., 2018).

T. cordifolia (Thunb.) Miers is a climbing plant with a woody structure featuring distinctive lenticels on its stems. It has widely been utilized in Ayurvedic medicine and it is commonly known as Gaduchi or Amrit (Bhakshu *et al.,* 2024).

T. cordifolia is a rapidly rising, broad-leaved, deciduous shrub with several winding branches that grow to a height of about 15 m by 0.3 m at a fast rate. It has succulent stems with long aerial roots that resemble threads. The plant's stem has a grayish-brown to black hue, a bitter feel, a soft and dry wooden texture and a cylindrical shape with a circumference ranging from 5 mm to 25 mm (Modi *et al.*, 2020).

When the stem is young, it has a green succulent bark covered in a thin brown bark studded with warty lenticels. When the stem dries, the stem shrinks and the bark separates from the wood. It is a membranous 7-9 nerved, 5-10 cm, roundish, cordate or heart shaped leaves with a 2.5-7.0 cm petiole (thus the name *cordifolia*). *T. cordifolia* has a calorie content of 292.54 calories per 100 grams including K, Cr, Fe and Ca, all of which are significant in numerous regulatory functions (Choudhary *et al.*, 2013).

1.2.1 Taxonomic Classification of *T. cordifolia*

Kingdom : Plantae
Sub-kingdom : Tracheophyta
Super- division : Spermatophyta
Division : Magnoliophyta
Class : Magnoliopsia
Sub-class : Polypeptalae
Series : Thalamiflorae
Order : Ranunculales
Family : Menispermaceae
Tribe : Tinosporeace
Genus : *Tinospora*
Species : *cordifolia*
Botanical name : *Tinospora cordifolia* (Bharathi *et al*., 2018)

Figure 1: a) T.cordifolia plant ***b) Stem of T.cordifolia***

1.2.2 Traditional and medicinal use

Tinospora cordifolia (Thunb.) Miers, also known as Guduchi/Gurjo, is a natural herbal shrub which is being used to treat a variety of ailments including jaundice, skin problems, gout, diabetes and others, according to traditional medicine (Tiwari *et al*., 2018).

Aromatic or medicinal plants are widely used due to high nutrient and medicinal values. The consumption of these plants is increasing day by day due to their antioxidant and

medicinal importance. These products are working as alternatives of synthetic antioxidants or chemicals (Kumar et al., 2018).

T. cordifolia is a well-known in classical Ayurvedic writings for its extensive use in treating a variety of medical diseases (Shrestha & Lamichhane, 2021).

T. cordifolia has its special use in folk and tribal medicine in different regions of the nation since time immemorial. It has been shown to possess anti-allergic, anti-diabetic, anti-hepatotoxic and anti-pyretic properties (Murshed *et al*., 2021).

1.2.3 Chemical Constituents of *T. Cordifolia*

T. cordifolia is a versatile medicinal plant that is the sole source of several chemicals with a variety of chemical structures (Reddy & Reddy, 2015) .

Numerous ailments such as arthritis, ulcerative colitis, asthma, allergies, parasite infections, cancer and infectious diseases are linked to immune system dysfunction. 1-hydroxymustakone, N-methyl-2-pyrrolidone, N-formylannonain, cordifolioside, magnoflorine, tinocordiside and syringin have immunomodulatory characteristics and may be involved in the plant's ability to treat a variety of illnesses (Sharma *et al*., 2012).

Plants have been recognized as a crucial component of traditional medicine owing to their remarkable phytoconstituents. It stands out as a plant with significant pharmacological functions and medicinal benefits attributed to its diverse constituents like terpenes, glycosides, alkaloids, steroids and flavonoids (Dhama *et al.*, 2016).

Alkaloids, terpenoids, lignans, steroids, and other chemical substances have been reported to be present in the plant, and these chemicals contribute to the phytochemical composition and pharmacological actions of *T. cordifolia* (Sharma *et al*., 2019).

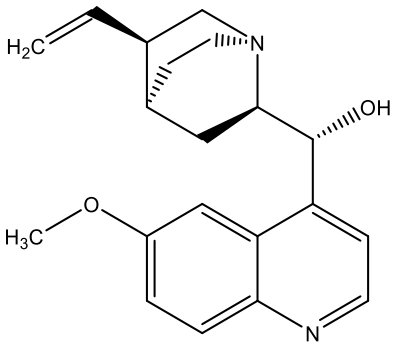
T. cordifolia stem extract contains four bioactive chemicals that have been identified as syringin, cordifolioside, magnoflorine, and tinocordiside (Bala *et al.*, 2015).

T. Cordifolia produces a variety of secondary metabolites including alkaloids such as tinosporine, magnoflorine, and berberine; terpenoids like tinosporide, furanolactone diterpene, cordifolioside; phenolics such as lignans, flavonoids, and phenylpropanoids; polysaccharides like glucose, xylose, and rhamnose; steroids including giloinsterol and β-sitosterol; essential oils; and aliphatic compounds. Additionally, it contains compounds like giloin, tinosporidine, sinapic acid, tinosporone, tinosporic acid among

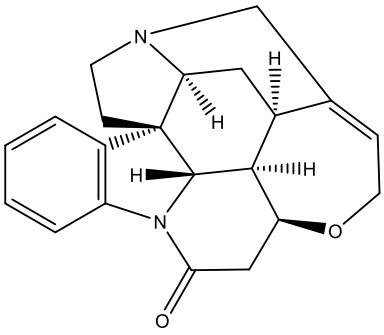
others. These phytochemicals contribute to a diverse array of medicinal properties in *T. Cordifolia*, including antioxidant, immunomodulatory, antimicrobial, antiviral, anticancer, antipyretic, anti-inflammatory, hepatoprotective, neuroprotective, anti-osteoporotic, antitoxic, antidiabetic, anti-arthritis, anti-ulcer and more. Notably, the terpenoid (tembetarine) is attributed to the anti-diabetic characteristics of *cordifolia* (Chandramohan *et al.*, 2024).

Some major compounds found in the *T. cordifolia* are shown below.

Alkaloids

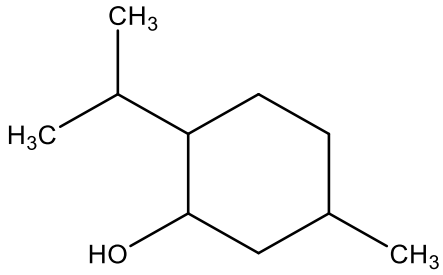


Quinine



Strychnine

Terpenoids



Menthol

1.2 Extraction Process

Extraction is a method of separating organic constituent from the mixture of solution. Extraction methods play a distinct role in the field of analytical chemistry and it is notable that many laboratories continue to rely on traditional extraction procedures that have been in use for decades. But new modern extraction techniques also have been developed (Raynie, 2006).

In order to evaluate the bioactivity of secondary metabolites, use natural products for medical purposes or isolate a specific combination of ingredients, natural product extraction is essential (Stéphane *et al.*, 2021). Some standard extraction techniques are:

a) Maceration: In this process, the whole or roughly powdered raw medicinal material is placed in a sealed container with the solvent and let to sit at room temperature for at least three days. During this time, the container is agitated often to guarantee that the soluble components dissolve. After that, the mixture is filtered, the remaining wet solid material is crushed and the liquids that are left behind are either filtered or decanted after being given time to settle (Singh, 2008).

b) Percolation: This is the technique that is typically used to extract the active ingredients from tinctures and fluid extracts. One tool used is a percolator which is usually a thin, cone-shaped jar with apertures on both ends. The solid components are moistened with an adequate quantity of the prescribed menstruum and left in a tightly sealed container for roughly 4 hours. The mass is then compressed, and the percolator's upper section is sealed. To form a thin layer atop the mass, more menstruum is added, and the closed percolator is left to macerate for a full day. After that, the percolator outlet is opened, letting the liquid flow gradually. Menstruum is added as needed until the percolate reaches approximately 75% of the volume needed for the finished product (Singh, 2008)**.**

c) Ultrasonic Extraction (Sonication): The technique makes use of ultrasonic frequencies between 20 and 2000 kHz in order to increase cell wall permeability and cause cavitation. The procedure's linked disadvantage is the infrequently harmful effects of ultrasonic energy (above 20 kHz) on the active ingredients in medicinal plants. This effect results from the production of free radicals, which causes the drug molecules to change in an unfavorable way (Singh, 2008).

1.3 Phytochemicals

The chemical constituents derived from plant species are regarded as phytochemicals. These are generated by the plant either through primary or secondary metabolism. These phytochemicals contributes great in plant growth or defense against diseases (Molyneux *et al*., 2007). Various medicinal and herbal plants contains the significant phytochemicals such as alkaloids, flavonoids, saponins, terpenoids, vitamin-C, phenolic compounds, carbohydrates, steroids and others. These ingredients in plants can be detected by using phytochemical screening tests (Yadav & Agarwala, 2011).

1.4 Separation of Compounds

Mixtures in solution form can be separated by using a variety of techniques, including simple distillation, chromatography, sublimation, separating funnels and filtration.

Chromatography is a general term for a group of scientific methods used to separate mixtures into their constituent parts. Thin-layer chromatography is an adsorption chromatography by the use of a solid –liquid interface. Every type of chromatography operates on the same principle. It involves a liquid or gas as a mobile phase, and a stationary phase that can be either solid or liquid. The mixture that needs to be separated is dissolved in the liquid mobile phase, which then moves it through a device that holds a different material called the stationary phase. The mixture's components are carried by the mobile phase as it moves through the stationary phase, and their different rates of movement help to separate the components. This distinction is based on the differential interaction between the stationary and mobile phases (Ramallo *et al.*, 2015).

When a sample contains colorless molecules, visible colored reactive products such as ninhydrin or black-light imaging, can be produced using fluorescence, radioactivity, or a specific chemical reagent. These molecules' locations on the chromatogram can be determined with the aid of this procedure (Bhusal, 2023).

1.5 Biological Study of the Compound

Many different types of plants create secondary metabolites, which enhance the intrinsic characteristics of the plant. Modern drug development techniques prioritize natural ingredients which also adhere to accepted medical norms.

1.5.1 Anti-bacterial Activity

A substance known as anti-bacteria eliminates or stops the growings of bacteria. Due to the presence of some phyto-constituents in plants such as alkaloids, flavonoids, steroids, phenol, tannins, carbohydrate and saponin, they exhibit anti-bacterial activity (Salama *et al.*, 2020). Antibacterial prevents the bacteria so can be useful in therapeutic treatment. So, their determination provides the information for developing new source for drugs (Anyamaobi *et al*., 2020).

Physicians have few choices when it comes to treating infections caused by newly resistant strains, as the number of people suffering from these diseases is increasing and treatment fails. In the last twenty years, the number of novel medications that have been

brought to the market has been relatively small in comparison to the increasing number of bacterial populations both gram-positive and gram-negative exhibiting resistance. Those few drugs which possess the capacity to eliminate the growth of bacteria are called antibacterial substance or antibiotics (Kagithoju *et al*., 2012).

1.5.2 Anti-oxidant Activity

Free radical reactions are known to have a significant impact on physiology and are implicated in many acute and chronic illnesses in humans such as diabetes, atherosclerosis, aging, immunosuppression, and neuro-degeneration. These free radicals are stabilized or neutralized by antioxidants usually prior to their damaging effects on biological cells. Natural antioxidants are very important in stopping the negative consequences of oxidative stress. The application of medicinal plants that have high antioxidant content has been proposed as a potential treatment approach for various diseases (Saeed *et al*., 2012).

1.5.2.1 Principle of DPPH Assay

Assessing the antioxidant capacity of a substance, extract or other biological origins starts with the α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method. This is a simple method that involves mixing the material or extract being studied with a DPPH solution then measuring the absorbance after a specific duration of time.

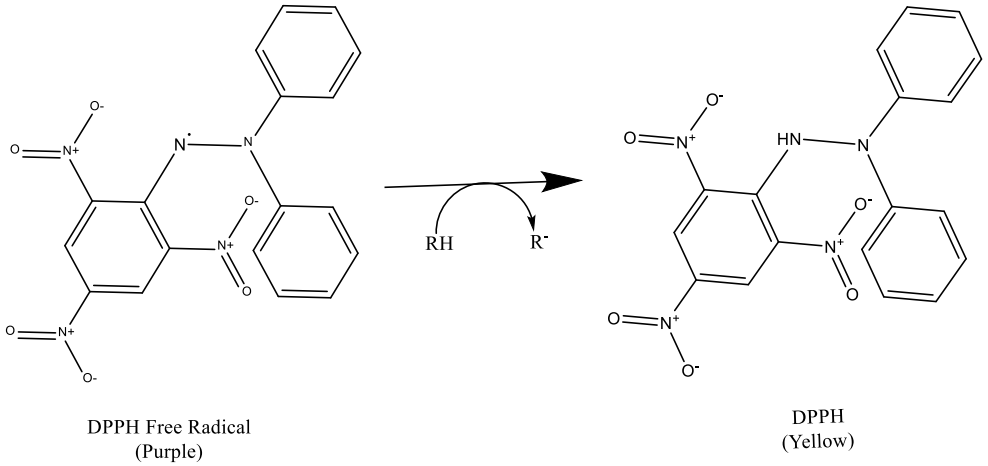


Figure 3: Mechanism of DPPH radical scavenging

The DPPH assay is based on evaluating antioxidants' capacity to scavenge free radicals. In this mechanism, antioxidants give a hydrogen atom to neutralize the unpaired

electron in the nitrogen atom of DPPH resulting in the creation of the equivalent hydrazine. Because

of the electrons' complete delocalization throughout the molecule, which inhibits the dimerization that is frequently seen in other free radicals, DPPH stands out as a stable free radical. Along with adding a deep violet hue, this delocalization produces an absorption peak in ethanol solution at about 520 nm. DPPH reduces when combined with an atom of hydrogen-supplying material; this causes the chemical to lose its violet color and change into pale color (Kedare & Singh, 2011).

A free-radical scavenger antioxidant reacts to DPPH to form DPPHH, which has a lower absorbance than DPPH because of the lower amount of hydrogen. In comparison to the DPPH-H state, this radical version causes decolorization (a yellow hue) as the number of electrons collected increases (Baliyan *et al.,* 2022).

1.5.3 Anti-diabetic Activity

Diabetes is a metabolic disease with a variety of origins that is a major global public health concern. It is typified by a disturbance of glucose homeostasis, which results in deficits in insulin secretion and/or insulin activity, which cause disturbances in the metabolism of proteins, fats, and carbohydrates. Inhibiting the activity of α-amylase enzyme, glucose level can be reduced. According to the International Diabetes Federation (IDF), high blood sugar is the third most important risk factor for dying young. Eighty to eighty-five percent of populations rely on medicinal plants which have been a major source of therapeutic supplies for people all over the world. To meet their basic healthcare needs, people frequently use extracts or active ingredients from medicinal plants as traditional medicine. Medicinal plants offer a wealth of active ingredients that can be used directly as medications or as lead compounds and pharmacological agents. (Tafesse *et al*., 2017).

1.5.4 Polyphenols and Flavonoids

The identification of plant phenolics and flavonoids is crucial due to their strong biological effects. An aromatic ring connected to one or more hydroxyl groups are the phenolic compounds and these compounds exhibit wide range of biological characteristics, including antimutagenic, carcinogenic, and gene-expression-modifying effects. Flavonoids, on the other hand, are the most abundant class of naturally occurring phenolic compounds and can be found in both free and glycoside forms in

different plant parts. Due to their polyphenolic composition, they can efficiently scavenge dangerous free radicals such as superoxide and hydroxyl radicals. When compared to monophenols, polyphenolic compounds have greater antioxidant effectiveness (Sulaiman & Balachandran, 2012).

1.5.5 Brine Shrimp Lethality Assay

One popular method for determining a substance's cytotoxicity is the Brine Shrimp Lethality Assay. This bioassay is distinguished by its short turnaround time (24 hours), simplicity (aseptic methods not required), cost-effectiveness and low test material requirement (2–20 mg or less). This in vivo lethality test has shown value since its introduction as a preliminary screening method that enables further confirmation through more complex and specialized bioassays after active chemicals have been identified (Apu *et al.*, 2010).

1.6 OBJECTIVE

1.6.1 General Objectives

- The general objective of this research work is to evaluate the medicinal properties of the stem of *T. cordifolia.*

1.6.2 Specific Objectives

- Ultrasonic extraction using various solvents like hexane, chloroform, acetone and methanol.
- Perform phytochemical analysis, TPC and TFC analysis of the extracts.
- Inspect the extracts through TLC and FTIR analysis.
- Study bioactivity of the extracts like antibacterial, antioxidant, antidiabetic and cytotoxicity.

CHAPTER II: LITERATURE REVIEW AND RESEARCH GAP

The entire plant, including the roots, stems and leaves are used extensively in Ayurvedic medicine. It is used in Ayurvedic medicine to treat a variety of conditions such as high cholesterol, diabetes, gout, cancer, gastrointestinal disorders, rhinitis, arthritis, hepatitis, ulcers, fever and STDs. Due to the plant's many medicinal qualities which include anti-inflammatory, anti-spasmodic, anti-stress, anti-allergic, antimalarial, anti-leprotic, hepatoprotective, immune-modulatory and antineoplastic effects researchers from all over the world have given it a great deal of attention. Different sections of the plant including the roots, stems and entire body, have been shown to contain a variety of active chemicals including steroids, glycosides, aliphatics, diterpenoid lactones and alkaloids (Banerjee *et al*., 2021).

The pharmacological activities of *Tinospora cordifilia* was because of its chemical constituents like diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, essential oils, a mixture of fatty acids and polysaccharides and is present in a different part of the plant body including root, stem and whole part (Sharma et al., 2019).

As a promising herb for medicinal purposes, *T. cordifolia* has long been used in ethnobotany as a natural supplement to treat a variety of ailments, most notably brain fever. Prior studies have demonstrated the several benefits of it including its neuroprotective and hepatoprotective qualities (Ali & Datusalia, 2024).

The stem of *T. cordifolia* was frequently used in traditional Indian folk medicine to treat diabetes by regulating blood glucose levels. According to research, it worked against diabetes through a number of methods such as lowering oxidative stress increasing insulin secretion and blocking the processes of gluconeogenesis and glycogenolysis which controls blood sugar levels. Alkaloids, tannins, cardiac glycosides, flavonoids, saponins, and steroids were the main ingredients in *T. cordifolia* that have been useful to the plant's anti-diabetic effects (Saha & Ghosh, 2012).

Meyer's toxicity index indicate that extracts that have LC_{50} values less than 1000 µg/ml are considered toxic whereas extracts that have LC_{50} values more than 1000 µg/ml are considered non-toxic. The methanol extract of *T. cordifolia* was found to have a fatal

concentration of 232.64 µg/ml, indicating that the extract was poisonous (Shrestha & Lamichhane, 2021).

The leaves and stem of *T. cordifolia* was used to extract the phytochemicals in different solvents as chloroform, methanol, ethanol and the total flavonoid content was also assessed. Phytochemicals such as amino acids, flavonoids, diterpines, proteins, saponin and carbohydrate were present. The total flavonoid content was found higher in the leaves of ethanolic extract but the flavonoid content was found higher in the stem of methanolic extract (Garg & Garg, 2018).

Mishra *et.al* studied on scientific validation of medicinal efficacy of *T. cordifolia* using its stem extracts. All extracts showed positive results for anthroquinones, terpenoids and phenol. Different contents of phenolics (8.75-52.5 mg/g) was present in all extracts (Mishra *et al*., 2013).

Owk and Lagudu studied on phytochemicals and antimicrobial studies of stem extracts of *Tinospora cordifolia*. The phytochemical studies revealed the presence of alkaloid, phenols, flavonoids, saponins, tannins, steroids, terpenoids in all extracts (hexane, chloroform, methanol and aqueous). By using agar well diffusion method, methanolic and aqueous extracts showed the higher anti-bacterial activity against Bacillus subtilis (Owk & Lagudu, 2016).

Agarwal *et al*. studied on assessment of anti-microbial activity of different concentrations of *T. cordifolia* against streptococcus mutans. By using agar well diffusion methods, the maximum anti-bacterial activity was observed at a volume of 40 µL at 2% concentrations with a zone of inhibition of 19mm (Agarwal *et al.*, 2019).

The extraction of *T. cordifolia* was carried out by Soxhlet extraction method and it was done using DPPH assay to compare the free radical scavenging activity in between ethanol and methanol extracts. The result revealed the higher antioxidant potential in ethanol extract than the methanol extracts (Upadhyay *et al*., 2014).

T. cordifolia stem extracts were prepared in both aqueous and alcohol in different doses. The experiment was carried out in streptozotocin diabetic albino rats. It was observed that the extracts showed a significant anti-diabetic activity and showed an efficacy of 40% to 80% compared to insulin (Puranik *et al*., 2010).

The entire *T. cordifolia* plant was extracted using methanol and the extract was used to identify its bio-active constituent by employing Gas Chromatography Mass Spectrometry (GC-MS) technique. Several components such as γ-Sitosterol, n-Hexadecanoic acid, 9-Octadecenoicacid(Z)-2,3-dihydroxypropylester, 2-Hydrazino-2-imidazoline and these compounds can have antioxidant, antitumor and antimicrobial potential (Akhilraj et al., 2024).

HPLC analysis was performed to identify the phenolic compounds. Catechin hydrate, Rutin hydrate, Ellagic acid and Catechins were detected and these compounds were useful for antioxidant and anti-inflammatory purposes. The extract contained higher amount of phenols in methanol than in ethanol. About 50% DPPH was scavenged by 9.36±1.75 mg/mL methanol extract whereas 27.40±1.09 mg/mL ethanol extract was required to scavenge the 50% DPPH (Khan et al., 2020).

The *T. cordifolia* leaf extract was used to assess the antidiabetic potential in diabetic rats. The ethanolic and hydroalcoholic extracts both showed the significant strong antidiabetic effects (Kumar et al.,).

Phytochemical analysis and biological investigations of *T. cordifolia* (Gurjo) in context of Nepal till date is found to be less. The detailed study on the biological activity has been tried to address from this study.

CHAPTER III: MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Chemicals Required

- Analytical-grade chemicals such as hexane, chloroform, acetone and methanol were used.
- 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, KOH, conc. H_2SO_4, conc. HCl, $AlCl_3$ and phenol were employed as chemicals and reagents of laboratory grade.
- Reagents like Mayer's, Dragendroff's, Fehling's, etc, were made in the lab using chemicals that are provided in the laboratory of laboratory grade.

3.1.2 Instrument and Equipment

- Rota Evaporator (IKA, RV 10 D S96)
- Sonicator, Refrigerator
- UV lamp (UV 2510TS)
- Heating Bath, Digital weighing balance, Hot air oven, Electric grinders
- Measuring cylinders, beakers, conical flasks, test tubes, burettes, micropipettes, pipettes, thermometers, water baths, and vial tubes.
- FTIR (PerkinElmer Spectrum IR; Version 10.6.2)

3.2 Methods

3.2.1. Sample Collection and Identification of Plant Materials

Stems (matured phase) of *T. cordifolia*, also called Gurjo in Nepali, were collected from Mandan Deupur in Kavrepalanchok, Nepal, at an elevation of 872 meters and coordinates of 27.7042° N latitude and 85.5661° E longitude in December, 2022.

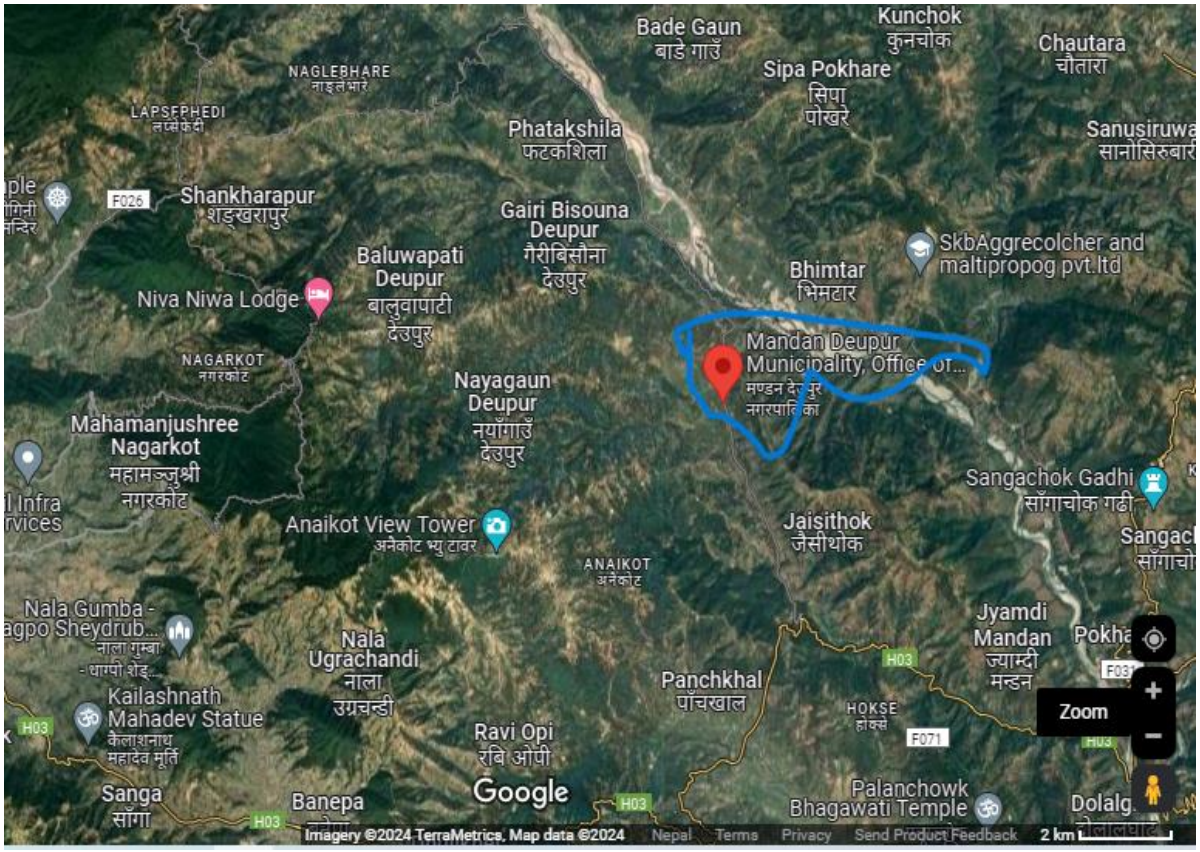


Figure 2: Sample collection area from google map

Associate Professor Dr. Sheela Singh from the Department of Botany at Amrit Campus, assisted to certify the plant which was collected from the Kavrepalanchowk district to represent the whole species.

3.2.2. Sample Preparation

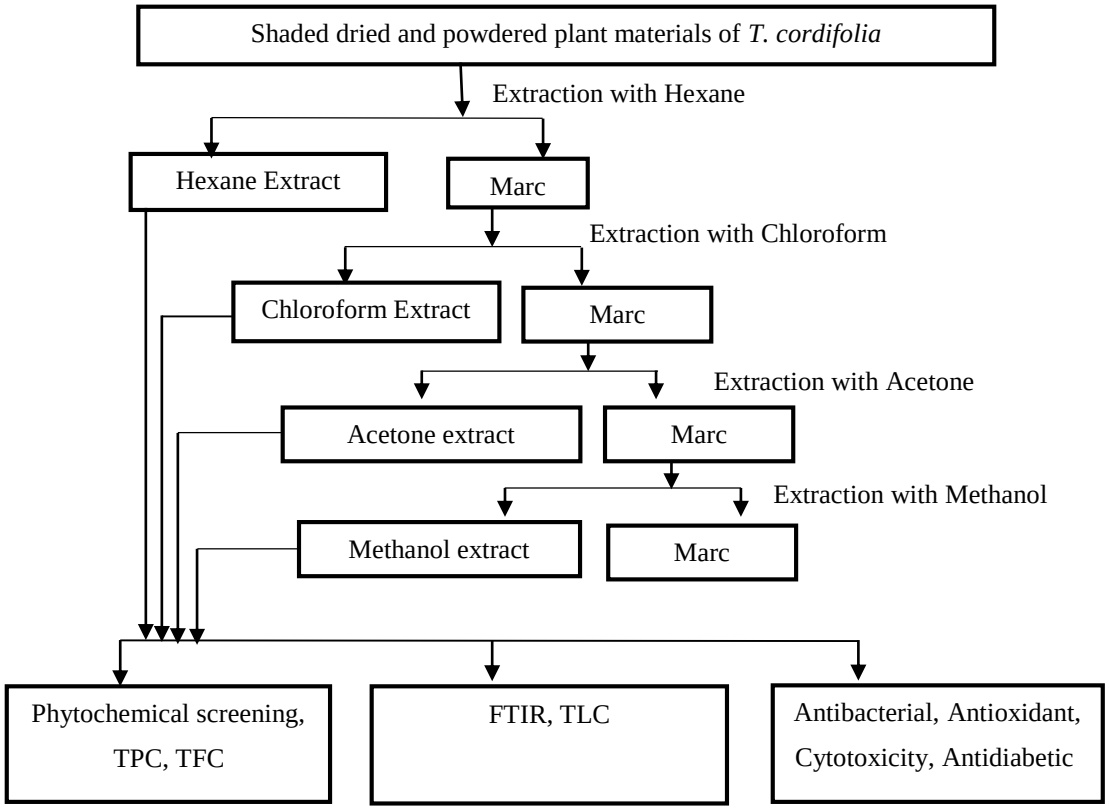
About five kilograms of the plant's stem were gathered, cleaned in water and allowed to air-dry for a few days at room temperature under cover. The stems were dried in the shade and then ground with an electric grinder to a fine powder. After that, the resulting powder which weighed about 500 grams was kept at lower temperature until it was needed.

3.2.3 Extraction Process

Out of numerous techniques, the ultrasonic extraction procedure was employed.
In this technique, 500 grams of powdered plant material was placed in a sterile and dry beaker for storage. After adding hexane and mixing it well, the beaker was put into a 30 x 15 x 20 cm ultrasonic cleaner tank. Distilled water filled the tank to around one-

third of its total capacity. The modules operated at 40 kHz and had a combined output of 350 watts. After being drained off, the extracted beaker was allowed to cool to room temperature before being filtered. The filtrate was concentrated using a Rota evaporator. After being measured and dried, the concentrated extract was kept for later use in airtight bottles. Before the sonication procedure, hexane was added to the filtered residue as well. The same procedure was replicated for three batches to obtain the crude extract. Various solvents such as chloroform, acetone, and methanol were generated using the identical method.

The below diagram in schematic form illustrates the entire technique.



Scheme 1: Research Process

3.2.4 Phytochemical Screening

The phytochemical screening test was carried out in all four extracts. And the procedure was based on standard protocol (Banu & Cathrine, 2015). The screening tests aids in the identification of bioactive substances in various extracts. This process includes the successive and selective extraction of phytochemicals with solvent in increasing polarity. The phyto-constituents were determined by the presence of specific color with specific reagents.

3.2.5 Total Phenolic Content (TPC)

Using an oxidation-reduction procedure, the Folin-Ciocalteu colorimetric technique was used to quantify the total phenolic content. The concentration of gallic acid is used as a benchmark for comparison (Balasundram *et al*., 2006).

3.2.5.1 Folin-Ciocalteau Reagent Preparation

A dilution ratio of 1:10 in distilled water was achieved by dissolving 1 mL of the Folin-Ciocalteau reagent in 10 mL of distilled water.

3.2.5.2 Gallic acid standard solution preparation

In the first step, 1 mg of gallic acid was dissolved in 1 mL of methanol to prepare a stock solution with a concentration of 1000 mg/mL (ppm). Gallic acid was prepared at different concentrations after the stock solution was diluted further at 500, 250, 150, 100, 50, and 25 µg/mL respectively**.**

3.2.5.3 Calibration curve construction

Initially, each test tube was filled with 20 µL of a solution containing gallic acid. The test tube was then filled with 20 µL of the extract and 100 µL (10%) of the Folin-Ciocalteu reagent (FCR). It was then allowed to incubate for five minutes at room temperature in total darkness. To make this mixture a final volume of 200 µL, 80 µL of a 7% Na_2CO_3 solution in water was added. After giving the blue solution a good shake, it was allowed to sit in the dark for two hours. At 765 nm, the reaction's absorbance was measured and compared to a control, which consisted of incubating 20 µL of methanol (rather than the extract), 100 µL of F-C reagent, and 80 µL of sodium carbonate for two hours.

3.2.5.4 Sample Solution Preparation

Firstly, 1 mg of the extract was dissolved in 1 mL of methanol to create a stock solution with an extract concentration of 1000 μg/mL (ppm). To create different extract concentrations, the stock solution was diluted again and again. The same method used for gallic acid was applied to determine the absorbance values of these various concentrations.

3.2.5.5 Total Phenolic Content (TPC) Calculation

To calculate the total content of phenolic substances in the sample, the given equation was used and it was represented as milligrams of gallic acid equivalent per gram.

$$C = \frac{c \times V}{m} \quad \ldots\ldots\ldots\ldots (1)$$

Where,

C = Total content of phenolic substances (mg/g) in gallic acid equivalent

c = Concentration of gallic acid derived from the calibration curve (μg/mL)

V = Volume of the sample extract (mL)

m = Weight of the plant extract (μg)

For every concentration, the average absorbance values were noted. These data points were then used to compute the linear correlation coefficient (R^2). The regression equation is shown below.

$$y = mx + c \quad \ldots\ldots\ldots\ldots...(2)$$

where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = concentration of the extract

c = Intercept

The extract's concentration was calculated by using the regression equation. Then, using the concentration of the extract, equation (1) was created to determine the substance's total phenolic content.

3.2.6. Total Flavonoid Content (TFC)

The total flavonoid content of the plant extract was determined using a colorimetric technique that used aluminum chloride. Quercetin served as the standard reference. This method induces a chemical reaction between the hydroxyl groups of flavonoids in the sample and aluminum chloride, generating a measurable result (Chandra et al., 2014).

3.2.6.1. Preparation of the Quercetin Standard Stock Solution

To prepare stock concentration of 1 mg/mL, 1 mG of quercetin was dissolved in 1 mL of pure methanol to create a quercetin stock solution. The concentrations of 250 µg/mL, 150 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL were then obtained by serial dilutions. A total of 200 µL was achieved by adding 100 µL of 2% aluminum chloride (in methanol) to a microplate well after each concentration was added in 100 µL aliquots. After that, the pink solution was incubated for 10 mins in total darkness.
Subsequently, utilizing a spectrophotometer, the absorbance at 425 nm was measured for both the control consisting of 100 µL of solvent and 100 µL of 2% aluminum chloride. The sample and the control were incubated simultaneously. The calibration curve was built using the average absorbance values found for different quercetin concentrations. The standard calibration curve's positive control was quercetin. By determining the quantity of quercetin equivalent (mg QE) per gram of dried plant material, the total flavonoid concentration was calculated.

3.2.6.2 Preparation of the Sample Solution

Initially, 1 mg of the extract was dissolved in 1 mL of methanol to create a stock solution with an extract concentration of 1000 µg/mL. The stock solution was then diluted several times to produce different extract concentrations, and the absorbance values of these extracts were measured utilizing the same method previously explained for quercetin.

3.2.6.3 Calculation of Total Flavonoid Content

Using the following formula, the amount of flavonoids in the extract can be determined.

$$C = \frac{c \times V}{m} \quad \ldots\ldots\ldots\ldots\ldots (3)$$

Where,
C = Total Flavonoid Content (in mg/g) in Quercetin Equivalent (QE)

c = Concentration of quercetin derived from calibration curve

V = Volume of the sample extract (in mL)

m = Weight of extract (in μg)

The average absorbance for each concentration was reported, and the linear correlation coefficient (R^2) value was calculated. Using these values, the regression equation was created: $y = mx + c$ ……...(4)

where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = concentration of the extract

c = Intercept

Utilizing the regression equation, the concentration of the extract was predicted. Consequently, the concentration of the extract was used as the input in equation (3), which was developed to measure the substance's total flavonoid content.

3.2.7 Antioxidant Activity

Antioxidants are compounds that effectively block or slow the oxidation process when present in less quantities than the material that is prone to oxidation. Because of their numerous health benefits, these compounds are highly significant in the field of nutraceuticals. They are also frequently used in the food sector to prevent lipid peroxidation. Antioxidant-containing materials have been found in varieties including fruits, vegetables, barks, roots, woodlands, leafy spices, and herbs (Shen *et al*., 2022). The antioxidant activity may reduce the oxidative stress by avoiding free radical from damaging protein, DNA and lipids. Besides, they also work against cancer, cardiovascular and stroke (Parham *et al*., 2020).

For antioxidant tests, the electron transfer-based DPPH free-radical technique is used. It first produces a violet solution when dissolved in alcohol. Nevertheless, the solution turns colorless when an antioxidant molecule is present (Otohinoyi *et al*., 2014).The following equation can be useful to calculate the percentage of DPPH free radical scavenging activity:

$$\text{Radical scavenging (\%)} = \frac{(Ao - As)}{Ao} \times 100 \quad \text{……..(5)}$$

where,

A_0 = Absorbance of the control solution (DPPH solution + methanol)

A_S = Absorbance of the extract sample.

50% of the DPPH free radicals can be neutralized by a sample amount equal to its IC_{50} (50% inhibitory concentration). The extraction concentration was plotted against the scavenging activity to create an inhibition curve. From this process, the IC_{50} values can be calculated.

3.2.7.1 Preparation of the DPPH Solution at 0.1 mM

DPPH has a molecular weight of 394.32 g/mol. A 0.1 mM DPPH solution was prepared by adding 0.0019716 g of DPPH in methanol and adjusting the volume to 50 mL. After that, the solution was kept in dark.

3.2.7.2 Ascorbic Acid Preparation (standard)

To make a stock solution with a concentration of 1000 μg/mL, 10 mg of ascorbic acid were measured and then dissolved in 10 mL of methanol. Then, ascorbic acid solutions with concentrations of 30 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL and 2.5 μg/mL were prepared by serial dilution method.

3.2.7.3 Preparation of Sample Solution

After weighing 10 mg of the methanol, ethyl acetate and chloroform extracts, each was diluted in 10mL of methanol to prepare a stock solution with a concentration of 1 mg/mL. Following this process, extract solutions were made by performing sequential dilutions at concentrations of 30 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL and 2.5 μg/mL for every extract.

3.2.7.4 Measurement of DPPH Radical Scavenging Activity

To prepare a total 200 μL volume, 50 μL of ascorbic acid from each concentration was pipetted into 150 μL of methanolic DPPH solution in a 96-well plate. The solutions were then left in the dark for twenty minutes. A spectrophotometer was then used to measure their absorbance value at 520 nm using methanol and DPPH as a blank.

Similar to the process for ascorbic acid, the absorbance values for the extracts and the DPPH solution were measured. The X-axis was used to plot the ascorbic acid and sample solution concentration whereas the Y-axis was used to plot the radical % scavenging activity. From the derived data, IC_{50} values were then calculated.

3.2.8 Antibacterial Activity

Plant extracts have antibacterial action so that they can stop some hazardous bacteria from growing. This feature describes their ability to prevent the growth of microorganisms. The paper disc diffusion method was used to assess the inhibition of microbial growth, and the results were reported as a ZOI (Zone of Inhibition) value (Kalemba & Kunicka, 2003).

3.2.8.1 Standard culture collection

Two standard strains of microorganisms were provided by the Bio-medical Research Lab in Gwarko, Lalitpur in activated cultures. The following two different kinds of microorganisms were investigated in this study:

Gram-positive bacteria: *Bacillus subtilis* ATCC 6051

Gram-negative bacteria: *Escherichia coli* ATCC 8739

3.2.8.2 Preparation of the media

By following the manufacturer's instructions, the below mentioned exact measures were taken in order to prepare the study's media.

A. Nutrient agar

For this preparation, 2.5 grams of agar were dissolved in 100 mL of distilled water. The solution was then autoclaved for 45 minutes at 121°C for sterilization. Then, the solution was autoclaved, allowed to cool to about 40°C and then uniformly poured into a petri dish. After 20 minutes, the petri dish was dried and solidified by placing it in a laminar flow hood.

B. Nutrient broth media (Liquid Media)

2 grams of nutrient-dense broth powder were dissolved in 100 mL distilled water. The mixture was then well mixed to guarantee full dissolution, and then it was autoclaved for 45 minutes at 121°C and 15 pounds of pressure. After then, it was let to cool.

3.2.8.3 Preparation of the working solution

A sterile container was filled with 15 mg of each unique crude extract and 100 mL of DMSO solution. After being sealed and capped, the vials were kept in a refrigerator between 2- 8 degrees Celsius until needed. 5 mg of kanamycin was dissolved in 1000 mL of distilled water to create the Kanamycin positive control.

3.2.8.4 Antimicrobial activity screening and assessment

At first, liquid nutrient broth medium was used to develop the microbiological microorganisms: Escherichia coli (ATCC 8739) and Bacillus subtilis (ATCC 6051). Then, each strain's 100 µL of culture broth was put on a nutritional agar plate and left to remain at 37°C for 15 minutes. The bio-plastic sample was placed on the nutrient agar plate, incubated for an additional 15 mins at 37°C and then left overnight. On the subsequent day, the bio-plastic's antibacterial effect on the microbiological strains B. subtilis and *E. coli* was evaluated. DMSO was used as the negative control and kanamycin as the positive control. After the plates were observed, a specified scale was used to evaluate the Zone of Inhibition arising from the antibacterial activity of plant extracts.

3.2.9 Anti-diabetic Assay

Diabetes is a category of metabolic diseases characterized by hyperglycemia or increased blood sugar levels, brought on by partial or total lack of insulin synthesis. Chronic hyperglycemia exposure over an extended period of time can cause microvascular problems that impact different organs like the kidneys, retina and peripheral nerves (Egan & Dinneen, 2019). Numerous compounds found in the plant have been identified through extensive phytochemistry research. However, it is noteworthy that no claims or assertions have been made about the efficacy of these drugs as anti-diabetic medicines (Wickramaratne *et al*., 2016).

3.2.8.3 A general procedure for α-Amylase inhibition

The 3,5-dinitrosalicylic acid (DNSA) method was used for the inhibition of α-amylase. The plant extract of *T. cordifolia* was diluted using at least 10% DMSO. The sample and DMSO combination were combined once more in a pH 6.9 buffer containing NaCl in order to obtain a wide range of concentrations. Next, 200 µL of the extract and α-amylase solution were mixed together. The mixture was then allowed to incubate for 10 minutes at 30 °C. After that, each tube was filled with 200 µL of starch solution, which was let to stand for three minutes. The addition of 200 µL of DNSA reagent stopped the process. After that, the mixture was boiled for 10 mins in a water bath between 85- 90 degrees. The combined sample was further diluted with 5 mL of distilled water once it had reached room temperature. A UV spectrophotometer was used to measure the absorbance at 540 nm and the results were compared to a blank

solution. To prepare a blank solution with 100% enzyme activity, 200 µL of buffer was added in place of the plant extract.

Using the following equation, the percentage of α-amylase inhibitory activity was determined. Plotting the extract concentration versus the percentage of α-amylase inhibition yielded a graph from which IC50 values were calculated.

$$\% \text{ α-amylase inhibition} = = \frac{(Ao - As)}{Ao} \times 100 \quad \text{……..(6)}$$

3.2.10 Brine Shrimp Lethality Assay (BSLA)

The BSLA is a useful technique for determining a plant extract's cytotoxicity towards laboratory-cultivated larvae. This technique is characterized by its simplicity, cost-effectiveness, and the smallest number of test material necessary for experimentation (Sarah *et al*., 2017).

3.2.10.1 General Process for Brine Shrimp Lethality Assay

Every piece of equipment used in the experiment was sterilized before use.

3.2.10.2 The Brine Shrimp Egg Hatching

In a beaker coated with aluminum foil, 50 mg of brine shrimp eggs were uniformly placed over simulated saltwater. Tiny pores were made in the foil to enhance heat and light conduction. The beaker was then exposed to radiation from a 60-watt light for 48 hours while remaining undisturbed at ambient temperature.

3.2.10.3 Sample Preparation

To prepare a stock solution with a concentration of 1000 µg/mL. 2 mL of DMSO (dimethyl sulfoxide) was added after 2 mg of the extract was measured. The original stock solution was serially diluted to get solutions at concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.0625 mg/mL respectively. There were 15 test tubes in total because each concentration was divided into three test tubes. Three test tubes were also given 2 mL of DMSO each in order to serve as blanks. After that, the tubes with labels were kept alone for a whole day.

3.2.10.4 Assay calculation

The lethality percentage of nauplii for each concentration and control was determined through calculations. The number of dead and live nauplii in each tube was counted in

order to calculate the percentage of mortality which was then calculated using the formula below.

$$\% \text{ Mortality} = \frac{No.\ \text{of dead shrimps}}{\text{Total No. of shrimps}} \times 100 \ldots\ldots. (7)$$

3.2.11 Thin Layer Chromatography

A Thin-Layer Chromatography (TLC) analysis is a method to isolate bioactive compounds and determine the potential quantity of substances within a fraction. The foundation of chromatographic technique is the idea that molecules can be separated from the solution by first arranging them on a surface. After that, these molecules are separated by using a mobile phase to help them move away from a solid or liquid stationary phase (Coskun, 2016) .

For the separation of components present in extract, thin layer chromatography (TLC) is employed to all extracts. The test samples that had been dissolved in the appropriate solvents were applied as spots by using a fine capillary on a 0.2mm aluminium plate. For every position, there were identifying markers placed on top of the plate. Chromatography was performed in glass chambers that were rectangular in shape to avoid undesired edge effects and inadequate saturation. In order to minimize edge effects and guarantee uniform solvent distribution, a U-shaped filter paper was placed within the TLC chamber such that it came into contact with the developing solvent. Throughout the investigation, a number of solvent systems were tested; the most illuminating and satisfying solution was selected as the ultimate solvent system. Then, the plates were subjected to a UV examination, let to air dry, and visualized. The generated chromatogram provides information about the different constituents present in the mixture. A UV lamp was used to observe the chromatogram.

The constant term which is used to determine how the component migrates relative to the solvent in a certain chromatographic system and also helps in the determination of substance properties is called the Rf value.

$$\text{Relative factor (Rf)} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

3.2.12 FT-IR Analysis

Fourier Transform Infrared (FTIR) spectroscopy is a very important spectroscopic technique which provides insightful information on the properties of organic molecules. It offers details on π-bond conjugate systems, functional groups and the presence of aliphatic and aromatic structures in the compounds under analysis. A (PerkinElmer Spectrum IR Version 10.6.2) FTIR spectrometer at the Amrit Campus in Kathmandu was used to collect the FTIR spectra of several extracts for this work. The spectra were taken within the range of 4000 to 450 cm^{-1}, with a scan interval of 4 cm^{-1}.

CHAPTER IV: RESULTS AND DISCUSSION

4.1 Confirmation of the Selected Plant

Associate Professor Dr. Sheela Singh from the Department of Botany at Amrit Campus, assisted in the verification of the plant, which was brought from Kavrepalanchowk district in Province-3, to serve as a representative specimen for the entire species.

Table 1: Collection of sample and identification

Identified Plant	Common Name	Family	Place of Collection
T. cordifolia	Gurjo	Menispermaceae	Kavrepalanchowk district

4.2 Yield

Table 2: Gram yield in various extracts

Extracts	Hexane	Chloroform	Acetone	Methanol
Yield (g)	0.50	9.35	2.93	10.07
%Yield	0.16	3.11	0.97	3.35

The *T. Cordifolia* plant was extracted by adopting the ultra-sonication method of extraction. The total yield in hexane, chloroform, acetone and methanol extracts are 0.16%, 3.11%, 0.97% and 3.35 % respectively.

4.3 Qualitative Analysis of Phytochemicals

As indicated in Table 3, the micro-chemical analysis of a raw extract obtained from the *T. cordifilia* plant using various solvent systems unveiled a set of phytochemical compounds. The phytochemical content of each *T. cordifilia* extract was assessed and the identification of phytochemicals was based on the observed colors during the analysis.

Table 3: Phytochemicals observed in the extract of *T. cordifilia*

S.No.	Class of Phytochemicals	Hexane Extract	Chloroform Extract	Acetone Extract	Methanol Extract
1.	Alkaloids	-	-	-	+
2.	Carbohydrates	-	-	-	+
3.	Phenolic Compounds	-	+	+	+
4.	Flavonoids	-	+	+	+
5.	Tannins	-	+	_	+
6.	Quinones	-	-	-	+
7.	Terpenoids	+	-	-	+
8.	Saponins	-	+	-	+
9.	Resin	-	-	+	+
10.	Steroids	-	+	+	+
11.	Triterpenoids	-	+	-	-
12.	Anthraquinones	-	-	-	-
13.	Proteins	-	+	-	-
14.	Glycosides	+	+	+	+

Where "+" means presence and "-" means absence.

The observation showed that most of the phytochemicals are extracted in polar solvents. It was found that flavonoids, glycosides and steroids were present in nearly all solvent extracts. The employment of an ultrasonic sonicator for extraction and concentration through a Rota-evaporator presents the possibility that the absence of alkaloids could be attributable to the possible disintegration of alkaloids generated by heat and molecular vibration.

There are some or all differences between the data on this plant that are recorded in the literature and the outcomes that are shown in the above table. These discrepancies can be linked to various elements, including the plant's elevation, a variety of environmental circumstances, the extraction technique utilized, the time of sample collection, the arrangement of the laboratory and the grades of chemical used. As a result, the results of phytochemical screenings directed towards certain phytochemical elements may differ from those of screenings conducted for other samples.

4.4 Quantitative Analysis of Phytochemicals

4.4.1. Estimation of Total Phenolic Content (TPC)

The amount of polyphenols found in plant extracts combine with a specific redox reagent (FCR) to generate a blue complex that absorbs light most efficiently at 765 nm. UV-visible spectroscopy can be used to find this absorption. There is an inverse relationship between the quantity of phenols present and the degree of light absorption at this wavelength. By measuring the absorbance at various standard gallic acid concentrations, a graphical absorbance was produced. In the figure showing the absorbance curve for standard gallic acid, the Y-axis denotes absorbance and the X-axis depicts concentrations such as 500, 250, 150, 100, 50 and 25 µg/mL.

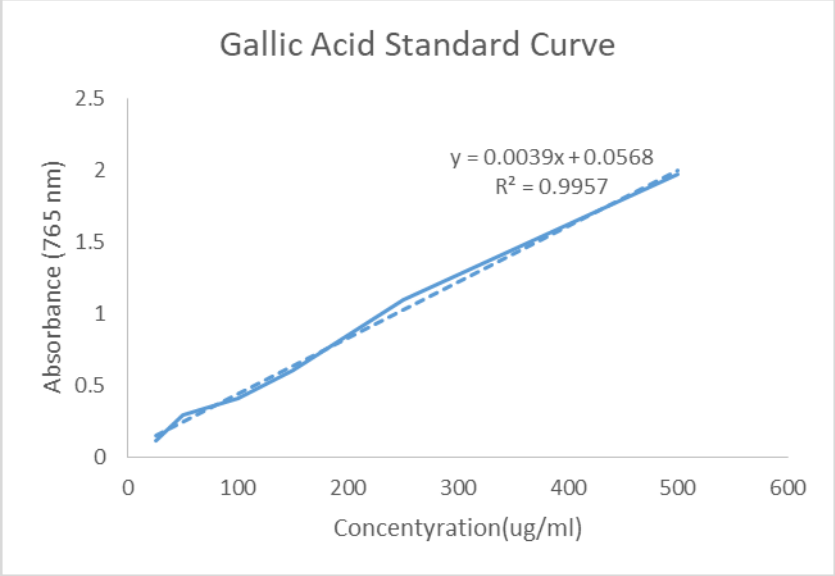


Figure 3: Standard gallic acid calibration curve

The above equation from standard Gallic acid in figure 3 was used to calculate the phenolic content of hexane, chloroform, acetone and methanol extracts.

4.4.2 Measurement of total phenolic contents in all extracts

Table 4: Total phenol content in hexane, chloroform, acetone and methanol extracts

Extracts	Concentration (µg/mL)	Observed Data $(O.D)_{sample}$	$O.D_{control}$	$O.D_{value}$	TPC (mg GAE /gm)
Hexane	1000	0.78	0.16	0.61	144.06
Chloroform	1000	0.48	0.16	0.32	68.25
Acetone	1000	0.43	0.16	0.27	55.77

Methanol	1000	0.70	0.16	0.54	125.35

The result depicted that *T. cordifolia* hexane extract had total phenolic content 144.06 mg GAE/g whereas its acetone extract had the least 55.77 mg GAE/g. Phenolic compounds are the secondary metabolites possessing defensive mechanisms to pathogens including bacteria, fungi and viruses. So, they exhibit antimicrobial and antioxidant properties (Kumar et al., 2020).

4.4.3 Estimation of Total Flavonoid Content (TFC)

4.4.3.1 Calibration curve construction

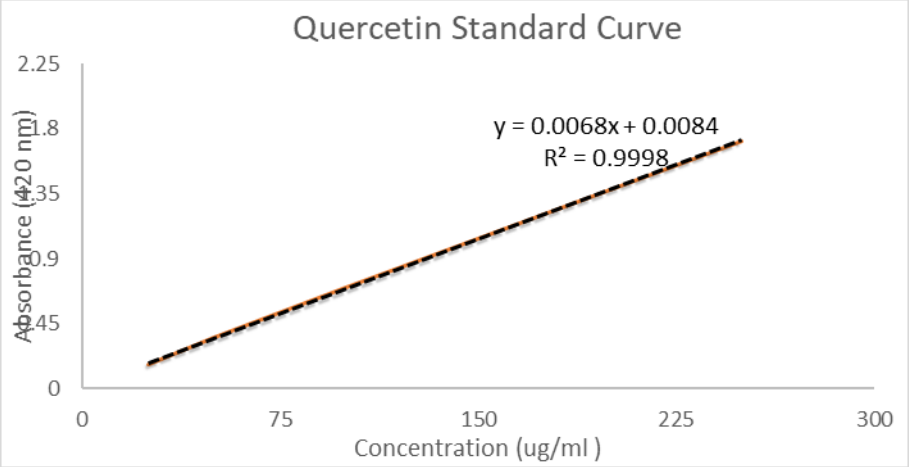


Figure 4*:* Standard quercetin calibration curve

The above equation from standard quercetin calibration curve in figure 4 was used to calculate the concentrations of hexane, chloroform, acetone and methanol extracts.

4.4.3.2 Measurement of total flavonoid contents (TFC) in all extracts

Table 5: Total flavonoid content in hexane, chloroform, acetone and methanol extracts

Extracts	Concentration (µg/mL)	Observed Data $(O.D)_{sample}$	$O.D_{control}$	$O.D_{value}$	TPC(mg Q /gm)
Hexane	1000	0.52	0.10	0.41	59.5
Chloroform	1000	0.23	0.10	0.13	27.14
Acetone	1000	0.31	0.10	0.21	17.88
Methanol	1000	0.30	0.10	0.19	29.69

Hexane extract contains higher amount of flavonoid contents whereas the acetone contains the flavonoid content in least amount. Flavonoids are the diverse group of secondary metabolites which have significant roles in plant defence against pathogens, herbivores and environmental stress (Treutter, 2005).

4.5 Antioxidant Assay

The DPPH free radical scavenging assay was used to determine the degree of antioxidant activity. The antioxidant capacity can be calculated using linear regression analysis on the inhibition percentage vs antioxidant activity. This value is inversely correlated with the IC_{50} value.

Following measurements of absorbance were taken for every solution's absorbance:

Table 6: Antioxidant activity of ascorbic acid

Sample	Concentration (µg/mL)	Absorbance (nm)			Average absorbance (nm)	% of scavenging
1	Control	0.48	0.52	0.47	0.49	
2	2.5	0.43	0.43	0.40	0.42	13.36
3	5	0.38	0.36	0.36	0.37	24.49
4	10	0.30	0.30	0.30	0.30	37.85
5	20	0.21	0.19	0.17	0.19	60.51
6	30	0.16	0.16	0.16	0.16	66.89
IC_{50}	15.33 µg/mL					

To neutralize free radicals, ascorbic acid was used and the observed absorbance was plotted as shown in below figure no. 5.

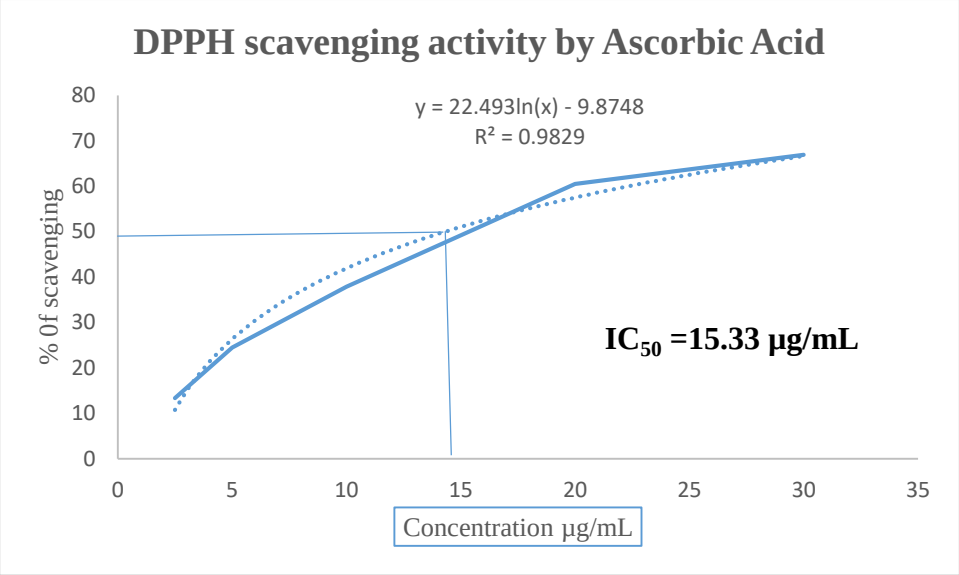


Figure 5*:* Anti-oxidant activity of ascorbic acid

From the above graph, the value of **IC_{50} was 15.33 µg/mL** and it was taken as a positive control.

At 520 nm, the absorbance values of different hexane extract concentrations were calculated. These absorbance measurements were used to compute the % inhibition of DPPH radicals for the sample as shown in the table below.

Table 7: Antioxidant activity of hexane extract

Concentration (mg/mL)	% of scavenging
1	85.31
0.5	63.37
0.25	49.21
0.125	39.9

IC_{50} =0.23 mg/mL

The IC_{50} value of hexane extract was found to be 0.23 mg/mL. The observed data of concentrations versus % scavenging of the extract was plotted in excel figure 6.

DPPH Scavenging by Hexane Extract

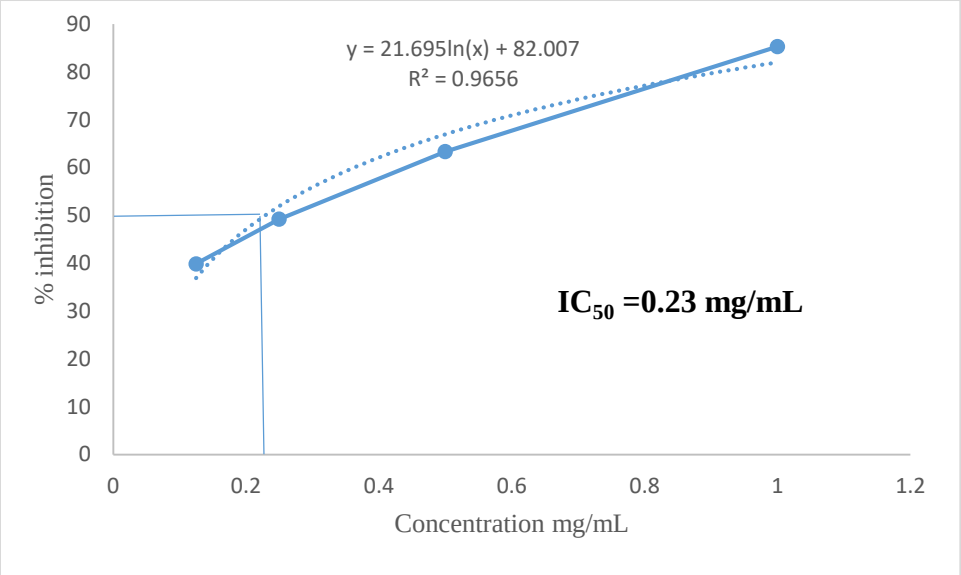


Figure 6: Anti-oxidant activity of hexane extract

At 520 nm, the absorbance values of different chloroform extract concentrations were calculated. These absorbance measurements were used to compute the % inhibition of DPPH radicals for the sample as shown in the table below.

Table 8: Antioxidant activity of chloroform extract

Concentration (mg/mL)	% of scavenging
1	79.72
0.5	59.99
0.25	44.26
0.125	33.20

IC_{50} = 0.38 mg/mL

The IC_{50} value of chloroform extract was found to be 0.38 mg/mL. The observed data of concentrations versus % scavenging of the extract was plotted in excel figure 7.

DPPH Scavenging by Chloroform Extract

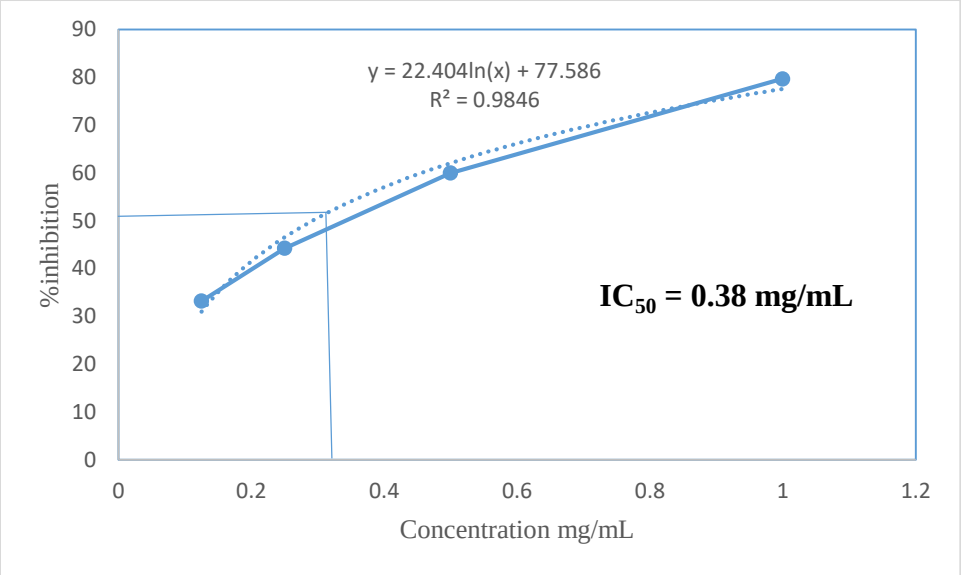


Figure 7: Anti-oxidant activity of chloroform extract

At 520 nm, the absorbance values of different acetone extract concentrations were calculated. These absorbance measurements were used to compute the % inhibition of DPPH radicals for the sample as shown in the table below:

Table 9: Antioxidant activity of acetone extract

Concentration (mg/mL)	% of scavenging
1	87.77
0.5	66.60
0.25	54.89
0.125	45.28

IC_{50} =0.19 mg/mL

The IC_{50} value of acetone extract was found to be 0.19 mg/mL. The observed data of concentrations versus % scavenging of the extract was plotted in excel figure 8.

DPPH Scavenging by Acetone Extract

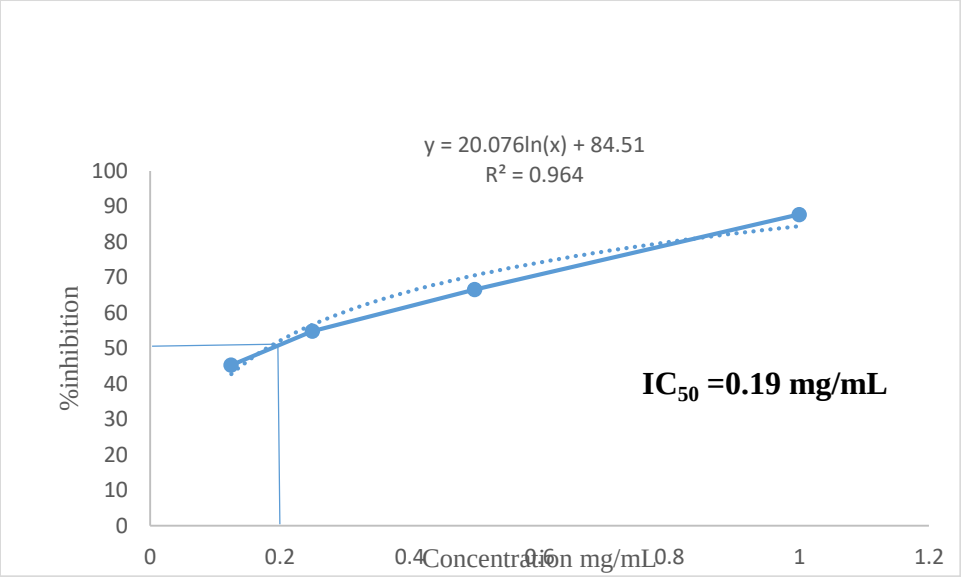


Figure 8: Anti-oxidant activity of acetone extract

At 520 nm, the absorbance values of different methanol extract concentrations were calculated. These absorbance measurements were used to compute the % inhibition of DPPH radicals for the sample as shown in the table below.

Table 10: Antioxidant activity of methanol extract

Concentration (mg/mL)	% of scavenging
0.25	75.01
0.12	62.22
0.06	45.33
0.03	40.67

IC_{50} =0.06 mg/mL

The IC_{50} value of methanol extract was found to be 0.06 mg/mL. The observed data of concentrations versus % scavenging of the extract was plotted in excel figure 9.

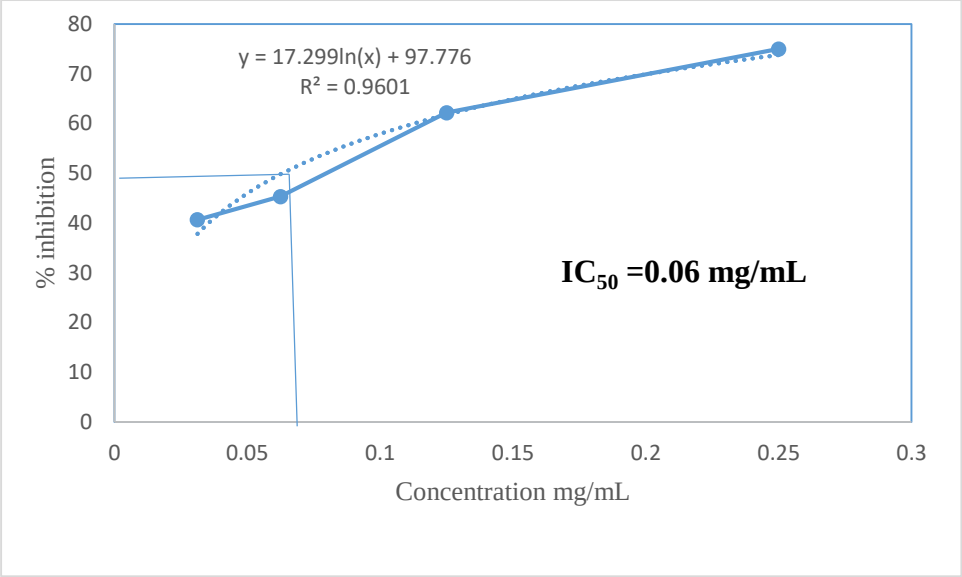


Figure 9: Anti-oxidant activity of methanol extract

Among all these four extracts, the methanol extract of *T. cordifolia* exhibited the highest antioxidant activity. In addition to this, acetone and hexane extract had also powerful antioxidant properties, but chloroform extract didn't exhibit good antioxidant properties. These values demonstrated the extraordinary antioxidant property as comparable to the typical ascorbic acid concentration (15.33 mg/mL).

4.6 Anti-bacterial Activity

By measuring the diameter of the zone of inhibition (ZOI) that plant extracts produced on bacterial cultures was measured in order to evaluate the antibacterial activities of the extracts. The ability of several *T. cordifolia* stem extract fractions to inhibit bacterial growth at a fixed concentration (100 mg/mL) was assessed by adopting the method described in this section. The inhibitory zone's diameter was used to interpretate the result.

A zone of inhibition (ZOI) measurement was taken and the result was in centimetres. The positive control was Kanamycin whereas the negative control was Dimethyl Sulfoxide (DMSO). Anti-bacterial action's efficiency depends on the kind of bacteria used.

Table 11: Different solvent extract of T. cordifolia showing anti-bacterial effect in diameter (cm) of the inhibition zone (ZOI)

Bacteria	Negative Control	Positive Control	Hexane Extract	Chloroform Extract	Acetone Extract	Methanol Extract
Bacillus (ATCC 6051)	0	3.0	0	0	0	1
Escherichia coli (ATCC 8739)	0	3.4	0.9	0.8	0.8	1.2

The antibacterial properties of each extract was observed, which is displayed in table 11. In this experiment, the methanol extract significantly inhibited *Bacillus*, as evidenced by a 1 cm zone of inhibition (ZOI) but the other extracts such as hexane, chloroform and acetone showed no anti-bacterial effects. Whereas, *E.coli* growth was found to be highly inhibited on methanol extract with 1.2 cm as zone of inhibition and hexane extract inhibited 0.9 cm as zone of inhibition. Similarly, chloroform and acetone extracts showed equal zone of inhibition with 0.8 cm as diameter.

4.7 Anti-diabetic Assay

The Amylase enzyme inhibition assay was done by using the 3,5-dinitrosalicylic acid (DNSA). As a measure of possible antidiabetic action, the correlation between the % of inhibition and the activity of α-amylase inhibition was examined using logarithmic regression analysis. The relationship between this potential and the IC_{50} value is inverse. To evaluate the effect of different extract concentrations on α-amylase enzyme, the absorbance values were measured at 540 nm.

The inhibition of α-amylase by the % of hexane extract is shown in the table below:

Table 12: α-Amylase inhibition by hexane extract

Concentration (mg/mL)	% of Inhibition
2	90.88
1	47.61
0.5	19.07
0.125	8.00
IC_{50} = 0.82 mg/mL	

T. cordifolia hexane extract had an IC_{50} value 0.82 mg/mL. The observed data of concentrations versus % inhibition of the extract was plotted in excel figure 10.

α-Amylase Inhibition Assay

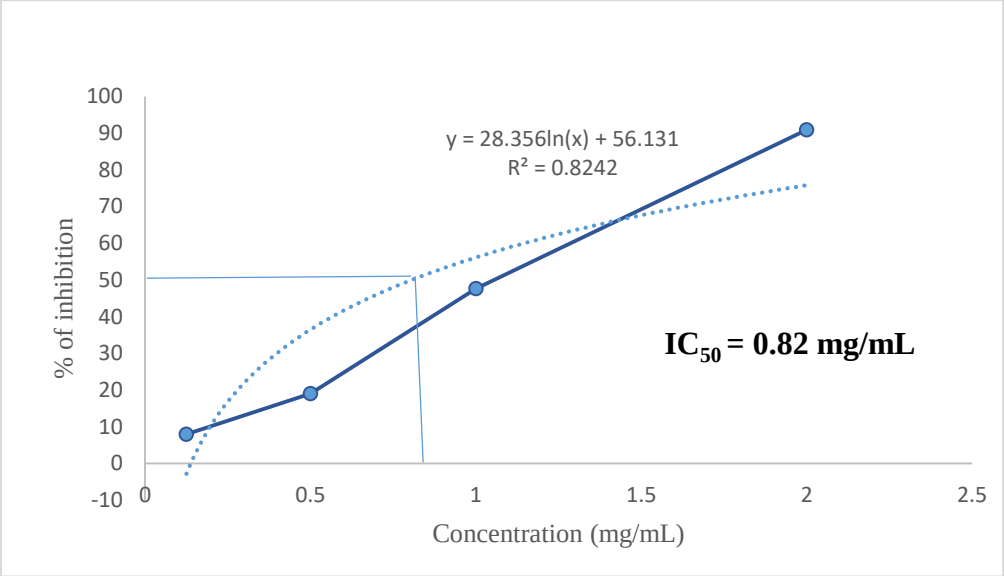


Figure 10: α-Amylase inhibition by hexane extract

The % level of chloroform extract –induced inhibition of α-amylase is displayed in the table below:

Table 13: α-Amylase inhibition by chloroform extract

Concentration (mg/mL)	% of Inhibition
2	81.33
1	48.71
0.5	38.97
0.125	32.83
IC_{50} = 0.72 mg/mL	

T. cordifolia chloroform extract had an IC_{50} value 0.72 mg/mL. The observed data of concentrations versus % inhibition of the extract was plotted in excel figure 11.

α-Amylase Inhibition Assay

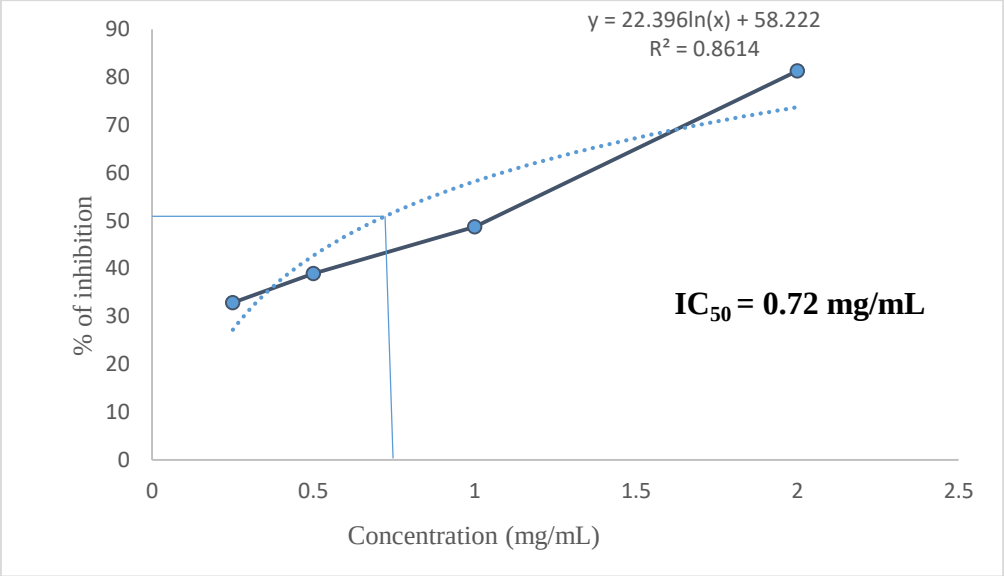


Figure 11: α-Amylase inhibition by chloroform extract

The inhibition of α-amylase by the % of acetone extract is shown in the table below:

Table 14: α-Amylase inhibition by acetone extract

Concentration (mg/mL)	% of Inhibition
2	89.87
1	70.17
0.5	46.32
0.125	33.14
IC_{50} = 0.50 mg/mL	

T. cordifolia acetone extract had an IC_{50} value 0.50 mg/mL. The observed data of concentrations versus % inhibition of the extract was plotted in excel figure 12.

α-Amylase Inhibition Assay

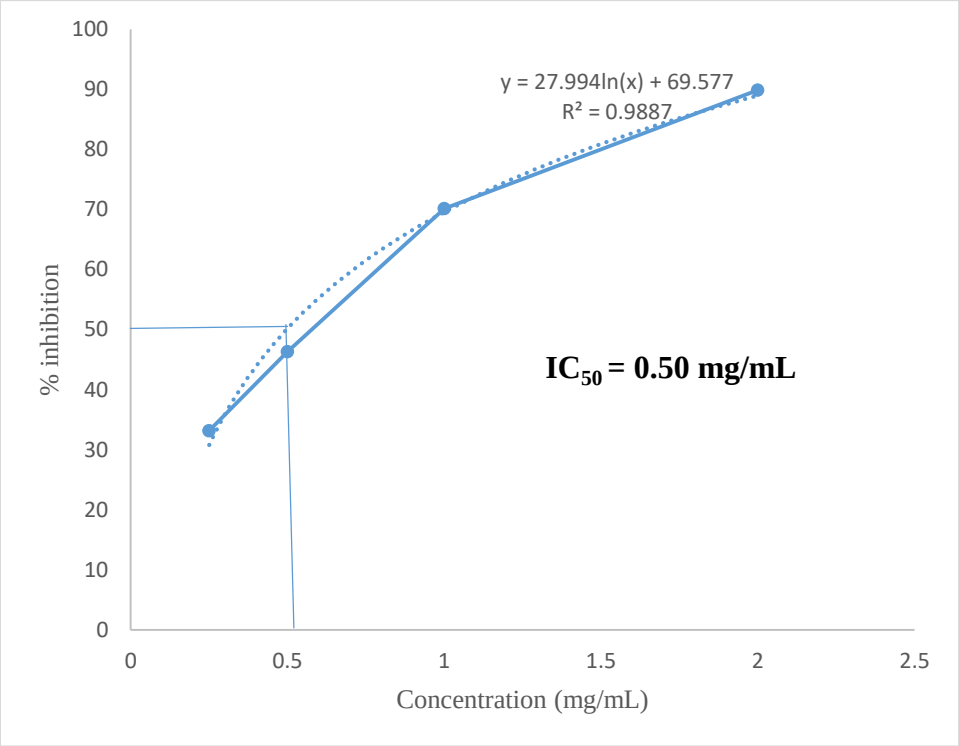


Figure 12: α-Amylase inhibition by acetone extract

The % level of methanol extract –induced inhibition of α-amylase is displayed in the table below:

Table 15: α-Amylase inhibition by methanol extract

Concentration (mg/mL)	% of Inhibition
2	95.08
1	61.63
0.5	35.34
0.125	19.18
IC_{50} = 0.56 mg/mL	

T. cordifolia methanol extract had an IC_{50} value 0.56 mg/mL. The observed data of concentrations versus % inhibition of the extract was plotted in excel figure 13.

α-Amylase Inhibition Assay

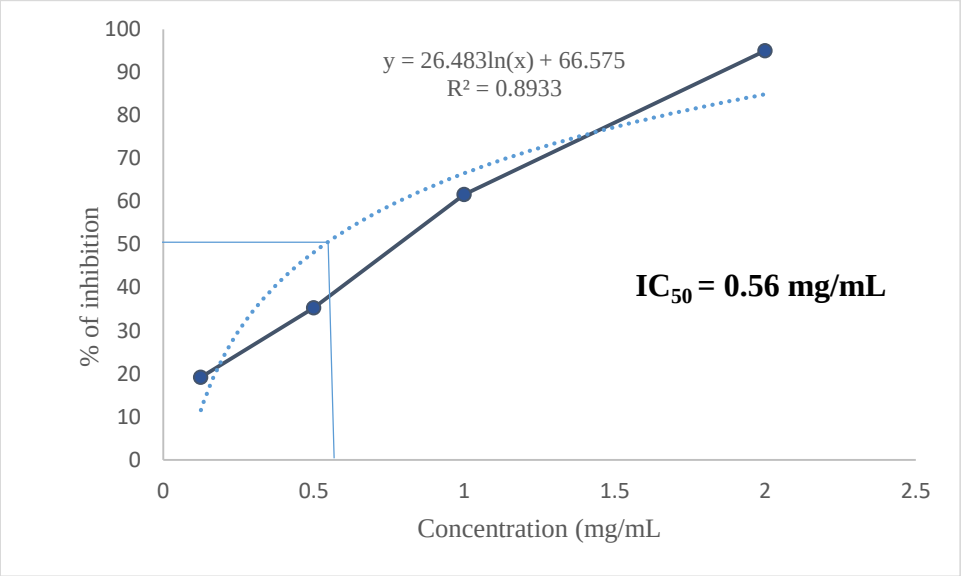


Figure 13: α-Amylase inhibition by methanol extract

Among all these extracts, acetone extract of *T. cordifolia* showed the highest α-amylase inhibition activity. Moreover, methanol and chloroform extracts had higher anti-diabetic properties than hexane extract. In a nutshell, the first three extracts collectively showed strong anti-diabetic effects.

4.8 Brine Shrimp Lethality Assay (BSLA)

LC_{50} is the lethal concentration for 50% mortality after 24 h of exposure. It is the measure of toxicity of the extract or compound (Tawaha, 2006).

The LC_{50} values for the hexane extract was calculated as given in the table below:

Table 16: Measurement of %mortality of hexane extract

Conc. (µg/ml)	No. of dead naupli			% mortality
1000	10	10	10	100
500	10	10	9	96.66
250	9	8	9	86.66
125	6	6	6	60
62.5	5	3	3	36.66
Control	0	0	0	0

The LC_{50} value of hexane extract of T. cordifolia was found to be 65.1 µg/ml. This is illustrated in the graph plotted below.

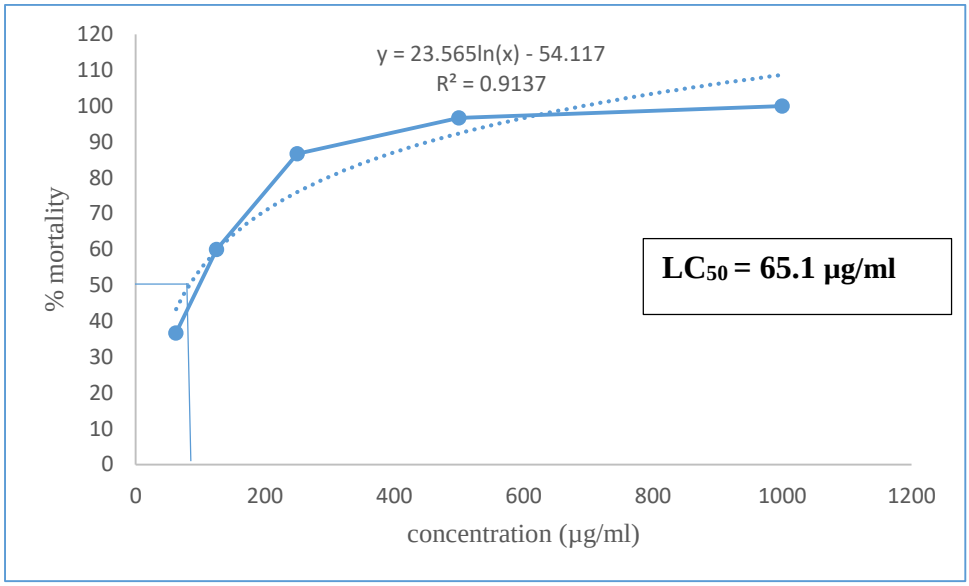


Figure 14: Cytotoxicity activity of hexane extract

The LC_{50} values for the chloroform extract was calculated as given in the table below:

Table 17: Measurement of %mortality of chloroform extract

Conc. (µg/ml)	No. of dead naupli			% mortality
1000	10	10	10	100
500	7	8	8	76.66
250	6	6	6	60
125	5	5	4	46.66
62.5	3	3	3	30
Control	0	0	0	0

The LC_{50} value of chloroform extract extract of *T. cordifolia* was found to be 150.5 µg/ml. This is illustrated in the graph plotted below.

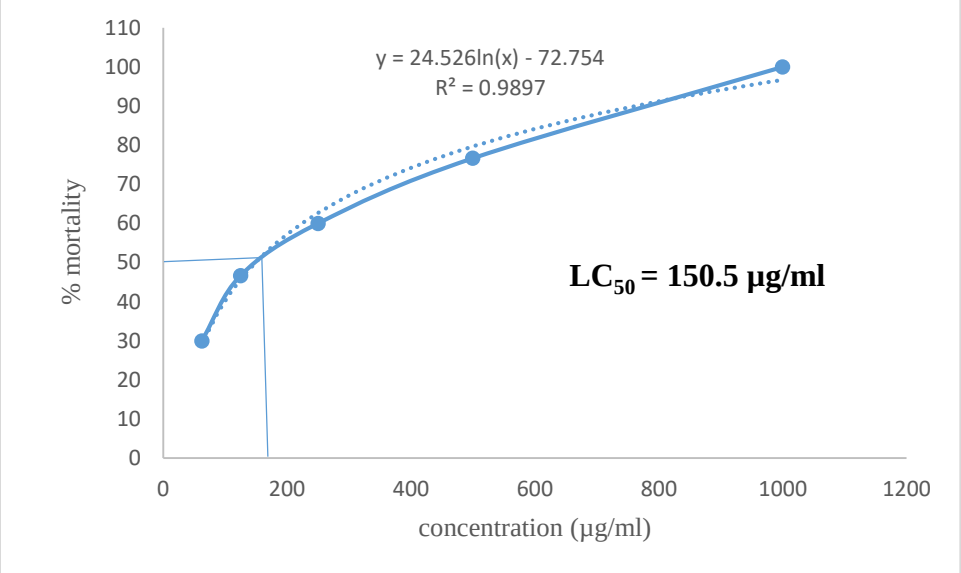


Figure 15: Cytotoxicity activity by chloroform extract

The LC_{50} values for the acetone extract was calculated as given in the table below:

Table 18: Measurement of %mortality of acetone extract

Conc. (µg/ml)	No. of dead Naupli			% mortality
1000	10	10	10	100
500	10	10	10	100
250	10	10	10	100
125	8	8	8	80
62.5	6	7	6	63.33

Control	0	0	0	0

The LC_{50} value of acetone extract of *T. cordifolia* was found to be 62.50 µg/ml. This is illustrated in the graph plotted below.

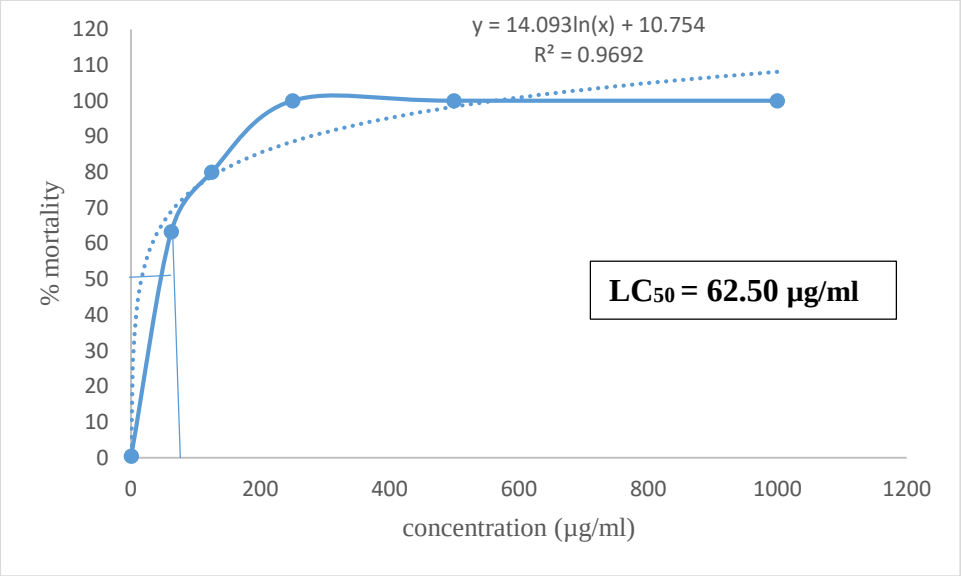


Figure 16: Cytotoxicity activity by acetone extract

The LC_{50} values for the methanol extract was calculated as given in the table below:

Table 19: Measurement of %mortality of methanol extract

Con. (µg/ml)	No. of dead Naupli			% mortality
1000	10	10	10	100
500	8	8	8	80
250	7	6	7	66.66
125	3	4	4	36.66
62.5	3	2	2	23.33
Control	0	0	0	0

The LC_{50} value of methanol extract of *T. cordifolia* was found to be 195.5 µg/ml. This is illustrated in the figure below.

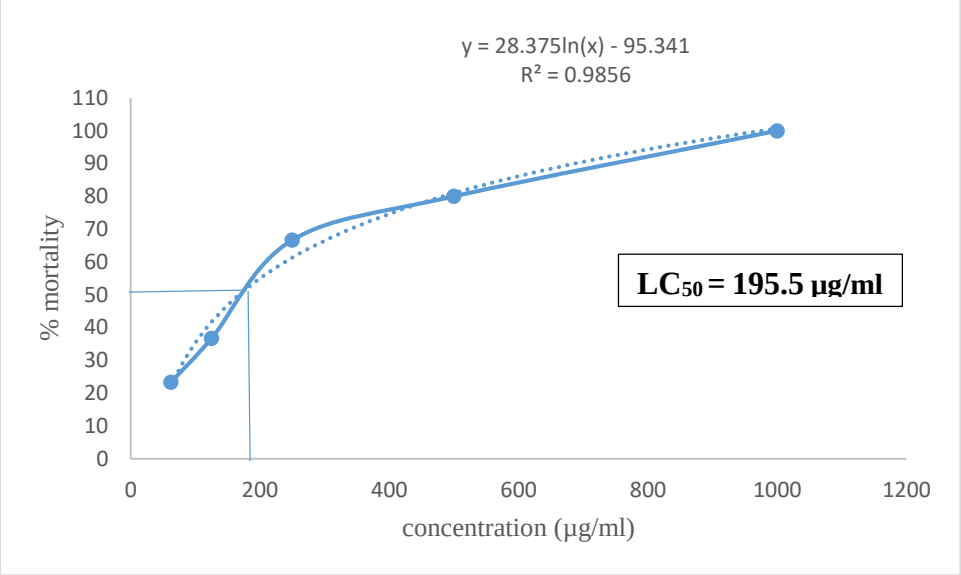


Figure 17: Cytotoxicity activity by methanol extract

In a nutshell, extracts were found to have pharmacological significance and harmful to brine shrimp larvae. Among all the four extracts, acetone extract with low LC_{50} value revealed high mortality rate i.e. the most cytotoxic whereas methanol extract with high LC_{50} value showed the least mortality rates i.e.it was the least toxic.

4.9 Thin Layer Chromatography

This technique was employed in three different solvent systems as mobile phase namely Chloroform: Hexane, Ethyl acetate: Hexane and Acetone: Hexane. All these mobile phase solvent systems were prepared in different concentrations at 5%, 10%, 20% and 50% respectively. The four different extracts were subjected to separation in this method. It means the qualitative separation of the four extract of *T. cordifolia* was done by TLC on increasing the polarity of solvents. The plates were first observed under naked eye and some spots of compounds were observed in it. But, for those compounds which are not visible to human eye, were studied under UV fluorescence cabinet. No separation was obtained at 5% concentration in all solvents. The separation of compounds was also not good i.e. no distinct spots were obtained at 10% concentration in all mobile phase. The best TLC was observed at 20% concentrations in all 3 mobile phases i.e. distinct spots were observed. Moreover, Blur spots and tailings were found on increasing the solvent concentration to 50%.

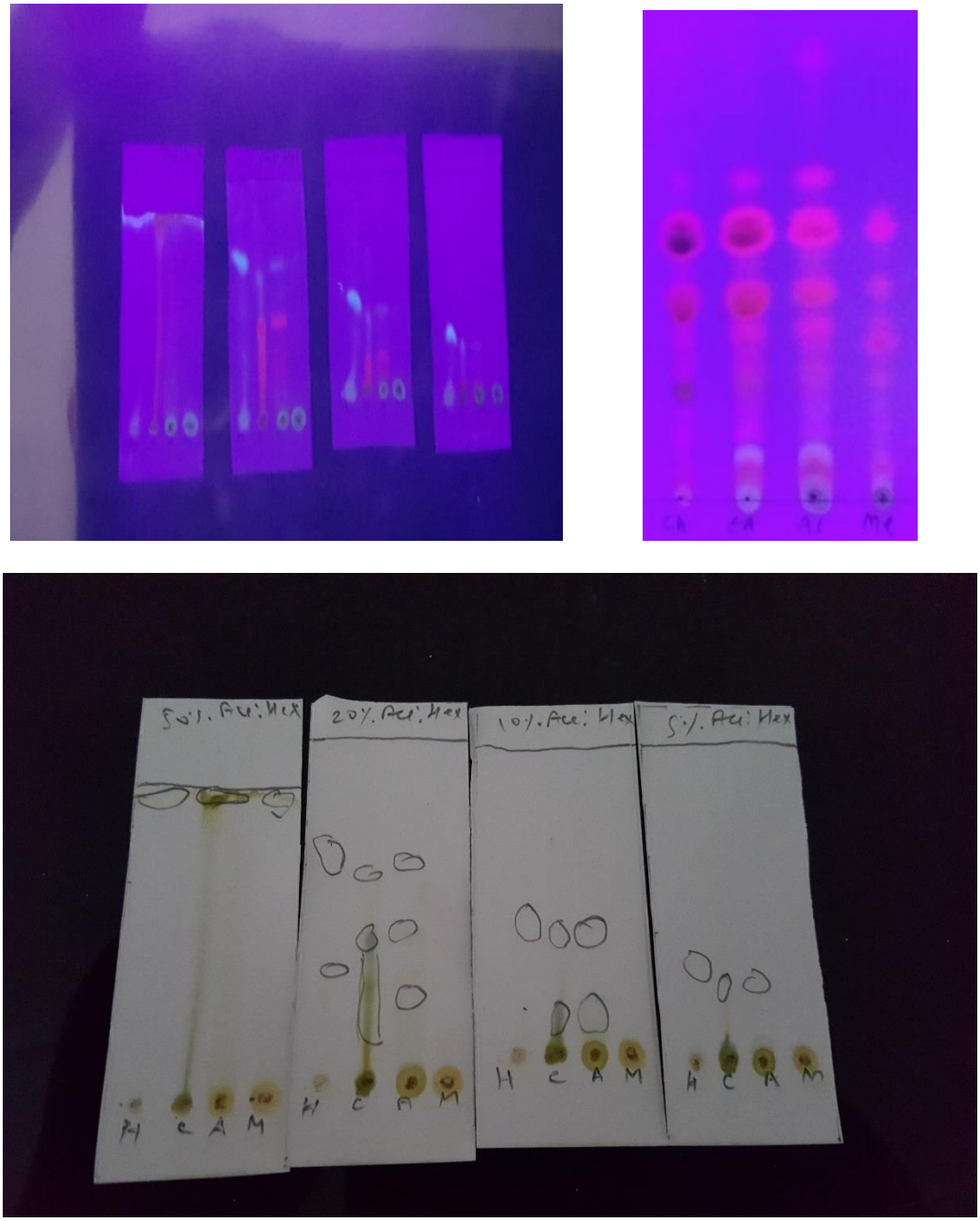


Figure 18: TLC plate at 5%, 10%, 20% and 50% in acetone: hexane, chloroform: hexane and ethyl acetate: hexane under UV fluorescence in a shorter and longer wavelengths

4.10 FT-IR Analysis

Using FTIR spectroscopic technique, various organic molecules can be analyzed to determine their bonding types, aromatic and aliphatic structures and most importantly the presence of functional groups in a molecule (Schmitt & Flemming, 1998). The FTIR spectrum is obtained in this technique which helps to verify the presence of a variety of functional groups and organic molecules. The bonding between heteroatoms and carbon can be determined by using the stretching frequency seen in the FTIR spectrum.

4.10.1 FT-IR spectrum of hexane extract

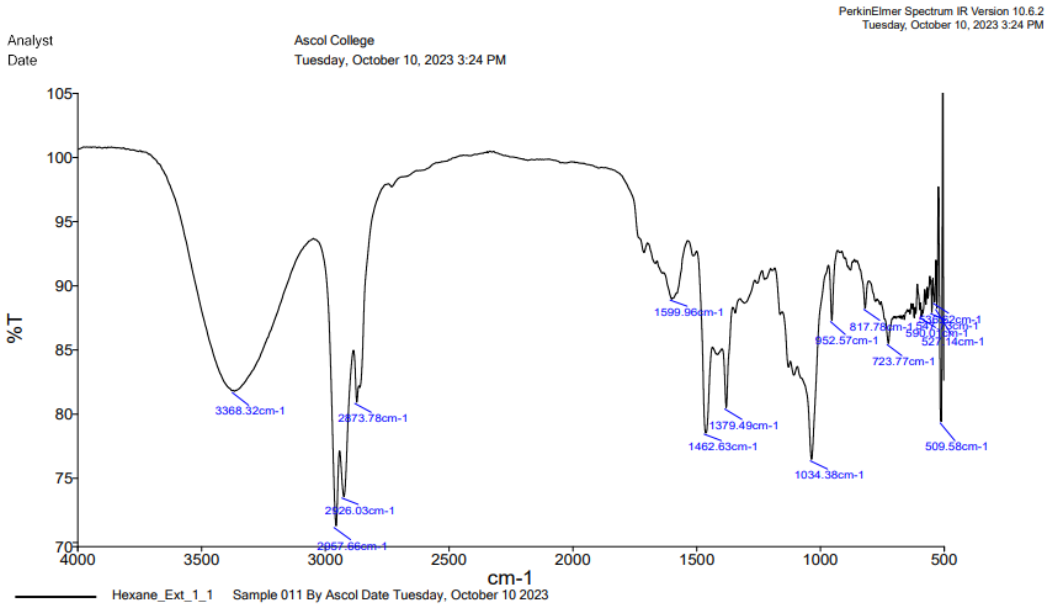


Figure 19: FT-IR spectrum of hexane extract

The types of stretching, absorption and appearance by hexane extract is shown in the table below:

Table 20: FTIR peak value of hexane extract

Absorption (cm^{-1})	Types of stretching	Appearance
3368	O-H	Strong
2957, 2926, 2873	C-H	Strong
1462, 1379	$-NO_2$	Weak
1034	-O-	Strong

The above results were verified by comparing with an infrared chart.

4.10.2 FT-IR spectrum of chloroform extract

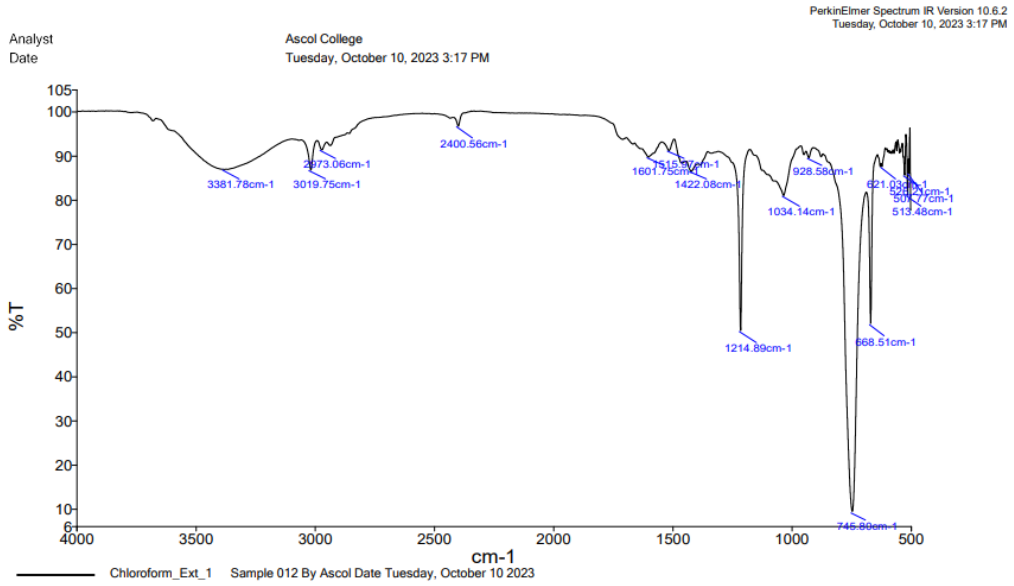


Figure 20: FT-IR spectrum of chloroform extract

The types of stretching, absorption and appearance by chloroform extract is shown in the table as:

Table 21: FTIR peak value of chloroform extract

Absorption (cm^{-1})	Types of stretching	Appearance
3381	O-H	Strong
3019, 2973	C-H	Weak
1601	N-O	Weak
1422	C-H	Weak
1214	-O-	Strong
745	C-H	Strong

The above results were verified by comparing with an infrared chart.

4.10.3 FT-IR spectrum of acetone extract

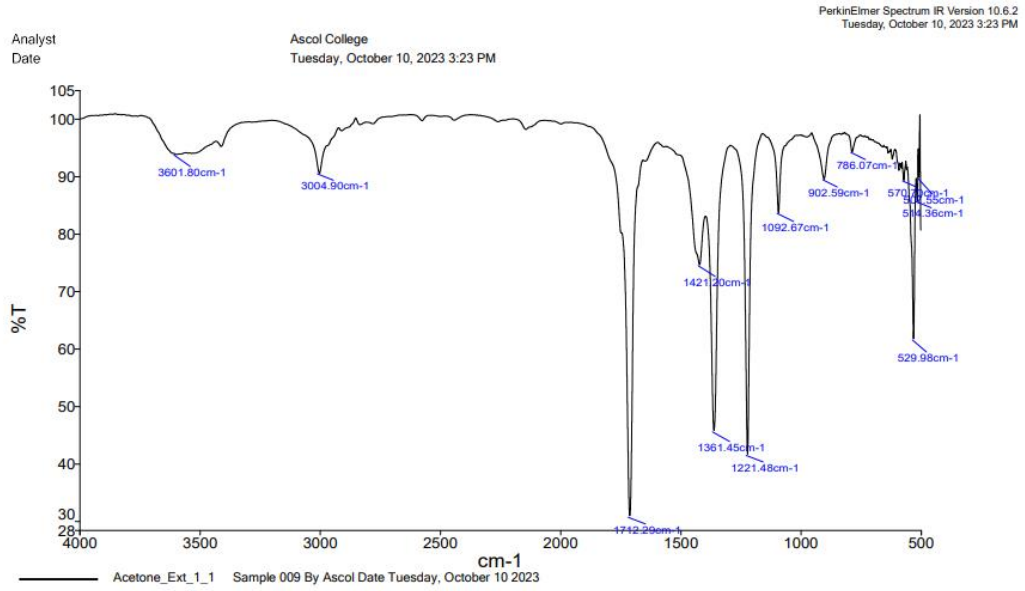


Figure 21: FT-IR spectrum of acetone extract

The types of stretching, absorption and appearance by acetone extract is shown in the table below:

Table 22: FTIR peak value of acetone extract

Absorption (cm^{-1})	Types of stretching	Appearance
3601	O-H	Weak
3004	C-H	Strong
1712	C=O	Strong
1361, 1221	$-NO_2$	Strong
1092	-O-	Weak

The above results were verified by comparing with an infrared chart.

4.10.4 FT-IR spectrum of methanol extract

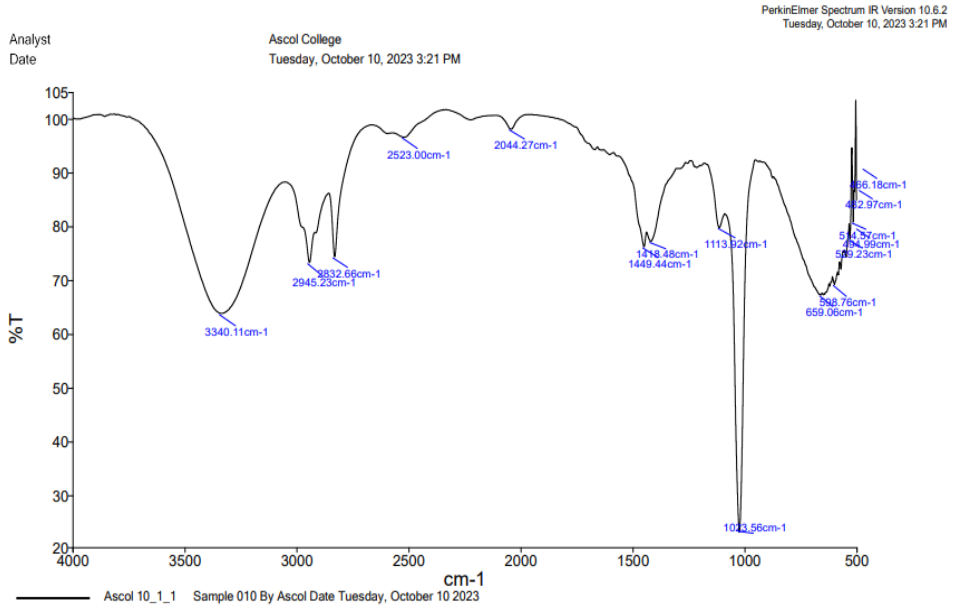


Figure 22: FT-IR spectrum of methanol extract

The types of stretching, absorption and appearance by methanol extract is shown in the table below:

Table 23: FTIR peak value of methanol extract

Absorption (cm^{-1})	Types of stretching	Appearance
3340	O-H	Strong
2945, 2832	C-H	Strong
1449	$-NO_2$	Medium
1023	-O-	Strong

The above results were verified by comparing with an infrared chart.

CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

For decades, the plant has been significant for its therapeutic properties and regarded as a powerhouse of health benefits. The phytochemical examinations showed that alkaloids, carbohydrates, polyphenols, flavonoids, tannins, quinones, terpenoids, saponins, resins, steroids, triterpenoids, anthraquinones, proteins and glycosides were present in different solvent extracts of *T. cordifolia*. Out of four extracts, the hexane extract (144.06 mg GAE/g) contained the highest content of phenolic compounds whereas the acetone extract (55.77 mg GAE/g) contained the least amount. Similarly, the highest content of flavonoid compounds (59.50 mg QE/g) was found in hexane extract but the lowest amount (17.88 mg QE/g) in chloroform extract.
In terms of biological activity potential of the plant extract, it showed the higher anti-oxidant potential on methanol extract than the acetone, hexane and chloroform extracts.

The α-amylase inhibition assay revealed higher potential on acetone extract in compare to other three extracts. Effective antibacterial qualities were shown by the extract against the two strains that were tested. *E. coli* in particular showed the highest inhibitory zone (ZOI) 1.2 cm. In comparison to the other strains tested, this finding implies that the extract is very effective against *E. coli*. In comparison to hexane extract (65.1 µg/ml), chloroform extract (150.5 µg/ml) and methanol extract (195.5 µg/ml), the cytotoxicity assay demonstrated a greater lethality concentration on acetone extract (62.50 µg/ml).

The best separation on TLC was obtained in 20% concentrations of different solvent system. There was the presence of different functional groups like O-H, C-H, C=O, -CH_2, -NO_2, C-O, N-H, C=C, etc in all the extracts from four solvents, which was verified by FT-IR.

5.2 Suggestions for further work

The biologically active components, as confirmed by phytochemical examinations, are important for the medical and pharmacological activities. Such extracted compounds have different medicinal values as discussed above. But, we can prepare plant extracts in different organic solvents by adopting different extraction techniques than the above

mentioned here, in order to extract a wider variety and quantity of phytochemicals in this plant. To extract a novel active compound from this plant, the extract can be prepared from other than stem. Phytochemical screening, TLC, Antibacterial, Antioxidant, Anti-diabetic, cytotoxicity and GC-MS analysis are all the topics of my dissertation. But, the interpretation of GC-MS spectra is still under process. Consequently, much effort needs to be done before the interpretation is complete.

The following subjects can be included as the possible future research areas:

a) For the anti-bacterial examinations, two micro-organisms were only taken. More examinations can be done on many organisms, which helps in creating antibiotics for the treatment of ailments.

b) The plant can constitute more important substances. So, it may be possible to carry out extraction, isolation, identification and the structure elucidations.

c) The extracts can be further subjected to Liquid Chromatography Mass Spectrometry (LC-MS) for better characterizations. Both volatile as well as non-volatile compounds can be analyzed with this technique.

d) Column Chromatography can also be carried out from which pure compounds can be separated and those separated compounds can be subjected to various bioactivities test for good results.

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APPENDICES

A. Phytochemical Screening Protocol

1. Test for Alkaloids

About 500 mg extract was dissolved in 3 mL of 2% (v/v) HCl. The solution was filtered, and the following tests were performed;

i. Mayer's Test

Few drops of Mayer's reagent were added to the first part. The formation of a pale yellow precipitate indicates the presence of alkaloids.

ii. Dragendroff's Test

Few drops of Dragendroff's reagent were added to the second part. The formation of an orange-red precipitate indicates the presence of alkaloids.

2. Test for Steroids

1 ml plant extract was taken in a test tube and dissolved with 10 ml chloroform and then an equal volume of concentrated sulphuric acid was added to the test tube by sides. The upper layer in the test tube should be turned red, and the sulphuric acid layer should show a yellow color with green fluorescence to show the presence of steroids.

3. Test for Terpenoids

To about 200 mg extract, 2 mL of chloroform ($CHCl_3$) and then 3 mL concentrated sulphuric acid (H_2SO_4) were added carefully. The formation of reddish-brown colouration at the interface indicates the presence of terpenoids.

4. Test of Flavonoids (Shinoda's Test)

About 200 mg extract was dissolved in 2 mL methanol. To this solution, a small piece of magnesium and 4-5 drops of concentrated hydrochloric acid (HCl) were added. The formation of orange colour indicates the presence of flavonoids.

5. Test for Phenolic Compounds/ $FeCl_3$ Test

To about 1 mL extract, 1 mL distilled water was added, followed by adding a few drops of 10 % (w/v) ferric chloride ($FeCl_3$) solution. The appearance of greenish blue coloration indicates the presence of phenolic compounds.

6. Test for Glycosides

About 500 mg extract was dissolved in 2 mL methanol and divided into two parts and the following tests were performed,

i. Molisch's Test

The first part was treated with 5 mL of Molisch's reagent and conc. H_2SO_4 was added drop by drop from the side of the test tube without disturbing the solution. The appearance of a violet ring at the junction of two liquids which on shaking turns the solution into a violet colour indicates the presence of glycosides.

ii. For the second part, 2 mL of 25 % (v/v) NH_4OH solution was added and shaken vigorously. The appearance of the cherry red colour indicates the presence of glycosides.

7. Test for Carbohydrates

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicates the presence of Carbohydrates.

8. Test for Quinones

To about 2 mL extract, 1 mL freshly prepared ferrous sulfate ($FeSO_4$) solution and a few crystals of ammonium thiocyanate (NH_4SCN) were added, and the solution was treated with conc. Sulphuric acid (H_2SO_4) drop by drop. The appearance of persistent deep red colouration indicates the presence of quinones.

9. Test for Saponins

About 500 mg extract was treated with hot water followed by shaking for 30 seconds. The formation of thick forth indicates the presence of saponins.

10. Test for Tannins

About 200 mg extract was boiled, adding 10 mL distilled water. The mixture was cooled and, filtered and a few drops of $FeCl_3$ solution were added to the filtrate. The appearance of blue-black precipitate indicates the presence of tannins.

11. Test for Proteins

The extracts were treated with a few drops of conc. Nitric acid. The formation of yellow color indicates the presence of proteins.

12. Detection of resins

To 0.5 ml extract, 3-4 ml $CuSO_4$ solution was added separately and the tubes were shaken vigorously for 1-2 minutes. The resulting solution was allowed to separate. The formation of green color precipitate indicated the presence of resins.

13. Test for Triterpenoids

The test for Triterpenoids is the same as that for steroids the appearance of red, pink color or violet color at the junction indicates the presence of Triterpenoids.

14. Test for Anthraquinones

5 mL. of extract was hydrolyzed with dil. H_2SO_4 and extracted with benzene, 1 mL. of dil. NH_3 was added to the above mixture. A pink color indicated the presence of anthraquinones.

B. Preparation of reagents

1. Mayer's Reagent

Mercuric chloride, $HgCl_2$ (0.679 g), was weighed in a 50 mL volumetric flask and dissolved in distilled water. To this solution, 2.5 g potassium iodide (KI) was added. The scarlet red precipitate was dissolved by shaking, and the volume was made up to the mark by adding distilled water.

2. Dragendroff's Reagent

Bismuth nitrate, $Bi(NO_3)_3$ (4.000 g) was dissolved in 5 N nitric acid (10 mL) to make solution A. Next, potassium iodide, KI (13.5 g) was dissolved in distilled water (20 mL) to make solution B. These two solutions were mixed in a 50 mL volumetric flask. Picric

acid (0.25 g) was dissolved in 50 mL of distilled water to make an aqueous picric acid solution. The solution was neutralized with sodium bicarbonate ($NaHCO_3$).

3. Molisch's Reagent

α-Naphthol (5.0 g) was dissolved in 50 mL methanol to prepare Molisch's reagent.

4. Neutral Ferric Chloride ($FeCl_3$) Solution

Ferric chloride crystals (1.0 g) were dissolved in 100 mL distilled water. To this solution, sodium carbonate crystals were added little by little with stirring until the slight turbidity was persistent. Finally, the mixture was filtered, and the colorless filtrate was used in neutral ferric chloride solution.

C. Photographs

Sample Collection

Extraction Process

Rota Evaporator

Extracts

Evaluation of Chemical and Biological Propertie...

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DISTRICT OF NEPAL A DISSERTATION SUBMITTED TO THE DEPARTMENT OF CHEMISTRY AMRIT CAMPUS INSTITUTE OF SCIENCE AND TECHNOLOGY TRIBHUVAN UNIVERSITY NEPAL FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER'S DEGREE OF SCIENCE IN CHEMISTRY By SURESH BISTA **Symbol No: CHE** 1904/076 **TU Regd. No: 5**

-1-33-164-2007 MARCH, 2024 CHAPTER I: INTRODUCTION 1.1 Background Nepal is surrounded by mountains and the Himalayan axis is approximately 800 kilometers long and 150–250 kilometers wide. The nation's total land area is roughly 147,516 square kilometers. On three sides, it borders India; on the other side, it borders China. As a border dividing Nepal from Bangladesh, the narrow Siliguri Corridor in West Bengal is limited in width (Nepal Tourism Board, 2018). Nepal is located between latitudes

26° 20'-30° 10' North and longitudes 80° 15'-88°

19'East. The country's unique geographic location combined with changes in altitude and climate, is the cause of the country's rich biodiversity (Bogs et al., 2006). Located in the heart of the Himalayas, Nepal makes up around one-third (800 km) of the full Himalayan range. It is home to eight of the highest peaks in the whole universe including the well-known Mount Everest (8,848m). Beyond the mountains, Nepal is home to flat terrain, river valleys and deep gorges that combine to provide a unique variety of habitats and a notable amount of biodiversity in a comparatively small region. The nation's 1,47,576 km2 constitute less than 0.1% of the world's land mass but despite this, it is home to an exceptionally diverse range of flora and fauna. More than 2% of the world's flowering plants, 3% of pteridophytes and 6% of bryophytes are found (Paudel et al.,