EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF LEAF EXTRACTS OF *Calotropis gigantea* (Linn) W.T. Aiton.

A DISSERTATION SUBMITTED TO THE

DEPARTMENT OF CHEMISTRY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

KATHMANDU, NEPAL

FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS OF MASTER OF SCIENCE DEGREE IN CHEMISTRY

BY

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Symbol No.: 2167/2077

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May, 2024

DECLARATION

The dissertation entitled **"EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF LEAF EXTRACTS OF *Calotropis gigantea* (Linn) W.T. Aiton"** is being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Nepal, for the partial fulfillment of the Master Degree of Science in Chemistry. It is a research work carried out by me under the supervision of **Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha**, and co-supervision of **Assoc. Prof. Dr. Bhushan Shakya**, Department of Chemistry, Amrit Campus, Kathmandu.

I declare that this dissertation has been composed by myself and has not been published or submitted elsewhere for the requirement of a master's degree program.

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RECOMMENDATION

This is to recommend that **Mr. Keshar Lal Chaudhary** has carried out research entitled **"EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF LEAF EXTRACTS OF *Calotropis gigantea* (Linn) W.T. Aiton"** for the partial fulfillment of the requirements of a Master of Science Degree in **Chemistry** under our supervision. To the best of our knowledge, this work has not been submitted for any other degree.

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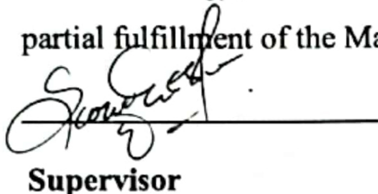

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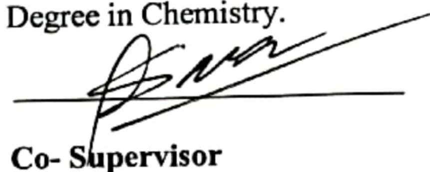

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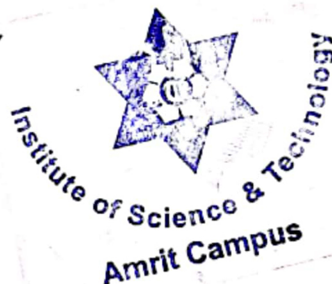

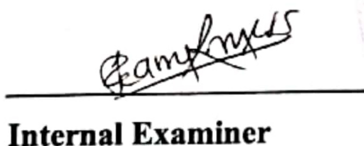

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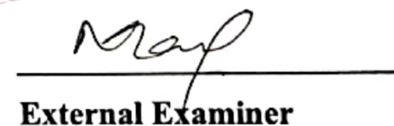

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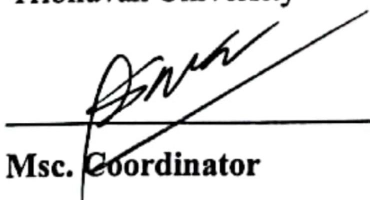

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LETTER OF FORWARD

On the recommendation of **Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha** and Assoc. Prof. Dr. **Bhushan Shakya**, this M.Sc. thesis submitted by **Mr. Keshar Lal Chaudhary** entitled **"EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF LEAF EXTRACTS OF *Calotropis gigantea* (Linn) W.T. Aiton"** is forwarded by the Department of Chemistry, Amrit Campus, Tribhuvan University to the Dean, IOST, T.U.

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ACKNOWLEDGEMENT

I want to start by sincerely thanking my supervisor, **Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha** of the Chemistry Department at the Amrit Campus, for inspiring and mentoring me and for igniting my enthusiasm for research. Throughout the process of investigating and writing my dissertation, I am incredibly appreciative of his enthusiasm, guidance, and continuous support. I feel fortunate to have had the chance to study and develop under his guidance. He has shown me love and support in addition to his supervisory duties, treating me as a valued part of his academic family.

My deepest gratitude goes out to my co-supervisor and M.Sc. coordinator, **Assoc. Prof. Dr. Bhushan Shakya** of the Chemistry Department at the Amrit Campus, and **Prof. Dr. Daman Raj Gautam**, the Head of the Chemistry Department at the Amrit Campus, for providing all the lab space required for this research as well as their gracious assistance.

I express gratitude to Kathmandu Valley College, Kalanki for supplying the laboratory facilities, Himalaya Research Institute of Biotechnology, Bhaktapur for the cytotoxicity and antimicrobial results, Research Institute of Bioscience and Biotechnology (RIBB), Lalitpur for the antidiabetic results, and the Department of Plant Resources for the acute oral toxicity result.

I acknowledge **Itahari Sub Metropolitan City**, **Sunsari** for providing a **support grant** for this research work and thesis.

Additionally, I would like to sincerely thank all of my esteemed lecturers for their advice and assistance throughout the research phase. My sincere appreciation goes out to the lab representatives **Mr. Nanda Krishna Manandhar** and **Mr. Mani Raj Budhathoki** for their assistance in making this research project a success.

Finally, I want to express my heartfelt gratitude to my **family**, soulmate **Sarika Pawe**, elders, and friends Sujan Dhital, Puja Thanait, Nirmal Parajuli, and Mukesh Singh for their ongoing support and encouragement.

Keshar Lal Chaudhary

May 2024

ABSTRACT

Calotropis gigantea (*C. gigantea*) also known as Aank is a flowering plant that belongs to the Apocynaceae family and is a glabrous and lactiferous shrub. This study focuses on investigating phytochemicals and biological activities of *C. gigantea*. The plant's dried leaf powder was ultrasonically extracted using three different solvents: hexane, chloroform, and methanol. Chloroform extract yielded the highest of 16.52 g (1.652%), and hexane and methanol yielded low. The phytochemical screening illustrated the presence of alkaloids, flavonoids, phenolic compounds, cardiac glycosides, terpenoids, tannins, steroids, proteins, carbohydrates, and quinones. The plant leaf extracts were tested for antimicrobial, antioxidant, anti-diabetic, and cytotoxic properties. The antimicrobial susceptibility test was carried out over different bacteria, and hexane extract showed potential antimicrobial activity on Gram-positive bacteria *Bacillus subtilis* (ZOI 1.6 cm). Chloroform and methanol extracts were found to contain high phenolic contents, i.e., 125.7±11.5 mg GAE/g and 48.5±5.1 mg GAE/g, respectively, compared to hexane extract (8.5±2.4 mg GAE/g), and methanol extracts were found to contain high flavonoid contents (51.58±2.0 mg QE/g). In the DPPH free radical scavenging assay, all extracts exhibited weak antioxidant potential. The 3,5-Dinitrosalicylic acid technique was used to assay the α-amylase inhibition properties, and no extracts showed a positive result. In the brine shrimp lethality assay, none of the extracts were found to be toxic (LC_{50}> 500 μg/mL). Thin Layer Chromatography showed different numbers of spots for various extracts. Fourier Transform Infrared Spectroscopy analysis of samples verified the presence of alcohols (-OH), Carbonyl group (=C=O), aromatic ring, nitro compounds (-O-N=O), alkenes, and ethers in the leaf extracts.

Keywords: *Calotropis gigantea*, Extraction, Antimicrobial, α-Amylase Inhibition, Antioxidant, Toxicity.

शोध सार

आँक (*Calotropis gigantea*) एक फूलफुल्ने बिरुवा हो जो एपोसिनासिआइ (Apocynaceae) परिवारमा सम्बन्धित छ र यो एक अस्तुल वा दुध जस्तो द्रव उत्पन्न गर्ने बिरुवा हो । यो अध्ययन बिरुवाको पातको एक्स्ट्र्याक्टको फाइटोकेमिकल र जैविक गतिविधिहरूको अनुसन्धानमा केन्द्रित छ । बिरूवाको सुकेको पातको पाउडरलाई तीन विभिन्न रासायनिक विलायकहरू हेक्सेन, क्लोरोफोर्म, र मेथेनोलबाट अल्ट्रासोनिक एक्स्ट्र्याक्टसन गरियो । क्लोरोफर्मको एक्स्ट्र्याक्टले सबैभन्दा बढी, १६.५२ g (१.६५२%) उत्पन्न गर्यो, र हेक्सेन र मेथेनोलले कम उत्पन्न गर्यो । बिरूवाको पातको एक्स्ट्र्याक्टहरूलाई एन्टीमाइक्रोबियल, एन्टि-अक्सिडेन्ट, एन्टी-डायाबेटिक, र साइटोटोक्सिक गुणहरूको लागि परीक्षण गरिएको थियो । फाइटोकेमिकल स्क्रीनिङले एल्कालोइड्स, फ्लाभोनोइड्स, फेनोलिक यौगिकहरू, कार्डिएक ग्लाइकोसाइड्स, ट्यानिन्स, स्टेरोइड्स, कार्बोहाइड्रेट्स, र क्विनोन्सको उपस्थिति देखायो । एन्टिमाइक्रोबियल संवेदनशीलता परीक्षण विभिन्न ब्याक्टेरियाहरूमा गरिएको थियो, र हेक्सेन एक्स्ट्र्याक्टले ग्राम-पोजिटिव ब्याक्टेरियामा सम्भावित एन्टिमाइक्रोबियल गतिविधि देखायो (१.६ cm ZOI) । क्लोरोफोर्म र मेथेनोल एक्स्ट्र्याकहरूमा उच्च फेनोलिक कन्टेन्ट पाइएको थियो, अर्थात, १२५.७±११.५ mg GAE/g र ४८.५±५.१ mg GAE/g, जसमा हेक्सेन एक्स्ट्र्याक्ट ती दुई एक्स्ट्र्याक्टहरूको तुलनामा निम्न देखियो (८.५±२.४ mg GAE/g), र मेथेनोल एक्स्ट्र्याक्टहरूमा उच्च फ्लाभोनोइड कन्टेन्ट पाइएको थियो (५१.५८±२.० mg QE/g)। डिपिपिएच फ्री रेडिकल स्क्याभेन्जिङ एसे अनुसार, सबै एक्स्ट्र्याक्टहरूले कमजोर एन्टिअक्सिडेन्ट क्षमता प्रदर्शन गरे । डिएनएसए विधि प्रयोग गरेर अल्फा एमाइलेज अवरोधन गुणस्तरहरूको मापन गरियो, र कुनैपनि एक्स्ट्र्याक्टहरूले सकारात्मक परिणाम देखाएन। ब्राइन श्रीम्प लेथालिटी एसेमा, कुनै पनि एक्स्ट्र्याक्टहरूले महत्वपूर्ण साइटोटोक्सिसिटी देखाएन (LC_{50}>५०० µg/mL) । TLC ले विभिन्न एक्स्ट्र्याक्टहरूका लागि विभिन्न स्पटहरू देखायो । नमुनाहरूको FT-IR विश्लेषणले पातको एक्स्ट्र्याक्टहरूमा अल्कोहल (-OH), कार्बोनिल ग्रुप (=C=O), एरोमेटिक रिङ, नाइट्रो कम्पाउन्डहरू (-O-N=O), अल्किनहरू, र इथरहरूको उपस्थिति प्रमाणित गर्यो।

कीवर्डहरू: आँक, एक्स्ट्र्याक्टसन, एन्टीमाइक्रोबियल, अल्फा-एमायलेज इन्हिबिसन, विषाक्तता ।

LIST OF ACRONYMS AND ABBREVIATIONS

mg	:	Microgram
µL	:	Microlitre
AST	:	Antimicrobial Susceptibility Test
B. subtilis	:	*Bacillus subtilis*
cm	:	Centimeter
Conc.	:	Concentrated
DMSO	:	Dimethyl Sulfoxide
DNSA	:	3,5-dinitro salicylic acid
DPPH	:	2, 2- Diphenyl-1-Picrylhydrazyl
E. coli	:	*Escherichia coli*
FCR	:	First Call Resolution
FTIR	:	Fourier Transform Infrared
g	:	Gram
GAC	:	Gallic Acid Concentration
GAE	:	Gallic Acid Equivalent
IC_{50}	:	Inhibitory Concentration 50% Inhibition
IR	:	Infrared
km	:	Kilometer
K. pneumoniae	:	*Klebsiella pneumoniae*
mg	:	Milligram
MIC	:	Minimum Inhibitory Concentration
min	:	Minute
mL	:	Millilitre
nm	:	Nanometer
NMR	:	Nuclear Magnetic Resonance
OD	:	Optical Density
QC	:	Quercetin Concentration
QE	:	Quercetin Equivalent
RSA	:	Radical Scavenging Activity
S. aureus	:	*Staphylococcus aureus*
s	:	Second

TFC	:	Total Flavonoid Content
TLC	:	Thin Layer Chromatography
TPC	:	Total Phenolic Content
UV	:	Ultra-Violet
WHO	:	World Health Organization
ZOI	:	Zone of Inhibition
TE	:	Trolox equivalent
RE	:	Rutin equivalent
FSC	:	Framycetin Sulphate Cream

LIST OF SYMBOLS

μ	:	micro
%	:	percentage
°	:	degree
α	:	alpha
β	:	beta
γ	:	gamma

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Nepal is surrounded by India to the south, east, and west, and China to the north. The Department of Surveys, Government of Nepal, has recently drafted political and administrative borders that put Nepal's entire area at 147,516 km^2. Nepal is primarily a mountainous country made up of three different natural zones: the Terai, the hills, and the mountains (Rai *et al*., 2023). Nepal has a diverse natural and biological environment. Regarding the richness of biodiversity, Nepal is rated 25th in the world and 11th in Asia. Nepal contains 1.1% and 3.2% of the world's known plant and animal species respectively, home to 118 distinct ecosystems (Thapa *et al.*, 2020). Different plant species are found in the country because of its distinct topography and bioclimate (Chaudhary *et al*., 2020), including around 7,000 floral plants (Kunwar *et al*., 2023; U. B. Shrestha *et al*., 2018) and 2,500 medicinal and aromatic plants (Pyakurel *et al.*, 2019). The abundance of medicinal plant species in Nepal increases at elevations of 1,000-2,500 meters above sea level (Bhattarai & Ghimire, 2006). Since ancient times, people have utilized plants to cure a wide range of illnesses. The earliest (4500 BC) medical traditions still in use today are Ayurveda, Traditional Indian Medicine (TIM), and Traditional Chinese Medicine (TCM). The selection, gathering, and preparation of medicinal plants have been well-versed by ancient cultures (Choudhari *et al.*, 2020). According to catalogs, Nepal has between 1515 and 2331 aromatic and medicinal herbs beneficial in easing human suffering. In Nepal, medicinal and aromatic plants have long been utilized for traditional remedies, household economics, and subsistence. These plants help sustain a market economy and are crucial for generating revenue. The nation's high biodiversity, more than 125 ethnic groups, and five distinct physiographic zones all favor the usage of medicinal herbs (Kunwar *et al*., 2022). Of the 300 medicinal plant species that have been sold in Nepal, 273 are employed in ethnomedicine, suggesting that these plants are used not just for basic healthcare but also for family economics and subsistence (Kunwar *et al*., 2022).

The identification of substances derived from plants has evolved over the last 200 years due to the wide variety of knowledge and expertise required. Botanists, ethnobotanists, ethnopharmacologists, and plant ecologists are the first to identify a plant. Before isolating the active component, a phyto chemist tests plant extracts and biological screening assays to determine potential therapeutic effectiveness. Finally, molecular biology research is required to determine the mechanism of action and appropriate

molecular targets. Pharmacognosy is an interdisciplinary approach that is based on the combining of many studies (Ahmed *et al*., 2024). Primary metabolism is the process by which all living things synthesize and break down the building blocks of life proteins, lipids, carbohydrates, and nucleic acids. The substances that are engaged in these pathways are referred to as "primary metabolites." Secondary metabolism refers to the process by which an organism biosynthesizes molecules known as "secondary metabolites" (natural products), which are frequently discovered to be specific to an organism or an indication of a species' originality. In general, secondary metabolites are not necessary for an organism's growth, development, or reproduction. As an alternative, they are either created as a result of the organism adjusting to its environment or as a possible predator defensive mechanism to help the organism survive. Primary biochemical processes like as photosynthesis, glycolysis, and the Krebs cycle yield biosynthetic intermediates, which ultimately result in the generation of secondary metabolites, which are also known as natural products (Dias *et al*., 2012).

Plants create bioactive molecules known as phytochemicals to defend themselves. Phytochemicals can be found in a wide variety of foods, such as whole grains, fruits, vegetables, nuts, and herbs. More than a thousand phytochemicals have been found thus far (Kumar *et al*., 2023). Some significant phytochemicals include carotenoids, polyphenols, isoprenoids, phytosterols, saponins, dietary fibers, and specific polysaccharides. In addition to having potent antioxidant properties, these phytochemicals have antiviral, antibacterial, antidiarrheal, anthelmintic, and antiallergic properties (Ahmed *et al*., 2024). The entire process of creating a new medication, from conception to commercialization, can take twelve to fifteen years and exceed one billion dollars. A target's concept may originate from the business sector, clinical and academic research, or from any combination of these sources. Before choosing a target for an expensive drug discovery effort, a body of supporting evidence may need to be gathered over a number of years (Hughes *et al*., 2011).

The understanding of the evolution of concepts surrounding the use of medicinal plants, along with the rise in consciousness, has made pharmacists and doctors more capable of addressing the issues that have arisen with the expansion of professional services aimed at improving the quality of life for humans (Petrovska, 2012). In industrialized and developing nations alike, plant-based drugs are becoming attractive options for preventive chemotherapy. One kind of secondary plant metabolite that has proven to be useful and tolerated in the treatment of cancer is alkaloids (Dhyani *et al*., 2022).

Medicinal plants or medicinal herbs include bioactive phytochemical components that have clear physiological and pharmacological effects on the human body or the bodies of other creatures (Saha *et al*., 2022). Analysis of the active substances found in herbal products with a natural origin was done to estimate plant potency. The quantitative and qualitative determination of chemicals may both be done using legally recognized and approved methods (Bairagi *et al*. 2021). It has been discovered that the greatest place to get a range of medications is from medicinal plants. In vitro studies have demonstrated the antimicrobial properties of bioactive compounds generated by medicinal plants, including tannins, terpenoids, alkaloids, and flavonoids. There is a rising interest in studying different extracts derived from traditional medicinal herbs as a potential source of novel antimicrobial agents due to the abundance of plants on the planet (Ahmad, 2020). In comparison to allopathic treatment, herbal medicine is growing more and more popular worldwide. Finding novel sources of natural antioxidants, functional foods, and nutraceuticals has drawn a lot of interest to the assessment of the antioxidant capabilities of diverse plants (Patel *et al*., 2014).

With an abundance of therapeutic plant species, Nepal is among the nations with the greatest genetic diversity of medicinal plant species. Its geography and climate vary greatly. There are domestic and international markets for medicinal and aromatic plants. The medicinal value of plants is significant among their many other applications since it is used in traditional medical practices including Ayurveda, Homeopathy, Unani, Chinese, and Tibetan medicines. According to the World Health Organization (WHO), traditional medicine is used as primary care of health by more than 80% of the world's population. The fact that “herbal medicines" are less expensive and safer than synthetic pharmaceuticals has sparked a surge in interest in drugs derived from plants. For thousands of years, natural resources have provided medical agents, and an astounding number of contemporary medications have known their chemical constituents and biological activities since prehistoric times (Timilsina *et al*., 2020).

Plants that naturally produce and accumulate secondary metabolites such as alkaloids, glycosides, tannins, volatile oils, minerals, and vitamins have been recognized for their therapeutic potential. Many necessary elements, including vitamins, minerals, proteins, carbohydrates, tannins, alkaloids, bitters, and flavonoids, are found in plants. Every plant part has distinct features and purposes (Singh *et al*., 2014). Finding commercially valuable compounds such as flavonoids, tannins, gums, essential oils, and precursors for

complex chemicals, as well as medicinal medicines, requires an understanding of the molecular structure of plants (Shrestha *et al*., 2015).

1.2 Medicinal Plants in Nepal

Nepal reported 8737 uses of 1762 different medicinal plant species. With district references, the first modern documentation of more than 1700 medicinal plant species utilized in Nepal is recorded. Of the species, 129 belonged to the Asteraceae family, followed by 114 Fabaceae, 65 Lamiaceae, 54 Rosaceae, and 53 Poaceae (Kunwar *et al*., 2022). Owing to considerable changes in temperature, geography, and altitude, Nepal displays a great floral variety with over 6500 species of blooming plants, 2000 of which are extensively utilized in traditional medicine. Therefore, Nepal is a country with diverse ethnic groups, unique topography, and rich floral diversity. The little nation holds a unique position in the globe for its ecological and cultural richness because of its broad altitudinal variance and different weather conditions (Pangeni *et al*., 2020). Medicinal and aromatic plants (MAPs) provide both economic and health advantages that enhance human well-being. About 3,000 of the 28,000 plant species that are now recognized to have medicinal applications are traded on a local, regional, and global scale. Natural materials, most of which are MAPs, provide the basis for more than 25% of recently approved medications. For example, medicinal and aromatic herbs are the source of more than 70% of anticancer medications (Shrestha *et al*., 2022).

1.3 Description of *Calotropis gigantea*

The Apocynaceae family contains the flowering plant *Calotropis gigantea*, which was originally described in 1810 (Deshpande *et al*. 2014).). Due to the latex they generate, they are sometimes referred to as milkweed (Julius *et al*., 2021). *C. gigantea* is a member of the Asclepiadaceae, often known as the Milkweed or Aak family, which has 2,000 species and 280 genera with a global distribution but rich in the sub-tropics and tropics and rarely found in cold places. *C. gigantea*, often known as milkweed or swallowwort, is a tiny tree or shrub that grows to a height of 3-4 meters and is lactiferous and glabrous or hoary. It has upright stems that can reach a diameter of 20 cm (Timilsina *et al*. 2020). The bark is corky and fissured, while the roots are simple, branched, and woody at the base. The leaves are exstipulate, simple, subsessile, and opposite-decussate, with an oval to fully obovate margin. Flowers have a pedicel that is 1-3 cm long, are bracteate, complex, antimorphic, pentamerous, hypogynous, and bisexual. Fruits range in shape

from simple, juicy, subglobose to obliquely oval follicles up to 10 cm in diameter (Gharge *et al*., 2017).

Native to much of South Asia, including Iran, Pakistan, Nepal, Sri Lanka, and India, C. gigantea is also found in most of South East Asia, including China, Laos, Thailand, Vietnam, Malaysia, Myanmar, and the Philippines (Al Sulaibi *et al*. 2020). Its natural plantation is found in semi-arid settings from sea level to a height of 1300 m, with annual rainfall ranging from 150 to 1000 mm (Deshpande *et al*. 2018).

1.3.1 Chemical Constituents

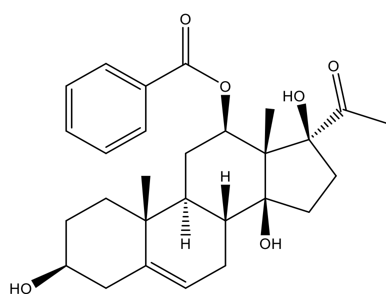
The compounds identified in the leaves included azulene, benalaxyl, cisvaccenic acid, levomenol, profenofos, β-tocopherol, and β-sitosterol (Sharma *et al*., 2016). Tests on human and rat cell lines were conducted to determine the cytotoxicity and efficacy of many isolated substances, including calotropin, frugoside, and 4'-O-β-D-glucopyranosyl frugoside. Calotropin (IC_{50} =15 mg/ml) showed the highest degree of action among them. Likewise, Calotropnaphthalene, Calotropone, Stigmasterol, 15β -hydroxycalactinic Acid, etc are some chemical constituents found in the plant (Kadiyala *et al.*, 2013). The chemical constituents extracted from *C. gigantea* are given in the table below (Negi & Bisht, 2021).

Table 1.1 : Chemical constituents of *C. gigantea.*

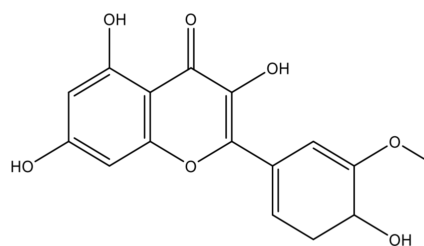
Class of Chemical Constituent	Name of Chemical constituent	Plant Part Used	Extract Taken
Triterpenoids	Di-(2-ethylhexyl) Phthalate	Flowers	Ethyl extract acetate
	Anhydrosophoradiol-3acetate		
	Lupeol	Aerial parts	Latex
	α -Taraxerol	Root barks	Ethyl extract acetate
Triterpene esters	γ -Taraxasterol	Aerial parts	Hexane and methanol soluble extract
Flavonol	Isorhamnetin	Aerial parts	Methanol extract
Cardiac glycosides	Calotropone	Roots	Ethanol extract
	Gofruside		
Steroids	Stigmasterol	Root barks	Methanol extract

	β -Sitosterol		
	β -Sitosterolacetate		Ethyl acetate extract
Resin	β -Amyrin	Root barks	95% Alcohol
	β -Amyrin acetate		
Fatty acids	Isovaleric acid	Root barks	95% Alcohol extract

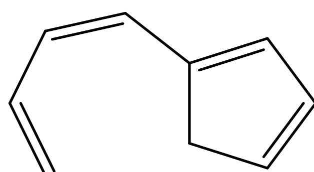
The structures of some chemical constituents are;



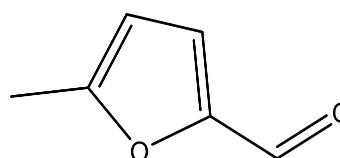
Calotropone



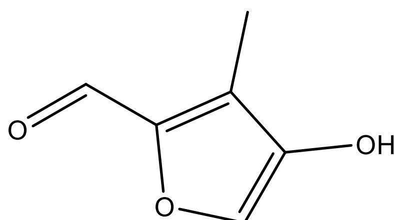
Isorhamnetin



Azulene



5- Methyl furfural



Hydroxymethyl furfural

Figure 1.1: Chemical constituents present in the *C. gigantea*

1.3.2 Medicinal and Traditional Uses

In Ayurveda; paralysis, swellings, and sporadic fevers are all treated with *C. gigantea* leaves. Flowers are used to treat inflammations, fevers, helminth infections, anorexia, asthma, and catarrh. The plant's root bark is useful for ascites, coughs, helminth infections, intestinal worms, and skin diseases. Asthma, bronchitis, dyspepsia, and other conditions can be treated with powdered root, which also encourages gastrointestinal secretions. In Siddha; the leaves of *C. gigantea* are used to cure ulcers, intestinal worms, vatha illnesses, periodic fever, and dangerous snake bites. By pressing hard over the bitten region, the plant's roots are properly pulverized and applied. This plant's latex is used to treat many rheumatic conditions, including gonococcal arthritis, swellings, rat bites, and dental issues. Flowers can be used as a treatment for bronchial asthma (Kumar *et al*. 2011). It is very crucial in medicine, and it is believed that different sections of this plant have several therapeutic effects. Dysentery and elephantiasis are treated with the root bark, while colds, coughs, asthma, and indigestion are relieved by using the blossoms sparingly (Singh & Javed, 2015). Analgesic, antibacterial, and cytotoxic properties are said to exist in the blooms. It has been stated that the plant's leaves and aerial parts have anti-diarrheal, anti-candida, antibacterial, anti-antioxidant, and antidiabetic properties (Gyawali *et al*., 2020). An application of root juice to the abdomen and vaginal region during childbirth reduces labor pains, and latex is used to treat a range of skin conditions. Asthma is treated by boiling the dried flowering powder (usually 2-4) in molasses. Fresh leaves were used to cure seizures and fit in youngsters. Leaf extracts were also used to treat rheumatic symptoms, together with rock salt, ear-ache-relieving oils, and fresh, warmed leaves. Used for burns, headaches, rheumatic pains, and as a tincture for fever the leaves are crushed, warmed, and applied (Timilsina *et al*. 2020). The whole plant is used as a tonic and purgative, especially for skin conditions including boils and ulcers. Crushed and heated, the leaves are given to burns, headaches, rheumatic symptoms, and as a fever medication. Even the flower infusion is used to cure epileptic seizures, intestinal worms, and rheumatism. Warm, fresh leaves were beneficial for rheumatic pains, and an extract of the leaf, along with rock salt, was used to cure convulsions. Oil was also used to treat earaches (Kadiyala *et al*., 2013).

1.3.3 Classification of Plant

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Subphylum	:	Euphyllophytina

Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Gentianales
Family	:	Apocynaceae
Subfamily	:	Asclepiadoideae
Tribe	:	Asclepiadeae
Genus	:	*Calotropis*
Species	:	*gigantea*
Botanical name	:	*Calotropis gigantea* (Kovendan *et al*., 2012)

(a) **(b)** **(c)**

Figure 1.2: *Calotropis gigantea* (a) flower and leaves (b) flowers (c) the whole plant

1.4. Rationale of Study

Chemists have long been interested in plant extracts because they have been used in traditional medicine for so long. There has been impressive evidence in this field, and many new biologically active compounds have been discovered as a result of recent advancements in the technologies for isolating and determining the structure of active principles, even small amounts of them were isolated, and their structure was determined. The goal of this study is to comprehend the most recent approaches, methodologies, and interest in finding bioactive compounds from the aforementioned plant and how their behaviors might extend the findings of previously unexplored species and well-known themes.

1.5 Objective

1.5.1 General Objective

Evaluation of phytochemical constituents and biological activities of *C. gigantea* leaf extract.

1.5.2 Specific Objective

- Extraction of constituents with different solvent systems (methanol, hexane, and chloroform) through ultrasonication of *C. gigantea* leaves.
- Phytochemical screening, thin layer chromatography, and FTIR of the extracts.
- Evaluate the total phenolic content and total flavonoid content of the extracts.
- Study of the bioactivities of the extracts like antimicrobial, antioxidant, antidiabetic, and cytotoxicity of the plant.

CHAPTER 2: LITERATURE REVIEW

From *C. gigantea*, chemicals like calotropin, coroglaucigenin frugoside, and glucoside were extracted and were found to be cytotoxic to multiple cell lines of human and mouse origin. Human small cell lung cancer (NCI-H187), human oral epidermal carcinoma cell lines, and human breast cancer cell lines were found to be cytotoxic by several substances isolated from *C. gigantea* leaves, including 18,20-epoxycalotropin, calactin, zarigenin, calotropin, 15hydroxycalotropin, and calactinic acid methyl ester (Jacinto *et al*., 2011).
A preliminary phytochemical study of stem bark using the Soxhlet extraction technique revealed the presence of alkaloids, flavonoids, glycosides, phenolic compounds, tannins, saponins, sterols, proteins, and amino acids. A standard analytical method was employed to examine the stem bark water extracts, chloroform, methanol, and petroleum ether extracts for the first phytochemical screening (Kumar *et al*. 2021).
Studies on the α-Amylase inhibition, antioxidant activity, and phytochemicals of *C. gigantea* were performed by different tests. The DPPH test was used to measure the antioxidant activity. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed on one of the eleven CC fractions. The methanolic extract was shown to have sufficient antioxidant activity (IC_{50} 268.80 µg/mL) when compared to ascorbic acid (141.82 µg/mL). Out of all the chemicals found by GC-MS of the ethyl acetate fraction, 5hydroxyl methyl furfural was the primary furan compound (59.49%). The identical fraction showed an IC_{50} value of 0.94 mg/mL for inhibiting α-amylase activity. *C. gigantea*, a plant native to Nepal, has antioxidant and amylase-inhibitory qualities (Gyawali *et al*., 2020).

When the phytochemical content of *C. gigantea* leaves extracted in methanol and petroleum ether was examined, a significant number of bioactive secondary compounds, including alkaloids, tannins, saponin, flavonoids, and glycosides, were found (Singh *et al*., 2014). A comparative study of the chemical composition of the essential oil of *C. gigantea* fruits, leaves, and flowers was carried out using mass spectrometry and gas chromatography. Using the total ion chromatogram of oil, a total of 21 compounds from the flower, 43 from the leaf, and 21 from the fruit were discovered (Singh and Javed, 2015).

Sethi used extracts of water, methanol, and chloroform to study the phytochemicals from *C. gigantea* leaves. The presence of alkaloids, steroids, tannins, flavonoids, carbohydrates, gums, and mucilages were all mentioned in the research (Sethi, 2014).

Ishnava *et al*., did a phytochemical analysis of chloroform latex extract of *C. gigantea*. To choose an appropriate solvent solution for the chromatogram development, analytical TLC was carried out. The chloroform: methanol (8:2) solvent solution was chosen as the finest of all and was utilized for further study. From the latex of *C. gigantea,* they isolated alkaloids, cardiac glycosides, phenolic compounds, steroids, and terpenes (Ishnava *et al*., 2012).

Dhivya and Manimegalai carried out a study on the ethanol flower extract of *C. gigantea* that revealed the presence of pharmacologically active constituents. The floral extract included alkaloids, tannins, phenols, flavonoids, sterols, anthraquinones, proteins, and quinones but lacked terpenoids and saponins. A mass experiment was carried out using a GC (T8000 Top CE) linked with a mass spectrometer (Md 800 FIS ONS). Every test was performed on the positive ion mode. The GC-MS analysis yielded 14 main peaks, each of which suggested the existence of different phytochemical substances with potential medical uses (Dhivya & Manimegalai, 2013).

The methanolic extract of the root bark of *C. gigantea* isolated stigmasterol and beta-sitosterol by identifying structures with different spectroscopic techniques. They used heat extraction on powdered root bark that had been dried. Thin-layer chromatography (TLC) was used to fractionate the crude methanol (MeOH) extract using petroleum ether, chloroform, and ethyl acetate. This resulted in the crystallization and naming of CG-1 (*Calotropis gigantea*-1) and CG-2 (*Calotropis gigantea*-2). Both had good results for steroids and alcohol. The melting points for CG-1(176) and CG2(133) agreed well with those for stigmasterol and beta-sitosterol. The chemical shifts of stigmasterol's H-22 and H-23 in the 1H-NMR spectra of CG-1 were similar. Similarly, the beta-sitosterol structure and the 1H-NMR data of CG-2 were in close agreement (Habib *et al*., 2007).

Studies by Dutta *et al*. showed that ethanolic extract of *C. gigantea* has antibacterial effects against *Bacillus subtilis*. Utilizing the agar well diffusion technique, the antibacterial activity of the crude extracts was assessed. *Bacillus subtilis* and *E. coli* cultures were spread out onto Muller Hinton agar plates. Different extractions were then put in a given volume (1 ml) together with controls (sterile water, methanol, ethanol, ethyl acetate). At 37°C, all of the plates were incubated for 24 hours. Additionally, *C. gigantea* ethanolic extract has shown notable antibacterial efficacy against *Escherichia Coli.* When leaf and stem extracts are combined in an identical ratio (1:1), the inhibitory zone is less pronounced than with other separate extracts. According to the findings,

several of the elements in the blend of leaf and stem extracts had a synergistic effect against bacteria (Dutta *et al*., 2015).

The leaves of *C. gigantea* ethanolic extract were evaluated by Alafnan and others in 2021 to determine phytochemicals, total antioxidant capacity, and wound healing capacity. The results demonstrated the substantial ability of the studied extract to scavenge DPPH (67.90 mg TE/g extract) and ABTS (89.67 TE/g extract) radicals. In the same way, the total antioxidant capacity of the examined extract for the phosphomolybdenum assay was provided with a value of 1.71 mg TE/g extract. The studied extract was found to have high levels of phenolic (33.71 mg GAE/g extract) and flavonoid (46.75 mg RE/g extract) compounds. Positive and negative ionization techniques of secondary metabolite UHPLC-MS analysis suggested the possible existence of 17 distinct phytocompounds, mostly sesquiterpene, alkaloid, and flavonoid derivatives (Alafnan *et al*., 2021).

The investigation was conducted by Deb *et al* to see the wound healing capacity of latex of *C. gigantea* on healthy, 150–250 g, Wistar albino rats of either sex and the same age. The animals were split into three groups, Group I received no therapy, Group II received Framycetin sulphate cream (FSC) (1% w/w), and Group III received *Calotropis gigantea* latex (200 mg/kg/day). The healing potential of *Calotropis gigantea* latex was examined using excision and incision wound models. The drug was shown to be safe up to a dosage of 2 g/Kg of the animal's body weight, according to acute toxicity testing. Animals treated with latex had wound areas that were 83.42% less than those of controls. The ability of the latex from *Calotropis gigantea* showed significant wound healing similar to FSC (Framycetin sulphate cream) (Deb *et al*., 2009).

C. gigantea has anti-inflammatory qualities in the body. Its inherent purgative and detoxifying effects facilitate the early healing of wounds, itchiness, irregularities of the skin, and gall bladder. Chloroform and ethanol extracts made from *Calotropis gigantea* flowers have been shown to have anti-inflammatory effects on sodium alginate oedema in transgenic mice. Its alkaloid component has a mild inflammatory effect. For a good cure of corn on the skin, plant milk and stem bark are both excellent treatments. When given orally, 400 mg/kg of *C. gigantea* was more effective in reducing inflammation than 100mg/kg of Ibuprofen (Saha *et al*., 2022).

Various human and mouse cell lines have reportedly been shown to be susceptible to the cytotoxic effects of cardenolide glycosides isolated from the *C. gigantea* root. The active ingredients were determined to be calotropin, frugoside, and 4'-O-

Dglucopyransylfrugoside. An ethanol extract of *C. gigantea* roots included two substances that were reported to have inhibitory effects on human gastric cancer and chronic myelogenous leukemia K562 cell lines. An ethyl acetate crude extract from *C. gigantea* flowers was shown to prevent Ehrlich's ascites cancer in mice. The number of viable tumor cells and the body weight gain brought on by the tumor burden and extended survival time are dramatically reduced by an intraperitoneal injection of the extract at dosages of 50, 100, or 200 mg/kg. The haematological and biochemical indicators (glucose, cholesterol, triglycerides, blood urea, ALP, SGPT, and SGOT) that were changed during tumor growth mitigate with a dosage of 200 mg/kg body weight of the extract (Kumar *et al*., 2011).

Pathak and Argal employed Swiss mice of either sex that weighed between 18 and 22 g as a test animal. Acetic acid-induced writhing and Eddy's hot plate technique were used to assess the analgesic efficacy. In the hot plate technique test, there was a considerable amount of activity 30 minutes after the dosage , and the greatest analgesic effect was seen 90 minutes later. At 500 mg/kg, the effects were similar to those of a popular medication. The plant's long history as a possible painkiller provided significance to the findings (Pathak & Argal, 2007).

In comparison to ascorbic acid (141.82 µg/mL), the antioxidant activity of the methanolic extract was determined to be adequate (IC_{50} = 268.80 µg/mL). The GC-MS of the ethyl acetate fraction found a total of 17 compounds, with 5-hydroxyl methyl furfural being the main furan compound (59.49%) (Gyawali *et al*., 2020).

Timilsina and others studied the phytochemical, antimicrobial, and ethnobotanical characteristics of *C. gigantea*. For *Escherichia coli* and *Staphylococcus aureus*, the ZOI shown by methanol extracts of *C. gigantea* leaves was measured at 8 mm and 13 mm, respectively. In a related study, the zones of inhibition induced by *C. gigantea* leaf hexane extract for *S. aureus* and *E. coli* were measured to be 9 mm and 11 mm, respectively, in an antibacterial experiment. Antimicrobial effectiveness against *Klebsiella pneumoniae* was not demonstrated by any of the extracts (Timilsina *et al*. 2020).

Leaf, flower, and root sections all had mildly detectable levels of alkaloids. Flowers were strongly identified with tannins, saponins, and flavonoids. Flowers, leaves, and roots all have proteins, but roots do not. Flowers and leaves have significant levels of diterpene, but roots have none. The antioxidant content of *C. gigantea* leaves was higher (45.2%)

than that of the roots (43.9%) and flowers (32.5%). Compared to the leaf extract (2.194 mg/100 g), the floral extract (2.2 mg/100 g) of *C. gigantea* contained higher quantities of flavonoid components (Rehman *et al*., 2023).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of Plant Materials

The plant species of *C. gigantea* were collected at an altitude of 185 meters in Ramdhuni Municipality, Sunsari District, Nepal, to preserve the distinctive chemical and biological characteristics of the species and to facilitate the botanist's examination and confirmation. After that, the extraction procedure was carried out after gathering the *C. gigantea* leaves.

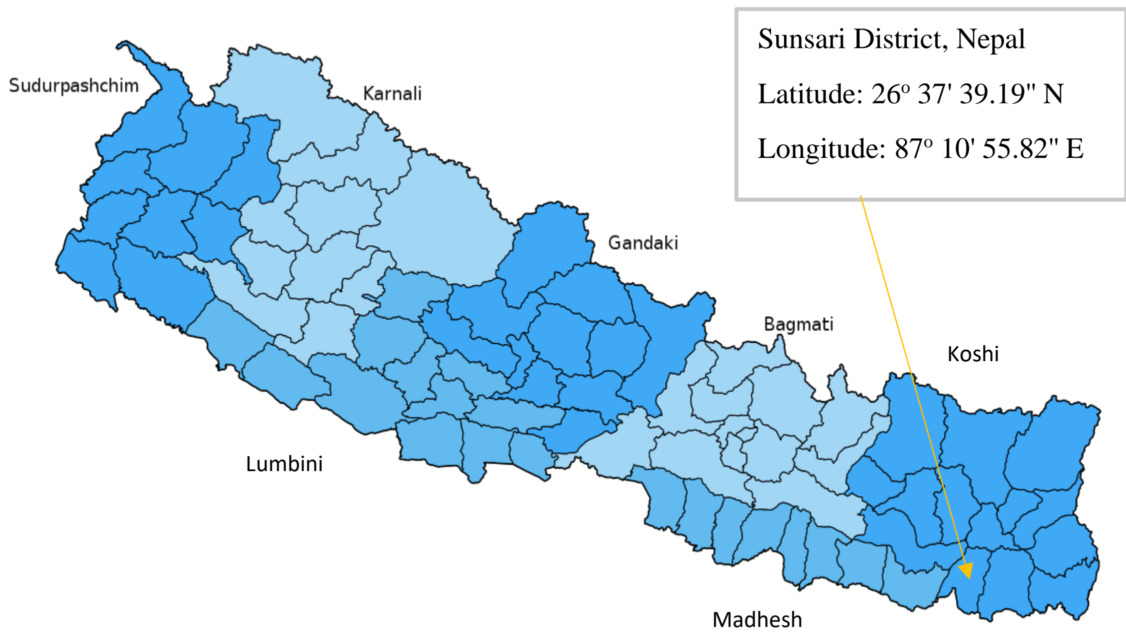


Figure 3.1: Collection site of *C. gigantea*

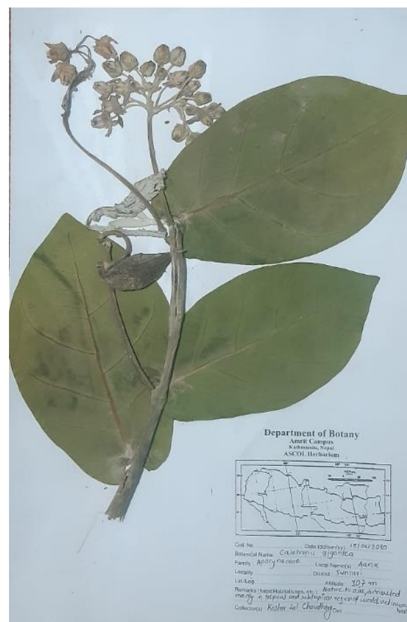


Figure 3.2: Identification of *C. gigantea*

3.1.2 Chemicals required

- Hexane (Fischer Scientific), chloroform (Fischer Scientific), and methanol (Fischer Scientific) used were of analytical grade. Distilled water was available in the laboratory.
- 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 provided in the laboratory.

3.1.3 Instrument and Equipment

The following instruments were used during the study;

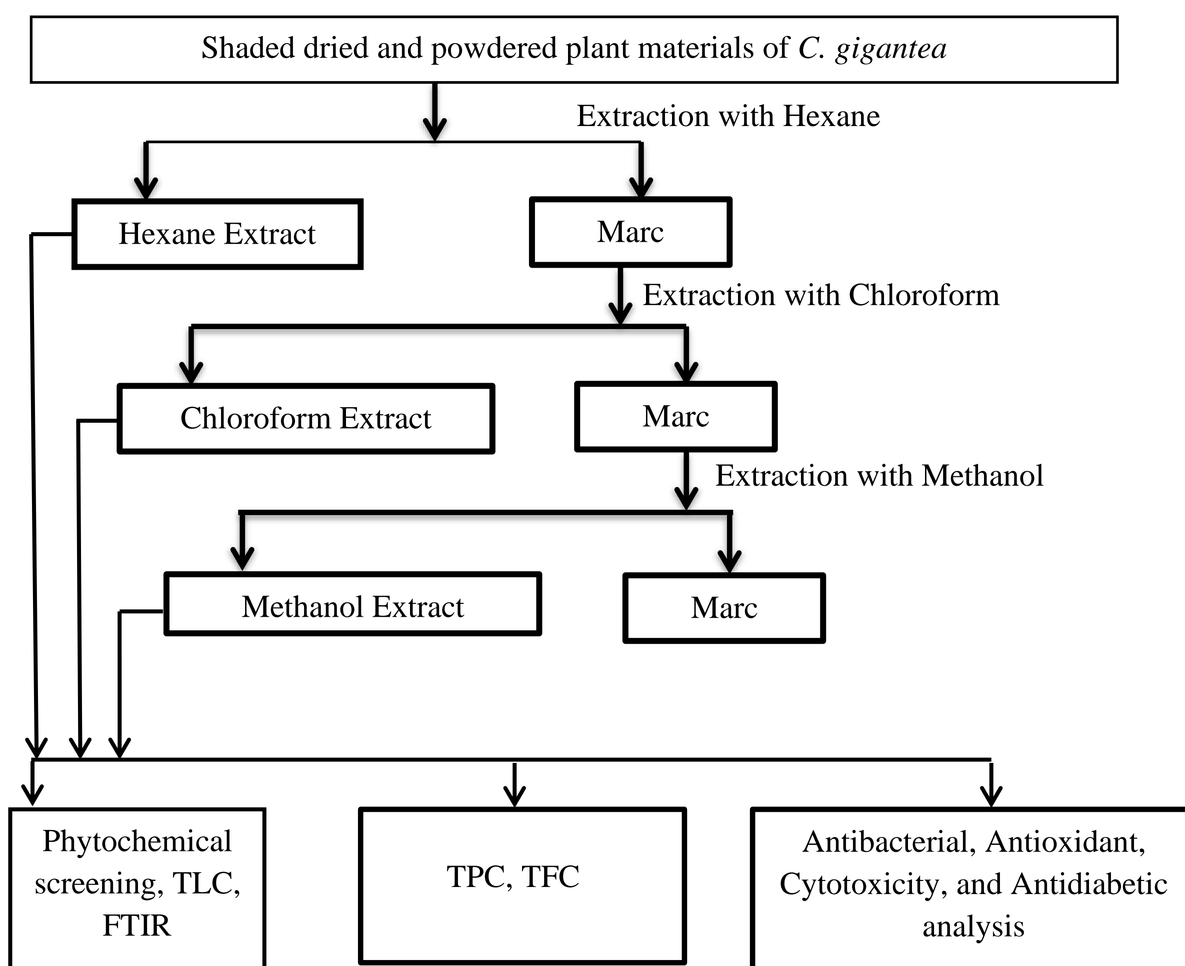
i. Digital balance
ii. Grinder
iii. Beakers
iv. Measuring cylinder
v. Sonicator
vi. FT-IR (PerkinElmer Spectrum IR; Version 10.6.2)
vii. UV lamp (UV 2510TS)
viii. Double beam UV-Vis spectrophotometer (Labtronics LT-2802)
ix. Micropipettes
x. Conical flasks
xi. Test tubes
xii. Vial tubes
xiii. Water bath
xiv. Rota evaporator (IKA, RV 10 D S96)
xv. Precoated TLC

3.2 Methods

3.2.1 Preparation of plant extracts

The entire *C. gigantea* plant material was rinsed with running water to eliminate dust. The plant samples were then air-dried for about 4 weeks in the shade at room temperature before being weighed and crushed to a fine powder with an herbal medicine disintegrator. The powdered plant components were kept in a clean container at a low temperature until they were needed. An ultrasonic extraction method was one of the several extraction

techniques used to create the numerous leaf extracts. During this procedure, a 5000 mL capacity conical flask that was dry and clean was used to hold 1000 g of powdered plant leaves. All of the solvents employed in the extraction procedure had a leaf powder to solvent ratio of around 1:3 after 2.5 L of hexane had been added to the flask and stirred. The bath was filled to one-third capacity with distilled water, and the flask containing the plant material was extracted in the ultrasonic cleaner bath for approximately 1.5 hours. The operating frequency was 40 kHz, with a total power output of 350 W. Following extraction, the flask was decanted and filtered. The filtrate was concentrated with a rotatory evaporator. To be used later, the concentrated extract was weighed, dried in a water bath, and sealed in an airtight container. Likewise, hexane was used to sonicate the filter residue. To obtain the crude extract, a similar procedure was used three times. The marc that was left over from each extraction was subsequently extracted using methanol and chloroform in the sequence specified by the solvent polarity.



Schemes 3.1: Research process for extraction, screening, chemical analysis, and biological activities.

3.2.2 Determination of percentage yield

Following the extraction procedure, the extract's % yield was computed using the formula given by Anokwuru *et al.*, 2011(Anokwuru *et al.*, 2011).

$$\%\ \text{Yield} = \frac{\text{Dry weight of ectract}}{\text{Dry weight of plant sample}} \times 100\ldots\ldots\ldots\ldots..(1)$$

3.2.3 Phytochemical Screening

Numerous plant chemicals, such as alkaloids, saponins, flavonoids, stimulants, tannins, and others, have been found through phytochemical investigations as potentially responsible for the pharmacological characteristics of plants. Finding the bioactive components of medicinal plants, which may help formulate new drugs and enhance existing ones, requires the phytochemical screening of plants (Khan *et al.*, 2024). Several extracts were the subject of a phytochemical investigation. The technique outlined by Banu & Cathrine (2015) served as the foundation for the phytochemical screening strategy. In general, phytochemical screening helps to discover the bioactive substances that are present in plants (Banu & Cathrine, 2015). The primary classes of natural components included in the various plant extracts were determined by utilizing a color reaction and a variety of specialized reagents. An extensive description of the reagent preparation process may be found in Appendix A.

3.2.4 Total Phenolic Content

Using Folin-Ciocalteu colorimetric technique, which is based on an oxidation-reduction process, the total phenolic content of the plant extract was determined (Du *et al.*, 2018).

3.2.4.1 Preparation of the Standard Gallic Acid Solution

To make a 1000 µg/mL stock solution of gallic acid, 50 mg of the acid was first dissolved in 50 mL of 30% DMSO. The stock solution was then serially diluted to produce various concentrations of gallic acid, including 250 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL.

3.2.4.2 Construction of the Calibration Curve

To establish the calibration curve, one milliliter of each concentration of the generated gallic acid solutions was collected and placed in a beaker, with one milliliter of DMSO serving as the blank. Each beaker was filled with one milliliter of Folin-Ciocalteu reagent (FCR), and the mixture was allowed to stand for five minutes. Each mixture was well shaken before 10 mL of a 7% Na_2CO_3 solution was added. 13 milliliters of distilled water

were then added. For ninety minutes, the final reaction mixture was incubated at 23°C. After the incubation period, the absorbance of each concentration and the blank solution were measured using a UV spectrophotometer set at 750 nm. To get triplicate data, the process was repeated twice more. Utilizing the average absorbance readings at various gallic acid dosages, the calibration curve was produced.

3.2.4.3 Preparation of the Sample Solution

A fixed volume of 100% DMSO was mixed with a little quantity of the component to make a stock solution that contained 5,000 µg/mL of the extract. To get 1000 µg/mL of extract, 1 mL of stock solution was diluted in 4 mL of 30% DMSO. The absorbance measurements were performed in triplicate using the same procedure as previously reported for gallic acid.

3.2.4.4 Calculation of the Total Phenolic Content (TPC)

The total phenolic content was calculated using Equation 3,

The total content of the phenolic (C) = $\frac{c \times V}{m}$ ………… (3)

Where,

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

c = Concentration of gallic acid established from the calibration curve (mg/mL)

V = Volume of extract (mL)

m = Weight of the plant extract (mg)

Data were recorded as a mean of three determinations absorbance determinations for each concentration, from which the linear correlation coefficient (R^2) value was calculated.

The regression equation is given as,

$$y = mx + c \quad \text{…………} \ (4)$$

Where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, the concentration of the extract was calculated. Thus, with the calculated value of the concentration of the extract, the total phenolic content was calculated from equation (2).

3.2.5 Total Flavonoid Content

The aluminium chloride colorimetric assay was used to measure the total flavonoid content of the plant extract (Pallab *et al.*, 2011).

3.2.5.1 Preparation of the Standard Quercetin Stock Solution

To make a stock solution with a concentration of 1000 μg/mL (1 mg/mL), 10 milligram of quercetin was weighed and dissolved in 10 mL of 100% DMSO. The stock solution was serially diluted to obtain quercetin solutions with concentrations of 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, and 3.125 μg/mL.

3.2.5.2 Construction of Calibration Curve

Separate test tubes were filled with 400 μL of each concentration of the produced quercetin solution, and 400 μL of DMSO was taken in one test tube for blank. 100 μL of 0.3M $AlCl_3$, 1.2 mL of ethanol, 2.2 mL of distilled water, and 100 μL of 10% sodium potassium tartarate solutions were added to each test tube. After thoroughly stirring the mixture, it was let to rest at room temperature for 30 minutes in the dark. After incubation, the UV spectrophotometer was used to determine the absorbance at 760 nm for each concentration and the blank solution. To get triplicate data, the procedure was repeated. The calibration curve was then plotted using the average absorbance values obtained at various quercetin doses.

3.2.5.3 Preparation of the Sample Solution

To generate a stock solution of 5,000 μg/mL, a tiny quantity of extract was dissolved in a determined volume of 100% DMSO. The extract was concentrated to 1000 μg/mL by dissolving 1 mL of stock solution in 4 mL of 30% DMSO. Absorbance was determined in triplicate using the same approach as for the standard quercetin.

3.2.5.4 Calculation of the Total Flavonoid Content

The following formula was used to calculate the total flavonoid content of the extract,

$$\text{Total Flavonoid Content (C)} = \frac{c \times V}{m} \quad \ldots\ldots\ldots\ldots (5)$$

Where,

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

c = Concentration of quercetin established from calibration curve in mg/ml

V = Volume of the extract (in mL)

m = Weight of the plant extract (in g)

3.2.5.5 Statistical Analysis

To compute the Linear Correlation Coefficient (R^2) value, the data were recorded as the mean of three absorbance readings for each concentration.

The regression equation is given as,

$$y = mx + c \text{ ……….. (6)}$$

Where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract,

c = Intercept

This regression equation was used to determine the extract concentration. Equation (4) was utilized to determine the flavonoid content based on the extracted concentration value that was computed.

3.2.6 Antioxidant Activity

The antioxidant strategy was developed using a technique that Blois M.S. (1958) detailed. The various techniques used to measure the antioxidant activity of plants may yield varied results, depending on the particular free radical that is employed as a reactant. One rapid, simple, and inexpensive method of determining antioxidant capacity is to use free radicals (DPPH). The effective sample concentration required to scavenge 50% of the DPPH free radicals is known as the IC_{50} value or 50% inhibitory concentration. By graphing the extract concentration against the associated scavenging effect, the inhibition curve was utilized to determine the IC_{50} values.

3.2.6.1 Preparation of the 0.1 mM DPPH Solution

The molecular weight of 2,2-Diphenyl-1-picrylhydrazyl-hydrate (DPPH) is 394.32 g/mol. 10mg of DPPH were weighed and then dissolved in a tiny volume of methanol to create 50 milliliters of 0.5 milligrams of DPPH. After that, the mark was filled with methanol and thoroughly shaken. Subsequently, 10 mL of the 0.5 mM DPPH solution was diluted and added to a 50 mL volumetric flask, which was then filled to the brim with 40 mL of methanol to create a 0.1 mM DPPH solution. The processes were carried out in a dark atmosphere, and the DPPH solution was kept until required.

3.2.6.2 Preparation Quercetin Solution (Standard)

To prepare a stock solution of 1000 µg/mL (1mg/ml), 10 milligrams of quercetin was weighed and diluted in 10 mL of 100% DMSO. Quercetin solutions with concentrations

of 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.56 µg/mL, and 0.78 µg/mL were obtained via serial dilution.

3.2.6.3 Preparation of Sample Solution

To prepare a stock solution of 5,000 µg/mL, a tiny quantity of extract was dissolved in an established volume of 100% DMSO. To obtain a concentration of 1000 µg/mL, a 1 mL stock solution was dissolved in 4 mL 30% DMSO. Extract solutions with concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, and 7.81 µg/mL were created using serial dilution.

3.2.6.4 Measurement of DPPH Radical Scavenging Activity

Separate test tubes were filled with serially diluted standard quercetin (1 mL), followed by 1 mL of 0.1 mM DPPH solution in a dark environment. Similarly, 1 mL of 30% DMSO was combined with 1 mL of DPPH and served as the blank. After 30 minutes of dark incubation at 37°C, the absorbance at 760 nm was measured with a UV spectrophotometer. Experiments were conducted in triplicate, and the percentage of DPPH free radical scavenging activity at each concentration was estimated using the following relation:

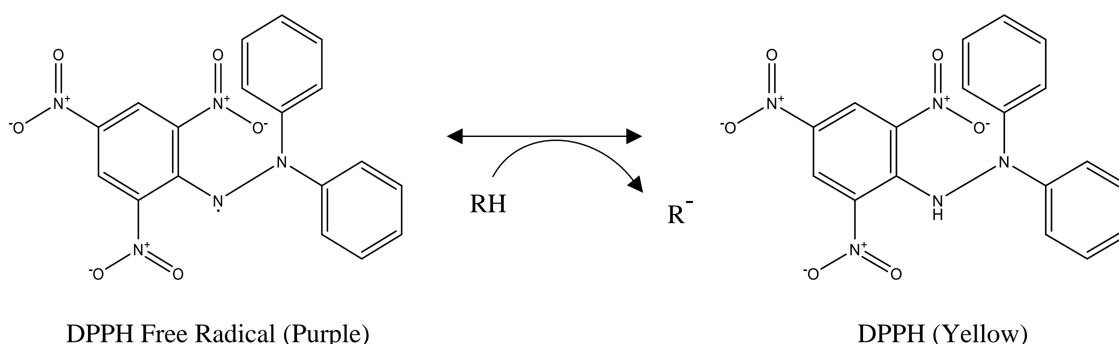


Figure 3.3: Mechanism of DPPH radical scavenging

Experiments were conducted in triplicate, and the percentage of DPPH free radical scavenging activity at each concentration was estimated using the following relation:

DPPH Scavenging Activity (%) = $\frac{Ao - As}{Ao} \times 100$………………..(7)

Where,

A_o = Absorbance of the control (DPPH solution + DMSO)

As = Absorbance of the test sample

Each plant extract was tested for free radical scavenging at a concentration of 500µg/mL using the same technique. Extracts having over 50% scavenging at 500µg/mL

concentration were serially diluted to assess percentage scavenging. The extract's IC_{50} value was determined by graphing % scavenging against concentration.

3.2.7 Antimicrobial Activity

Bacterial growth inhibition was examined using an agar well plate technique and assessed using the zone of inhibition (ZOI) given by Dingle et al. Biological screening investigates the impact of a certain dose level of a crude plant extract or fraction on the species of organisms. Antibacterial tests were conducted in this investigation. The antibacterial activity of crude plant extracts was tested using the agar well diffusion method.

The extracts were dissolved in 50% DMSO in water. Tubes were sealed and refrigerated at 4°C until use. The normal methodology was followed to prepare Muller Hilton Agar (MHA) plates, nutritional broth (NB) solution, standard culture inoculums, transfer bacteria to Petri plates, screen for antibacterial activity, and evaluate results. After 24 hours, petri plates were examined for the zone of inhibition (ZOI) caused by the antibacterial activity of plant extracts. Inhibition zones were evaluated using a scale (Timilsina *et al*., 2020).

3.2.7.1 Collection of Test Organism

The Himalaya Research Institute of Biotechnology at Suryabinayak-5, Bhaktapur, Nepal provided the microbial strains for this study. The strains studied comprised two bacteria and one fungus.

Gram positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus*

Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumonia*

Fungus: *Candida albicans*

3.2.7.2 Preparation of microbial culture media

The liquid broth (LB) media was prepared by dissolving 13 g of LB powder (Sisco Research Laboratories Pvt. Ltd, India) in 1 L of water. The mixture was autoclaved at 15 psi pressure at 121 °C for 25 minutes. The sterilized media was cooled down to 40-50°C, followed by transferring into sterilized 15 mL falcon tubes (5 mL each). The prepared media was used to co-culture bacterial seed culture in each tube separately and was incubated for 24 hours.

3.2.7.3 Preparation of Mueller-Hinton media plates and antimicrobial assay:

To prepare the Mueller-Hinton Agar (MHA) plates, 39 g of MH agar powder (Sisco Research Laboratories Pvt. Ltd, India) was dissolved in 1 L of water. The mixture was

then autoclaved at 15 psi pressure at 121 °C for 25 minutes. After the sterilized media was cooled to 40–50 °C, it was transferred into Petri dishes (25 mL each) and kept in the refrigerator until needed. The media plates were appropriately labeled with the names of the samples, and a sterile cotton swab was used to apply 150 µL of liquid bacterial seed onto the media plates' surface. The agar surface was used to create the wells, and each sample aliquot (100 µL, 100 mg/mL in DMSO) was placed into the well along with standard kanamycin (5 mg/mL, 10 µL). After that, the media plates were incubated at 37 °C for 24 hours. After a whole day, the antimicrobial test results were reviewed.

3.2.8 α-Amylase Inhibition Assay

Developing antidiabetic medications from medicinal plants is crucial in combating the global prevalence of diabetes. Current synthetic medications have drawbacks, including limited effectiveness and adverse effects such as hypoglycemia, weight gain, and persistent tissue damage. Scientific approaches are being used to validate the traditional use of some plants as antidiabetics. Certain plants have been shown to reduce blood glucose levels and have traditional uses. Research on the pancreatic alpha-amylase inhibitors found in antidiabetic plants or their phytoconstituents has been limited thus far (Mohd *et al.*, 2019).

3.2.8.1 A General Protocol for α-Amylase Inhibition

The investigation used the 3,5-dinitro salicylic acid (DNSA) technique for inhibiting α-amylase. The sample is dissolved in a minimum amount of 10% DMSO. Again, samples with DMSO were further dissolved in buffer and NaCl at pH 6.9 to give concentrations of varying ranges. A volume of 200 µL of α-amylase solution was mixed with 200µL of the extract and incubated for 10 minutes at 30ºC. Then, 200 µL of the starch solution (1% in water w/v) was added to each tube and incubated for 3 minutes. The reaction was terminated by the addition of 200 µL of DNSA reagent. The mixture sample was boiled for 10 minutes in a water bath at 85-90ºC. The mixture was cooled to ambient temperature and diluted with 5ml distilled water. The absorbance was measured at 540nm using a UV via spectrophotometer. The blank with 100% enzyme activity was prepared by replacing the plant extract with 200 µL of buffer. A blank reaction is similarly prepared using the sample plant extract at each concentration in the absence of an enzyme.

The α-amylase inhibitory activity of the extract and acarbose was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \text{ x } 100 \ldots\ldots\ldots\ldots(8)$$

The concentrations of individual extracts resulting in 50% inhibition of α-Amylase activity (IC_{50}) were determined graphically.

3.2.9 Brine Shrimp Lethality Test

The Brine Shrimp Lethality Assay is a bioassay test used to determine the toxicity of plant extracts. Brine Shrimp Lethality Assay is a practical method for keeping track of different plant species' biological activity. This approach is particularly helpful for determining the potential toxicity of different plant extracts, even if it does not give enough information on the mechanism of toxic action. This approach yields preliminary screening results that, if the active compounds are isolated, may be supported by more focused bioassays. Monitoring can last up to 60 hours, however, most LC_{50} estimates require data from 24 hours of exposure (Hamidi *et al.*, 2014). A substance is considered harmful if its LC_{50} value is less than 1000 g/mL and non-toxic if it is more than 1000 g/mL (Olowa & Nuneza, 2013).

3.2.9.1 Preparation of Artificial Seawater

30 grams of rock salt were crushed, weighed, and dissolved in 1 L of purified water.

3.2.9.2 Hatching of Brine Shrimp Egg

Brine shrimp eggs hatched at 22-29°C with constant air pump supply. The naupliis that hatched after 30-35 hours were used for the cytotoxicity test. For each sample concentration, 20 naupliis were placed on 96 well plates. The data shown here were acquired after 24 hours of experiment.

3.2.9.3 Preparation of Samples

10 mg of material was carefully weighed into a clean e-tube and then dissolved in 1 mL of DMSO. It was then further diluted with 9 mL of distilled water to achieve a concentration of 1000 ppm. Serial dilution was performed to achieve the lower concentration required for analysis. From 1000 ppm solution, lower concentrations of 800 µg/mL, 500 µg/mL, 100 µg/mL, 5 µg/mL, and 10 µg/mL solutions were derived. Cytotoxicity analysis was performed using a 96-well plate with a volume capacity of 0.5 mL in each well. Each well was infected with 20 naupuliis, and each diluted sample (0.4 mL) was placed into it. Each sample was examined three times. The experiment was continued for 24 hours with close observation for 8 hours.

3.2.9.4 Calculation of Lethality Percentage

The bioassay defines death as the lack of regulated forward propulsion in brine shrimp nauplii within a 30-second observation interval. To calculate the lethality % for each concentration and control, the number of dead and alive nauplii in each test tube is counted. To determine the percentage of mortality, divide the number of dead nauplii by the total number of nauplii. This tool evaluates death rates and compares the impact of various concentrations and controls on nauplii.

$$\% \text{ Mortality} = \frac{\text{Number of dead shrimps}}{\text{Total Number Shrimps}} \times 100 \ldots\ldots\ldots\ldots\ldots\text{..(9)}$$

3.2.10 Thin Layer Chromatography (TLC)

Mixtures are separated using chromatography using a technique known as thin-layer chromatography (TLC). Chromatography was discovered by M. Tswett in the year 1906. Thin-layer chromatography is done with a sheet of glass, plastic, or aluminum foil. A thin coating of adsorbent material, such as cellulose, silica gel, or aluminum oxide (blotter paper), is draped over this sheet. That layer of adsorbent in this instance is the stationary phase. A solvent or solvent combination known as the mobile phase is drawn up the plate by capillary action after the sample has been applied. The different rates at which different analytes rise on the TLC plate account for the separation (Bele & Khale., 2011).

Thin Layer Chromatography (TLC) was carried out on an aluminum TLC sheet that had a 0.2 mm thickness. Different solvent ratios were used to achieve the chemical separation by altering the polarity of the liquids. This change in solvent polarity results in different chromatographic patterns on the TLC plates, allowing for the identification and separation of certain components within a combination. An Eppendorf tube was filled with a little amount of extract that had been dissolved in the appropriate solvent. To apply the sample spots, a tiny pencil mark was formed at the bottom of the TLC plate. Following that, identical distances of sample solutions were applied to the locations indicated on the line. The TLC technique was used in several solvent systems, including acetone hexane systems at 5%, 10%, 20%, and 25%. Within the TLC chamber was the plate that had been prepared with the sample location. Spots appeared on the TLC plate after a while, and the chamber was covered with a lid. The solvent's travel distance was noted after removing the plates and letting them dry. The procedure was then carried out exactly for each solvent system after the sample spots were seen in both UV and visible light. There were the most spots in the chromatogram in the 25% acetone-hexane solvent

system. Afterward, distinct extracts of *C. gigantea* leaves showed distinct numbers of spots on their chromatograms.

Using a constant called the Rf value, one may ascertain the characteristics of a material in a particular chromatographic system, including how it moves about the solvent. The Rf value (Retention factor), or relative factor, is calculated by dividing the moved by the compound by the distance moved by the mobile phase.

$$\text{Rf value} = \frac{\text{Distance moved by the molecule}}{\text{Distance moved by the mobile phase}} \quad \ldots\ldots\ldots\ldots\ldots\ldots(10)$$

In chromatography, the Rf value is useful for identifying and comparing substances based on their migration on the plate. Rf values are unique to each substance and can help identify and characterize them (Gyawali *et al*., 2020).

3.2.11 FT-IR Analysis

One of the most effective, non-destructive methods for determining the kinds of chemical bonds and functional groups found in phytochemicals is the Fourier Transform Infrared Spectrophotometer (FT-IR). Plants possess unique characteristics and attributes due to the presence of several phytoconstituents such as flavonoids, alkaloids, phenol and tannins, carboxylic acids, terpenes, amino acids, and inorganic acids. Consequently, identifying the different biological activities of plants would be aided by the examination of their chemical compounds. Furthermore, FTIR spectroscopy is a well-researched, time-saving technique for identifying and characterizing functional groups (Rawat & Garg, 2021).

The FTIR spectra of three distinct extracts (hexane, chloroform, and methanol) were collected using a PerkinElmer Spectrum IR spectrometer in this specific investigation, which was carried out at the Amrit Campus in Kathmandu. The chemical makeup and structural traits of the chemicals found in the *C. gigantea* extracts were ascertained by analyzing the acquired spectrum.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Percentage Yield

Leaf extracts from the *C. gigantea* plant were made using the ultrasonic-mediated extraction technique. There were 4.16 g, 16.52 g, and 6.89 g of hexane, chloroform, and methanol yields respectively.

Table 4.1: Yield of extracts of *C. gigantea*.

Extracts	Hexane	Chloroform	Methanol
Yield (g)	4.16	16.52	6.89
% Yield	0.416	1.652	0.689

4.2 Qualitative Analysis of Phytochemicals

The phytochemical class shown in Table 4.2 is the result of the micro-chemical examination of a crude extract of *C. gigantea* leaves in various solvent systems. Following the phytochemical screenings of each of the *C. gigantea* extracts, the presence of phytochemicals was verified by the emergence of certain colors. The appendix part contains the test figures.

Table 4.2: Phytochemical analysis of extracts of *C. gigantea*.

S.N.	Class of phytochemicals	Hexane extract	Chloroform extract	Methanol extract
1	Alkaloids	_	_	+
2	Flavonoids	_	_	+
3	Steroids	+	_	_
4	Quinones	+	+	+
5	Tannins	_	_	+
6	Phenolic	_	+	+
7	Carbohydrates	_	+	+
8	Proteins	_	_	_
9	Cardiac Glycosides	+	_	_
10	Saponins	_	_	_

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

4.3 Estimation of Total Phenolic Content (TPC)

4.3.1 Construction of Calibration Curve

Using gallic acid as a reference, the Folin Ciocalteu (combination of phosphomolybdic acid and phosphotungstic acid) reagent calorimetric (FCR) technique was used to evaluate the total phenolic content of plant extracts. This method is based on the oxidation-reduction method. The reference chemical for the calibration curve in this process was gallic acid. Using a UV Visible spectrometer, the absorbance of gallic acid at 750 nm wavelength was measured at different concentrations (25 ppm, 50 ppm, 100 ppm, 200 ppm, 250 ppm, and 500 ppm) in order to build the calibration curve.

By reacting with a specific redox reagent (FCR), polyphenols in plant extracts create a blue complex that has a maximum light absorption of 760 nm, which can be detected by UV-visible spectroscopy. The degree of light absorption at that wavelength is directly correlated with the quantities of phenols. Plotting the concentration on the X-axis and the absorbance on the Y-axis allowed for a visual representation of the absorbance at various concentrations of standard gallic acid. The absorbance curve for standard gallic acid is displayed in Figure 4.1.

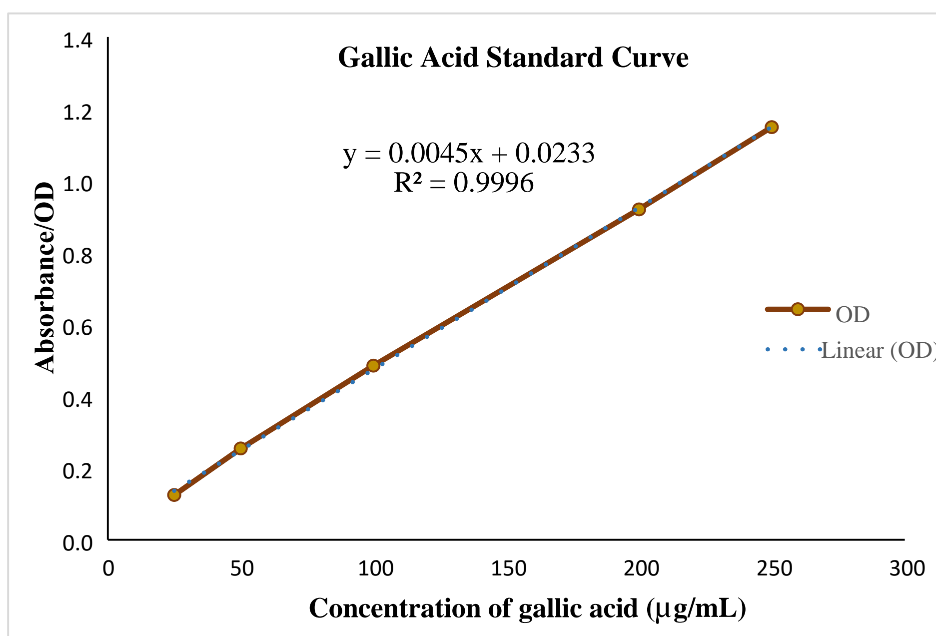


Figure 4.1: Calibration curve for standard

4.3.2 Calculation of Total Phenolic Content in Different Extracts

By using a calibration curve and absorbance values (triplicate of 1000 µg/mL), The total phenolic content in different extracts was calculated, which is shown in Table 4.3.

Table 4.3: Total phenolic content in different extracts

Extracts	OD of Samples			OD of Control	TPC (mg GAE/g)	Std Dev
	I	II	III			
Hexane	0.123	0.133	0.145	0.072	8.5	2.4
Chloroform	0.608	0.602	0.681	0.072	125.7	11.5
Methanol	0.291	0.312	0.337	0.072	48.5	5.1

The total phenolic contents of hexane, chloroform, and methanol extracts were 8.5±2.4 mg QE/g, 125.7±11.5 mg QE/g, and 48.5±5.1 mg QE/g respectively. The bar graph presentation of TPC in various extracts of *C. gigantea* is displayed below:

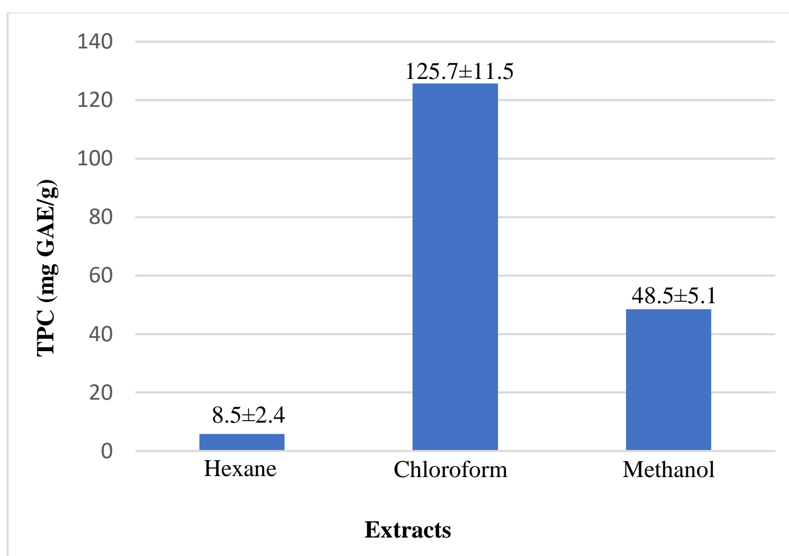


Figure 4.2: Total phenolic content in different extracts

4.4 Estimation of Total Flavonoid Content (TFC)

4.4.1 Construction of Calibration Curve

To find the total flavonoid concentration in plant extracts, a colorimetric test using aluminum chloride was utilized. The calibration curve for this approach was constructed using quercetin, the standard chemical. A UV spectrophotometer measuring 415 nm was used to detect the strong yellow fluorescence that the flavonoids in plant extracts create when aluminum chloride is present. Flavonoid content is directly correlated with the strength of light absorption at that wavelength. Several different biological and pharmacological effects are displayed by the flavonoid content. At several concentrations, including 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL the absorbance was measured.

Figure 4.3 shows the quercetin calibration curve.

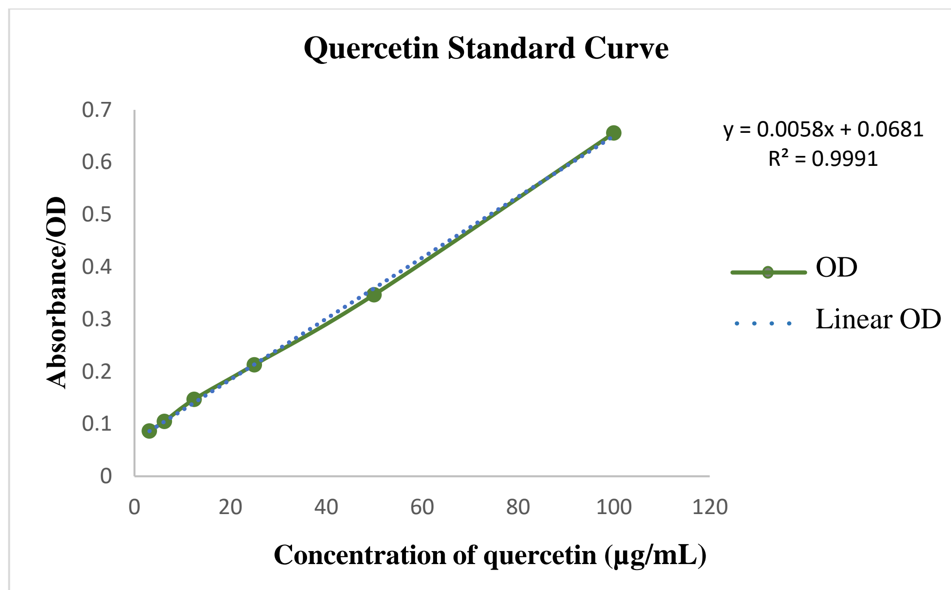


Figure 4.3: Calibration curve for standard quercetin

4.4.2 Calculation of Total Flavonoid Content in Different Extracts

Table 4.4 displays the total flavonoid content of the various extracts and was estimated using the calibration curve and absorbance readings (triplicate of 1000 µg/mL).

Table 4.4: Total flavonoid content in different extracts

Extracts	OD of Samples			OD of Control	TPC (mg GAE/g)	Std Dev
	I	II	III			
Hexane	0.633	0.686	0.695	0.06	37.47	2.3
Chloroform	0.674	0.691	0.693	0.06	38.48	0.7
Methanol	0.902	0.844	0.882	0.06	51.58	2.0

The total flavonoid contents of hexane, chloroform, and methanol extracts were 37.47±2.3 mg QE/g, 38.48±0.7 mg QE/g, and 51.58±2.0 mg QE/g, respectively. The bar graph presentation of TFC in various extracts of *C. gigantea* is displayed below:

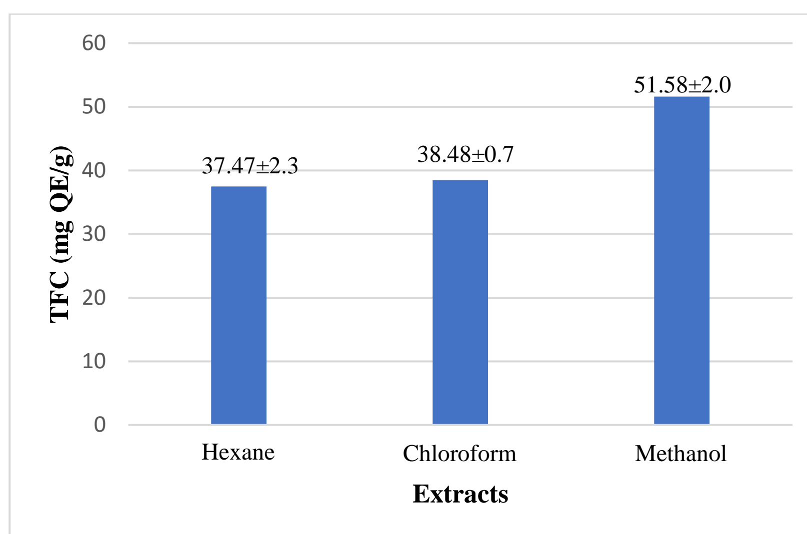


Figure 4.4: Total flavonoid content in different extracts

4.5 Antioxidant Activity

The activity of "free radical scavenging enzymes" (superoxide dismutase, catalase, peroxidase, etc.) and the presence of antioxidant substances—mostly phenolic compounds, carotenoids, tocopherol, and ascorbic acid are linked to a plant's potential for antioxidants. The IC_{50} value, which is determined by linear regression of percent inhibition vs antioxidant activity, shows an inverse connection with the antioxidant capacity. The antioxidant activity increases with decreasing IC_{50} value. Plotting the percentage of free radical scavenging vs concentration allowed researchers to assess the antioxidant activity of many *C. gigantea* preparations. Utilizing the absorbance readings recorded at 520 nm, the IC_{50} value of each extract was computed together with the % inhibition of DPPH radicals against the sample shown in the table below.

The % inhibition of DPPH radicals for the sample was computed using the absorbance values obtained at 520 nm for various concentrations of the hexane extract. The table below displays the findings:

Table 4.5: Percentage scavenging of different extracts at 500 µg/mL concentration

Extracts	Concentration (µg/mL)	OD of samples	OD of control	% Scavenging
Hexane	500	0.857	0.941	8.93
Chloroform	500	0.641	0.941	31.88
Methanol	500	0.681	0.941	27.63

4.5.1 Antioxidant Activity of Standard Quercetin

The antioxidant activity of quercetin was assessed by diluting the concentration and calculating its percentage scavenging value. Table 4.6 displays the % suppression of DPPH radicals at each concentration.

Table 4.6: Percentage inhibition of DPPH radical by standard quercetin

Sample	Concentration (µg/mL)	OD of Samples			OD of Control	% Scavenging			Average % Scavenging
		I	II	III		I	II	III	
Quercetin	25	0.176	0.242	0.248	0.957	81.60	74.71	74.08	76.79
	12.5	0.218	0.218	0.23	0.957	77.22	77.22	75.96	76.8
	6.25	0.37	0.372	0.25	0.957	61.33	61.12	73.87	65.44
	3.125	0.621	0.631	0.501	0.957	35.10	34.06	47.64	38.93
	1.56	0.779	0.791	0.711	0.957	18.59	17.34	25.70	20.54
	0.78	0.858	0.86	0.815	0.957	10.34	10.13	14.83	11.76

The IC_{50} value of standard quercetin was found to be 4.76 µg/mL and is shown in Figure 4.5.

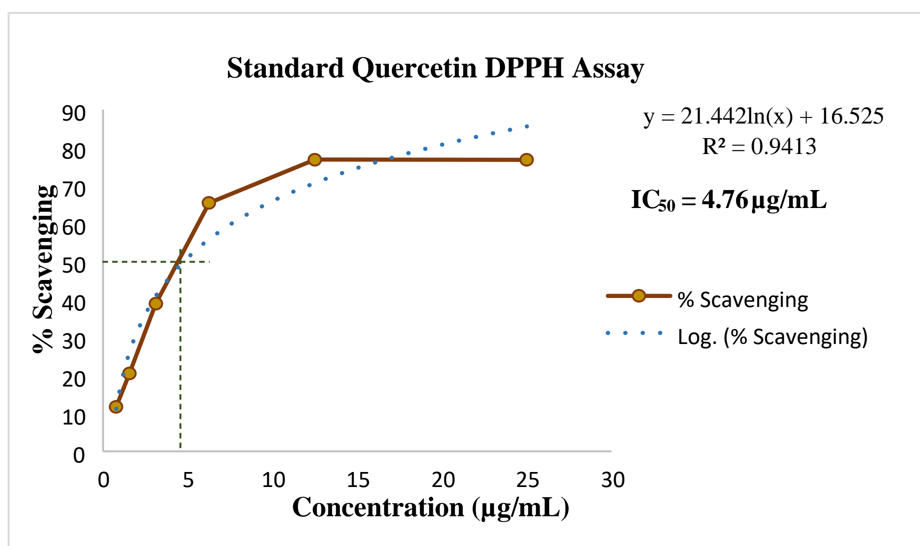


Figure 4.5: Antioxidant activity of standard quercetin

4.5.2 Antioxidant Activity of Hexane Extract

At 500 µg/mL, the hexane extract had only 8.93% free radical scavenging capacity. The study found that the hexane extract of *C. gigantea* had low antioxidant activity. The data showed that the hexane extract had a limited ability to eliminate free radicals.

4.5.3 Antioxidant Activity of Chloroform Extract

At a concentration of 500 µg/mL, the chloroform extract of *C. gigantea* demonstrated only 31.88% free radical scavenging activity. This suggests that it can neutralize 31.88% of the DPPH radicals in the test solution. As a result, the chloroform extract has a relatively low antioxidant activity.

4.5.4 Antioxidant Activity of Methanol Extract

Methanol extract of *C. gigantea* demonstrated 27.63% scavenging activity at 500 µg/mL concentration. It can be said that the methanol extract also had minimal antioxidant activity. Therefore, no extracts exhibited the significant DPPH free radical scavenging potential.

4.6 Antimicrobial Activity

The measurement of antimicrobial activity involved analyzing the width of the zone of inhibition (ZOI) that plant extracts created on certain bacteria. The method outlined in the preceding section was used to evaluate the capacity of various fractions of *C. gigantea* leaf extract to inhibit microbial growth at a constant dose (50 mg/mL). The findings were represented in terms of the width of the zone of inhibition. Microorganisms cannot grow in the area around the antimicrobial disk, which is known as the zone of inhibition. The term "minimum inhibitory concentration" refers to the lowest plant extract concentration required to prevent the development of microbes.

The antimicrobial activity of hexane, chloroform, and methanol extracts of *C. gigantea* was performed towards two Gram-positive bacteria i.e., *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram-negative bacteria i.e., *Escherichia coli* and *Klebsiella pneumoniae*. The zone of inhibition (ZOI) was measured in centimeters. The Kanamycin (5 mg/mL) was taken as a positive control, and DMSO was used as a negative control.

Table 4.7: Antimicrobial activity of different extracts

Microbial Strain	Reference culture	Type	Zone of inhibition (cm)				
			Positive control (c^+)	Negative control (c^-)	Hexane extract	Chloroform extract	Methanol extract
Escherichia coli	ATCC8739	Gram-ve	2.6	0	1.5	1.5	0

Bacillus subtilis	ATCC 6051	Gram +ve	2.5	0	1.6	1.5	1.3
Staphylococcus aureus	ATCC 6538P	Gram +ve	2.7	0	1.1	1.1	1.1
Klebsiella pneumonia	ATCC 700603	Gram-ve	2.1	0	1.5	1.5	1.3
Candida albicans	ATCC 2091	Fungi	2.6	0	1.6	1.5	1.5

The antimicrobial property of hexane, chloroform, ethyl acetate, acetone, methanol, and distilled water extracts in 50 mg/mL concentration was observed, which is shown in Table 4.7. Hexane extract showed the maximum ZOI of 1.6 cm against *Bacillus subtilis* and *Candida albicans*. While chloroform extract showed the highest ZOI of 1.5 cm against four microorganisms except *Staphylococcus aureus*. Likely methanol extract showed the highest ZOI of 1.5 cm against fungi *Candida albicans.*

4.7 α-Amylase Inhibition Potential Evaluation

The experiment for α-amylase inhibition utilized the 3,5-dinitro salicylic acid (DNSA) method. The logarithmic regression equation of percentage inhibition against α-amylase inhibition activity may be used to evaluate antidiabetic potential, which is inversely linked to the IC_{50} value. The extracts' absorbance values were measured at 540 nm at various concentrations.

4.7.1 α-Amylase Inhibition of Standard Acarbose

The table below displays the % inhibition of normal α-amylase function by conventional acarbose drugs.

Table 4.8: α-Amylase inhibition of standard acarbose

Sample	Concentration (µg/mL)	% Inhibition
Acarbose	62.5	52.48
	31.25	45.7
	15.62	35.02
	7.81	29.08
	IC_{50}	**49.57 µg/mL**

The IC_{50} value of the standard acarbose drug was found to be 49.57 µg/mL as exhibited in Figure 4.6.

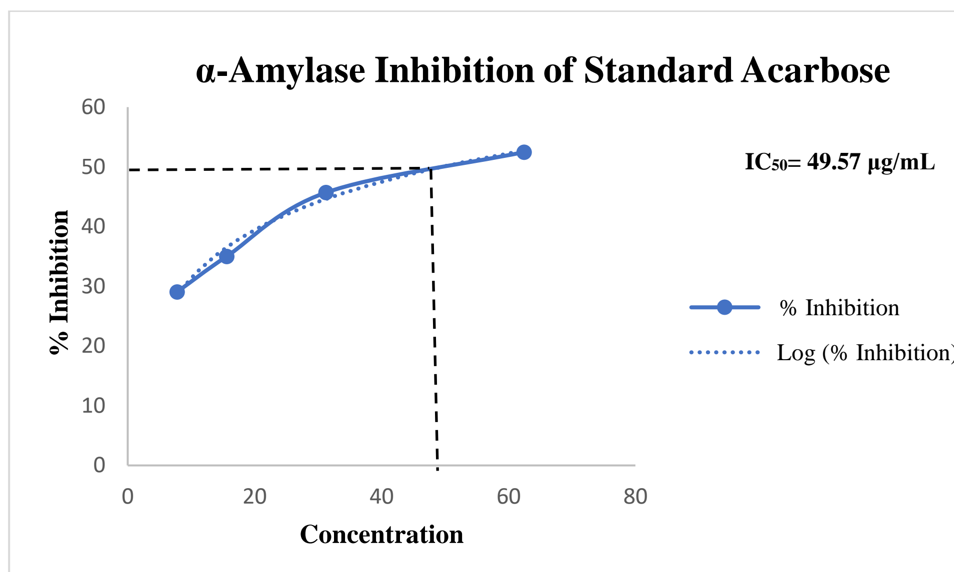


Figure 4.6: α-Amylase inhibition of standard acarbose

4.7.2 α-Amylase Inhibition of Hexane Extract

The IC_{50} value obtained in the hexane extract of *C. gigantea* showed negative result. The outcome suggested that the hexane extract had no effect on the α-amylase inhibitory activity.

4.7.3 α-Amylase Inhibition of Chloroform Extract

The table below illustrates the percentage of inhibition of α-amylase induced by the chloroform extract.

Table 4.9: α-Amylase inhibition of chloroform extract

Sample	Concentration (µg/mL)	% Inhibition
Chloroform extract of *C. gigantea*	2000	54.02
	1000	43.68
	500	35.06
	250	31.61
	125	27.59
	IC_{50}	**1719.86 µg/ml**

C. gigantea extract in chloroform had an IC_{50} of 1719.86 µg/mL. This is represented

in the figure below:

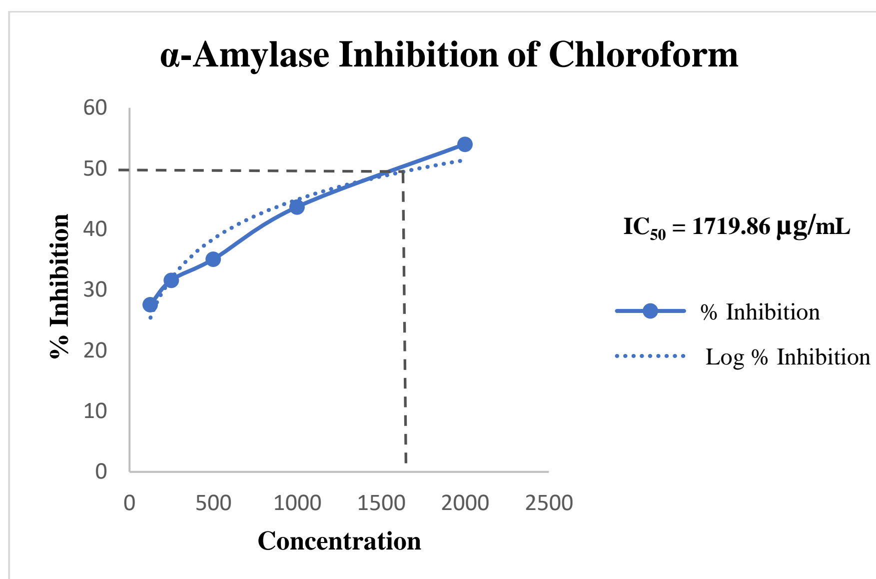


Figure 4.7: α-Amylase inhibition of chloroform extract

4.7.4 α-Amylase Inhibition of Methanol Extract

The following table illustrates the degree of inhibition of α-amylase caused by the Methanol extract.

Table 4.10: α-amylase inhibition of methanol extract

Sample	Concentration (µg/mL)	% Inhibition
Methanol extract of *C. gigantea*	2000	52.13
	1000	37.30
	500	30.56
	250	18.43
	IC_{50}	**1900.74 µg/mL**

The figure below depicts the IC_{50} value of 1900.74 µg/mL for the *C. gigantea* extract in methanol.

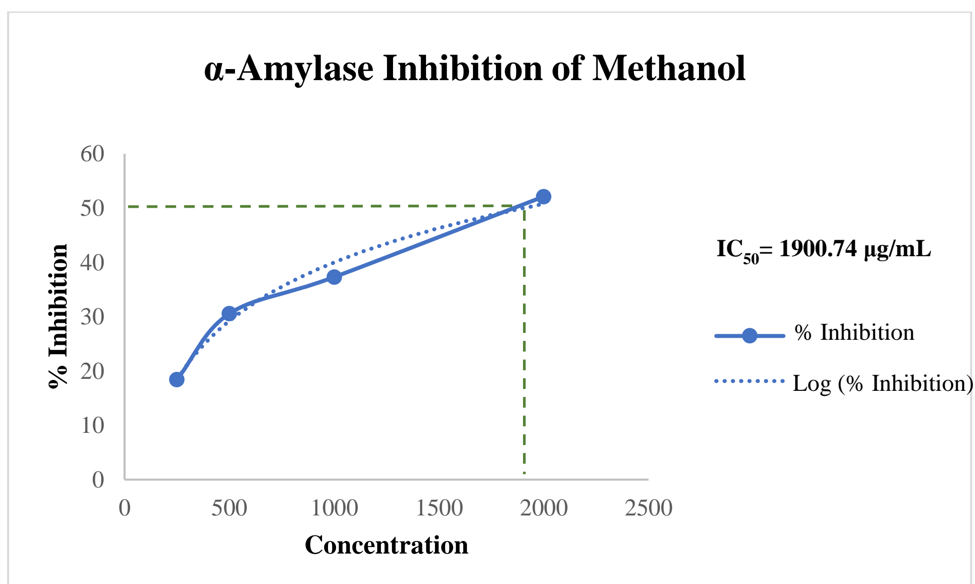


Figure 4.8: α-Amylase inhibition of methanol extract

Among the three extracts, chloroform extract was found to be highly effective in controlling diabetes being a strong α-amylase inhibitor whereas hexane extract was observed to have extremely lower α-amylase inhibitory properties.

4.8 Brine Shrimp Lethality Analysis

Freshly hatched live Brine Shrimp nauplii were exposed to concentrations of 1000 µg/mL, 800 µg/mL, 500 µg/mL, 100 µg/mL, 50 µg/mL, and 10 µg/mL to assess the LC_{50} values of hexane, chloroform, and methanol extracts. The extract concentration was shown to have a clear association with lethality levels. The tables and figures below show how lethality levels vary with extract concentrations.

The LC_{50} value for the hexane extract was determined as shown in the table below:

Table 4.11: Percentage mortality of hexane extract

Sample	Concentration (µg/mL)	No. of alive nauplii after 24 hours			% Mortality
Hexane extract of *C. gigantea*	1000	18	18	17	11
	800	20	20	20	0
	500	20	20	20	0
	100	20	20	20	0
	50	20	20	20	0
	10	20	20	20	0
	LC_{50}	**>1000 µg/mL**			

The hexane extract of *C. gigantea* has an IC_{50} value of more than 1000 µg/mL, with no death rate at the highest concentration. This finding confirms the extract's non-toxic nature.

The calculation of the LC_{50} value of the chloroform extract is summarized in the table below:

Table 4.12: Percentage mortality of chloroform extract

Sample	Concentration (µg/mL)	No. of alive nauplii after 24 hours			% Mortality
Chloroform extract of *C. gigantea*	1000	4	6	7	71
	800	10	9	9	53
	500	18	19	18	8
	100	20	20	20	0
	50	20	20	20	0
	10	20	20	20	0
	LC_{50}	**806.64 µg/mL**			

The L_{50} value of the chloroform extract of *C. gigantea* was 806.64 µg/mL. This is shown in the figure below:

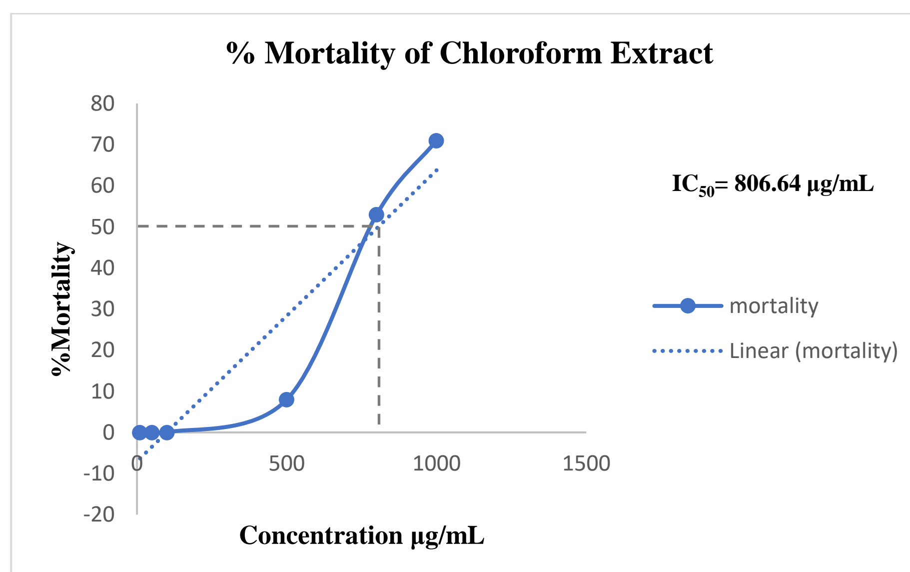


Figure 4.9: Calculation of IC_{50} value of chloroform extract

The calculation of the IC_{50} value of the methanol extract is summarized in the table below:

Table 4.13: Percentage mortality of methanol extract

Sample	Concentration (µg/mL)	No. of alive nauplii after 24 hours			% Mortality
Methanol extract of *C. gigantea*	1000	6	5	5	53
	800	8	7	9	60
	500	9	8	11	73
	100	20	20	20	0
	50	20	20	20	0
	10	20	20	20	0
	LC_{50}	**648.73 µg/mL**			

The IC_{50} value for the methanol extract of *C. gigantea* was 648.731µg/mL, indicating its moderate toxicity relative to the other two extracts.

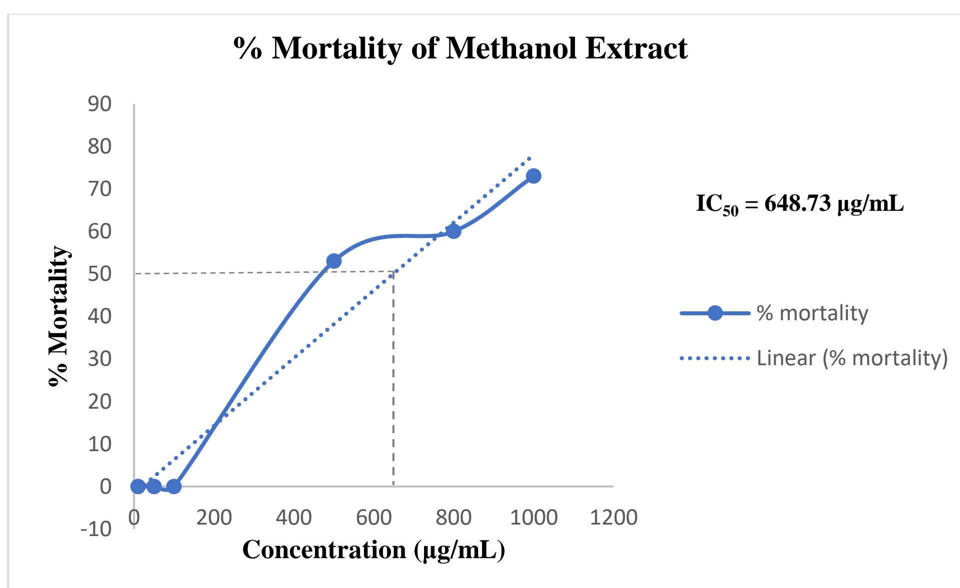


Figure 4.10: Calculation of IC_{50} value of methanol extract

The Brine Shrimp experiment showed that the toxicity was not strong in any of the extracts. The LC_{50} value of all three extracts was greater than 500 µg/mL which is non-toxic. While methanol exhibited a minimal LC_{50} of 648.73 µg/mL.

4.9 TLC Analysis

TLC executed a qualitative examination of several extracts of *C. gigantea*. A solvent solution containing several concentrations of ethyl acetate in hexane and acetone in hexane was used for TLC. UV light was used to develop and see the plates.

The background of the TLC plate remained clean and colorless, minimizing interference with spot visualization. Visualization methods such as UV light effectively highlighted the spots, aiding in their identification. The result showed the presence of many phytoconstituents in all three extracts (hexane, chloroform, and methanol) and it can be further utilized for column chromatography for isolation of pure compounds. It was confirmed by the TLC report that the solvent containing 25% ethyl acetate in hexane and 35% acetone in hexane caused several spots on the TLC plate to contain various phytochemicals.

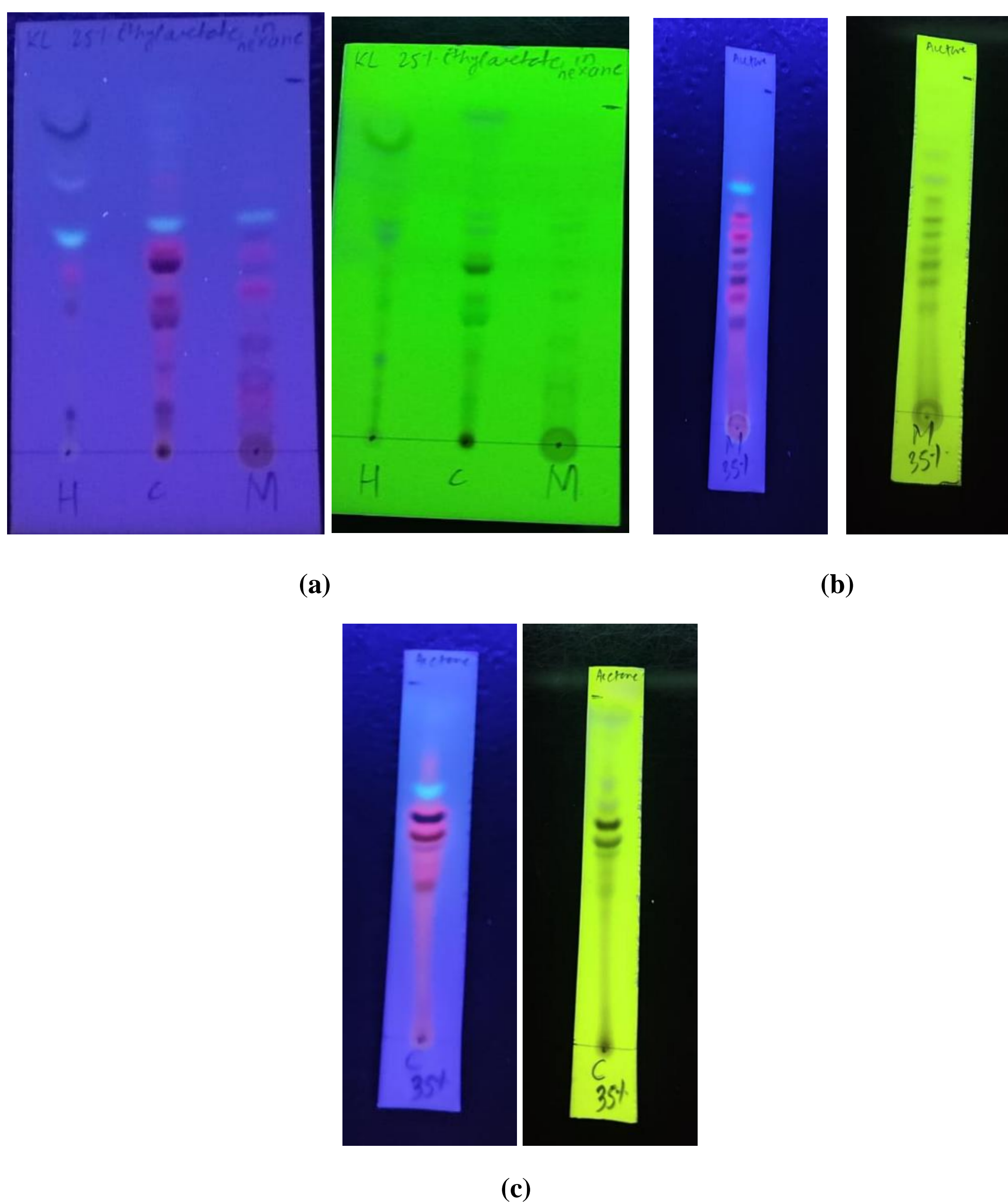


Figure 4.11: TLC spots of *C. gigantea* of (a) Hexane, chloroform, and methanol extract in 25% ethyl acetate: hexane solvent system (b) Methanol extract in 35% acetone: hexane solvent system and (c) Chloroform extract in 35% acetone: hexane solvent system

4.10 FT-IR Analysis

Fourier Transform Infrared (FTIR) is a method for simultaneously and quantitatively identifying molecule structure. The FTIR analyzes peaks and spectra to identify molecular structure and chemical bonds (Nandiyanto *et al.*, 2023). The resultant spectrum captures the sample's molecule absorption and transmission, forming a molecular fingerprint. Unique chemical structures do not create the same IR spectra, comparable to how two fingerprints cannot match. This makes infrared spectroscopy practical for a variety of analyses. For more than 70 years, IR spectroscopy has been a reliable tool for material investigation in the laboratory. An IR spectrum is a sample's unique fingerprint, with absorption peaks corresponding to the frequencies of vibrations between atom bonds. Each substance produces a distinct IR spectrum due to its unique composition and atom arrangement. IR spectroscopy can positively identify and qualitatively analyze all types of materials. The magnitude of a peak in the spectrum reflects the amount of material present. Modern software techniques make IR an ideal tool for quantitative analysis (Dutta, 2017).

4.10.1 FT-IR Spectrum of Hexane Extract

The table shows the absorbance, stretching type, appearance, functional group, and frequency range of hexane extract wavenumbers. The graphic shows the peak values for several functional groupings.

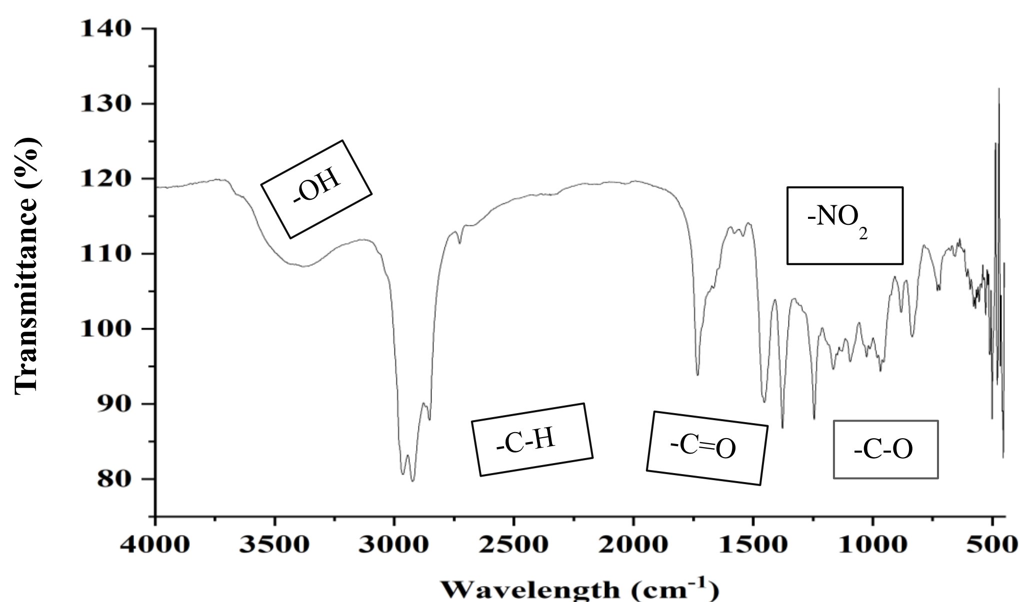


Figure 4.12: FTIR spectrum of hexane extract

Table 4.14: FT-IR peak values and functional groups of hexane extract

Sample	Absorption (cm -1)	Types of Stretching	Appearance	Functional Group	Frequency Range (cm^{-1})
Hexane extract of *C. gigantea*	835.25,	Out-of-plane bending	Strong	C=C or Aromatic C-H	1000-650
	881.31	Out-of-plane bending	Strong	Aromatic C-H	900-690
	968.00	Out-of-plane bending	Medium	Trans-C-H	970-960
	1025.35	Cyclohexane ring stretching	Medium	C-H	1055-1000
	1094.43, 1164.98	Out-of-plane Stretching	Medium	C-N	1350-1000
	1244.93	Alkyl aryl ether stretching	Strong	C-O	1300-1000
	1377.16, 1453.22	Nitro stretching	Strong	N=O	1550-1350
	1732.50	Stretching	Strong	C=O	1740-1720
	2728.50	Aldehyde Stretching	Medium	C-H	2800-2700
	2852.70, 2923.56, 2963.50	Alkane Stretching	Medium	C-H	3000-2840
	3377.71	Alcohol stretching	Broad	O-H	3550-3200

The aforementioned data produced strong bands like 1732.50 cm^{-1} of (C=O) stretch, 1244.02 cm^{-1} of (C-O) stretch, 1377.16 cm^{-1}, and 1453.22 cm^{-1} of (N=O) stretch. 2728.50 cm -1 of -C-H stretching vibration of aldehyde was observed. Three medium peaks 2852.70 cm^{-1}, 2923.56 cm^{-1}, and 2963.50 cm^{-1} represent C-H alkane stretching. The -OH functional group is shown by a wide peak at 3377.71 cm^{-1}. It indicated the presence of carbonyl compounds, primary or secondary alcohol, aliphatic nitro compounds, aliphatic alkane, etc. in the hexane extract.

4.10.2 FT-IR Spectrum of Chloroform Extract

The table and image below demonstrate the chloroform extract's FT-IR properties. The table includes absorption wavenumber, appearance, stretching type, and associated functional group.

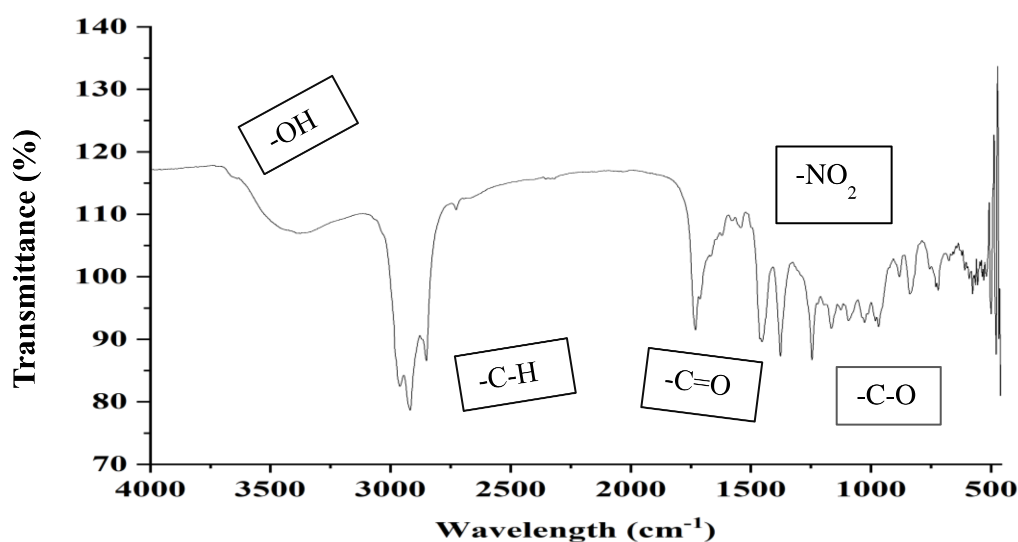


Figure 4.13: FTIR spectrum of chloroform extract

Table 4.15: FT-IR peak values and functional groups of chloroform extract

Sample	Absorption (cm^{-1})	Types of Stretching	Appearance	Functional Group	Frequency Range (cm^{-1})
Chloroform extract of	837.82	Out-of-plane bending	Sharp	C=C or Aromatic C-H	1000-650

C. gigantea	968.07	Out-of-plane bending	Medium	Trans C-H	970-960
	1026.51, 1092.78	Cyclohexane ring vibration	Medium	Cycloalkane	1100-1000
	1164.54	Tertiary alcohol stretching	Medium	C-O	1205-1124
	1245.38	C-O stretching	Strong	C-O	1275-1200
	1376.85, 1453.27	N-O Stretching	Strong	N-O	1550-1350
	1542.80	Aromatic nitro compound	Medium	N-O	1555-1485
	1730.04	Aldehyde stretching	Strong	C=O	1740-1720
	2850.71, 2918.21, 2961.08	C-H stretching vibration	Medium, Strong, Medium	C-H	3000-2800
	3376.44	O-H stretching	Broad	O-H	3400-3200

The data presented above resulted in strong bands such as 1730.04 cm^{-1} of stretch (C=O), 1245.38 cm^{-1} of C-O stretching, 1376.85 cm^{-1} and 1453.27 cm^{-1} of N-O stretching. The sharp peaks at 1376.85 cm^{-1} and 1453.27 cm^{-1} represent N-O stretching. At 968.07 cm^{-1} out-of-plane bending was seen with the trans-C-H group. The aromatic nitro compound was observed at 1542.80 cm^{-1} with a medium peak. The -OH functional group is shown by a wide peak at 3376.44 cm^{-1}. It indicated the presence of carbonyl compounds, primary or secondary alcohol, aliphatic and aromatic nitro compounds, aliphatic alkane, etc. in the chloroform extract.

4.10.3 FT-IR Spectrum of Methanol Extract

The table and figure below show the FTIR spectroscopy report for the methanol extract, including its different distinctive peaks.

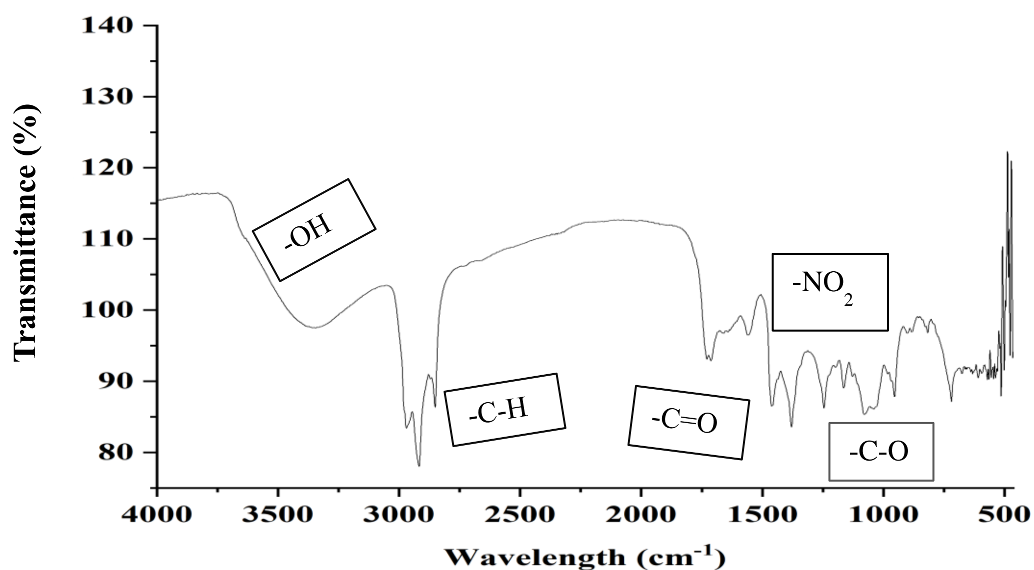


Figure 4.14: FTIR spectrum of methanol extract

Table 4.16: FT-IR peak values and functional groups of methanol extract

Sample	Absorption (cm -1)	Types of Stretching	Appearance	Functional Group	Frequency Range (cm^{-1})
Methanol extract of *C. gigantea*	953.76	Out-of-plane Bend	Sharp	Alkene or Aromatic C-H	1000-650
	1079.11	Out-of-plane stretching	Medium	C-N	1350-1000
	1163.79	C-O Stretching	Sharp	Ester	1210-1163
	1245.44	C-O Stretching	Sharp	Alkyl aryl ether	1275-1200

	1379.23	Aliphatic nitro stretching	Sharp	N-O	1380-1350
	1461.56	Assym/Sym bending	Sharp	C-H	1470-1430
	1558.75	Aromatic stretching	Medium	C=C	1650-1555
	1712.33, 1731.10	Carbonyl Stretching	Weak	C=O	1706-1740
	2851.00, 2916.92, 2969.74	Alkane Stretching	Small, Small, Medium	C-H	3000-2850
	3345.24	Alcohol Stretching	Broad	O-H	3550-3200

When the result was compared to the FT-IR standard chart, prominent bands were discovered in the methanol extract such as 953.76 cm^{-1} of C=C bending, 1163.79 cm^{-1} of C-O stretching, and 1379.23 cm^{-1} of N-O aliphatic nitro stretching. The weak peaks at 1712.33 cm^{-1} and 1731.10 cm^{-1} represent C=O stretching. At 1558.75 cm^{-1} aromatic stretching was seen with the C=C group. The -OH functional group is shown by a wide peak at 3345.24 cm^{-1}. It indicated the presence of carbonyl compounds, primary or secondary alcohol, aliphatic nitro compounds, aliphatic alkane, alkene, etc. in the methanol extract.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The ultrasonic extraction method was applied for the extraction of phytoconstituents from the dried and powdered leaves of *C. gigantea* by the use of three types of solvents (hexane, chloroform, and methanol). Phytochemical screening revealed the presence of flavonoids, terpenoids, alkaloids, phenolic compounds, carbohydrates, and quinones. Chloroform and methanol were found to contain high phenolic compounds i.e., 125.7±11.5 mg GAE/g and 48.5±5.1 mg GAE/g compared to Hexane extract (8.5±2.4 mg GAE/g). The methanol extracts were found to contain high flavonoid contents, i.e., 51.58±2 mg QE/g, compared to hexane and chloroform extract (37.47±2.3 mg QE/g and 38.48±0.7 mg QE/g) respectively.

In the DPPH scavenging assay, the % scavenging of all three different extracts was not found to be strong. The evaluation of the antibacterial activity of hexane extract showed significant inhibitory activity against the growth of Gram-positive bacteria *Bacillus subtilis* (ZOI, 1.6 cm) and *Candida albicans* (ZOI, 1.6 cm). Chloroform and methanol extract also exhibited significant activity in all microorganisms with a maximum ZOI of 1.5 cm. In the determination of the α-amylase inhibition test, all three extracts showed the negative result indicating weak α-amylase inhibition action.

The methanol extract had the maximum toxicity among others, with an LC_{50} value of 648.731 µg/mL which is also non-toxic, whereas the hexane and chloroform extracts were non-toxic to brine shrimp larvae. The existence of various chemicals in the extracts was verified by TLC, which displayed several spots in hexane, chloroform, and methanol, respectively. The FTIR analysis of different extracts verified the presence of primary and secondary alcohol (-OH), aldehyde group (=C=O), aromatic ring, aliphatic, and aromatic nitro compounds(-O-N=O), alkene, and ether as the major functional groups.

5.2 Recommendation

A preliminary phytochemical study shows several physiologically active phytoconstituents in the plants. Medicinal plants include secondary metabolites that contribute to their therapeutic powers. To extract more phytochemicals, plant extracts should be prepared in various solvents and extraction procedures than those given here. LCMS, Column Chromatography, and various spectroscopic techniques can be used for

isolation and identification of pure compounds. Furthermore, various *in-vitro* and in-vivo biological activity tests can be done to find the potentiality of the compounds. The plant's great extraction capacity allows for the production of active ingredients for various biological processes, potentially leading to the development of strong drugs. Further study is needed to extract unique active chemicals from this plant, perhaps leading to a new treatment option for many ailments.

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APPENDIX A

A. Phytochemical Screening Protocol

1 Test for Alkaloids

Mayer's test: 1 ml of the filtrate was treated with Mayer's reagent (Potassium Mercuric Iodide). Yellow color formation indicates the presence of alkaloids.

Dragendroff's test: 1ml of extract and 0.2ml dilute HCl was taken in a test tube. 1 ml of Dragendroff's reagent was added and left for a few minutes. Orange-brown ppt. indicate the positive result.

Wagner's test: 3 drops of freshly prepared Wagner reagent and 1ml of extract were added to the test tube. Reddish brown ppt indicates the presence of alkaloids.

2. Test for flavonoids

Alkali reagent test: Extract was treated with a few drops of sodium hydroxide solution. The intense yellow color formation which on the addition of dil. acid becomes colorless indicating the presence of flavonoids.

Lead acetate test: Extracts were treated with a few drops of lead acetate solution. The formation of yellow color indicates the presence of flavonoids. Shinoda test: A few pieces of magnesium were mixed with 3 ml extract followed by dropwise addition of concentrated HCl and boiled for 5 min. The Magenta color indicates the presence of flavonoids.

3. Test for Steroid Compound

Salkowski's test: A test tube containing 1 milliliter of plant extract was filled with 10 milliliters of chloroform, and then the test tube was topped off with an equivalent volume of concentrated sulfuric acid by the sides. The test tube's top layer needs to be made red, and the presence of steroids should be shown by a yellow color with green fluorescence in the sulphuric acid layer.

4. Test for Quinones

Add a few drops of concentrated H_2SO_4 or aqueous NaOH solution to 2 ml of extract. When the quinoid component is present, color creation occurs.

5. Test for Saponins

Foam test: After diluting the extracts with 20 mL of distilled water, they were agitated for 15 minutes in a graduated cylinder. Saponins are present when a layer of foam forms, about 1 cm in thickness.

5. Test for tannins

$FeCl_3$ test: 1 ml of 5% ferric chloride solution was added to five ml of extract solution. The presence of tannins was suggested by the greenish-black coloring.

Potassium dichromate test: 1 ml of a 10% aqueous potassium dichromate solution was added to 5 ml of the extract. The development of a precipitate with a yellowish-brown color indicates the presence of tannins.

Lead acetate test: Five ml of extract were combined with a few drops of freshly produced 1% lead acetate. The yellow precipitate indicates a successful outcome.

6. Detection of carbohydrates

Benedict's test: Benedict's reagent was applied to the filtrates, and they were then gently heated. Orange-red precipitate suggests that reducing sugars are present.

Fehling's test: Filtrates were heated using Fehling's A and B solutions, neutralized with alkali, and hydrolyzed with diluted HCl. Red precipitate development is a sign that reducing sugars are present.

7. Detection of resins

Separately, 3–4 ml of $CuSO_4$ solution was added to 0.5 mL of extract, and the tubes were rapidly shaken for one to two minutes. It came down to letting the resultant solution separate. The presence of resins was revealed by the production of a green precipitate.

8. Test for proteins

Xanthoproteic test: A few drops of concentrated nitric acid were applied to the extracts. The development of a yellow color signifies the existence of proteins.

9. Detection of amino acids

Ninhydrin test: Ninhydrin solution was added to the test solution, brought to boil, and the production of a violet color was monitored.

10. Test for phenols

Ferric chloride test: One percent ferric chloride solution and one percent potassium ferrocyanide were combined in equal parts. Three drops of this recently made mixture were added to two ml of extract. The development of a dark green or bluish-green coloration was interpreted favorably.

11. Test for cardiac Glycoside

Killer-Killani test: After dissolving 1 mL of the extracts in 1 mL of glacial acetic acid and allowing it to cool, add 2–3 drops of ferric chloride. Carefully 2 mL of concentrated H_2SO_4 was poured along the test tube sides into this solution. The presence of glycosides

is indicated by the emergence of a reddish-brown color ring at the intersection of two layers.

B. Preparation of reagents

1. Mayers Reagent

A volumetric flask with a capacity of 50 milliliters was used to weigh and dissolve 0.679 grams of mercury chloride, or HgCl2. This combination was supplemented with 2.5 g of potassium iodide (KI). After the crimson-red precipitate was dissolved by shaking, distilled water was added to bring the volume up to the necessary level.

2. Dragendroff's Reagent

To create solution A, 4.00 g of bismuth nitrate, $Bi(NO_3)_3$ was dissolved in 10 mL of 5 N nitric acid. To create solution B, potassium iodide, or KI (13.5), was then dissolved in 20 mL of distilled water. The 50 mL volumetric flask was used to combine these two solutions.

An aqueous solution of picric acid was prepared by dissolving 0.25 g of picric acid in 50 mL of distilled water. Sodium bicarbonate was used to neutralize the solution ($NaHCO_3$).

3. Molisch's Reagent

Molisch's reagent was made by dissolving 5.0 g of α- Naphthol in 50 mL of methanol.

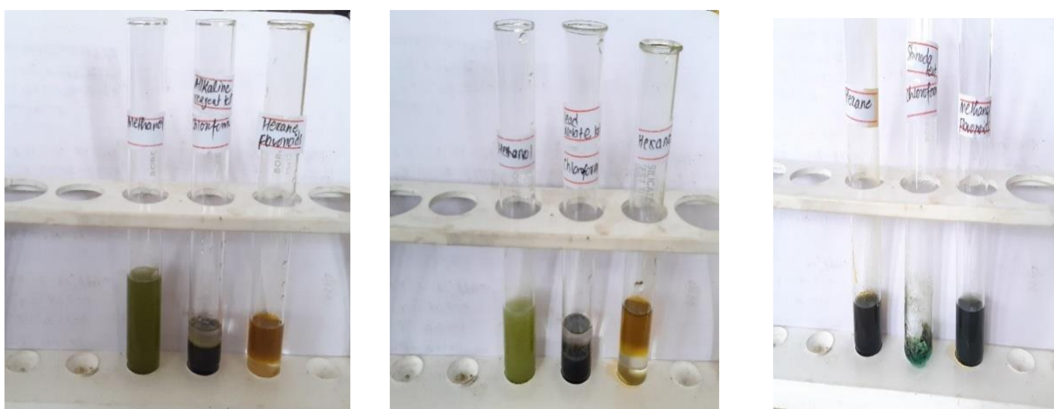
4. Neutral Ferric Chloride ($FeCl_3$) Solution

In 100 mL of pure water, 1.0 g of ferric chloride crystals were dissolved. Crystals of sodium carbonate were gradually added to this solution while being stirred until slight turbidity persisted. The mixture was then passed through a filter and its colorless filtrate was added to a solution of neutral ferric chloride.

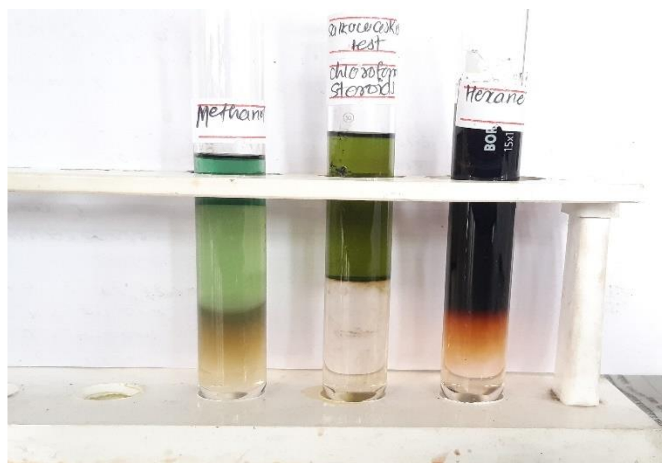
Figures of Phytochemical screening test

A. Test for Alkaloids

B. Test For Flavonoid



C. Test for Steroids



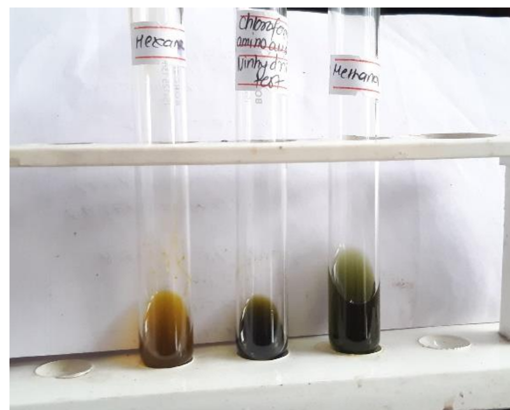
D. Test for Quinones

E. Test for Tannins

F. Test for Carbohydrates

G. Test for Proteins

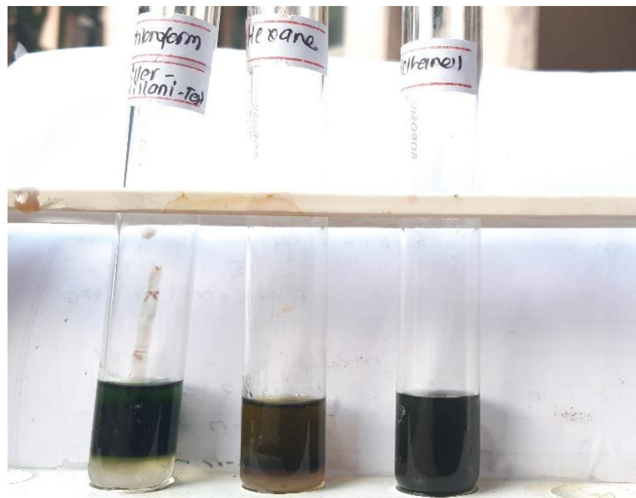
H. Test for Amino acids

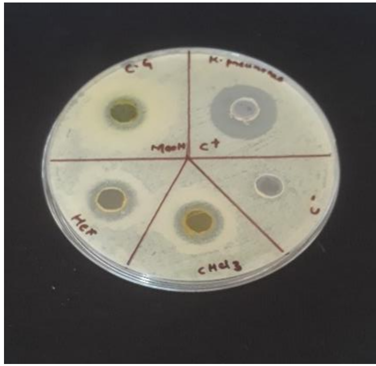


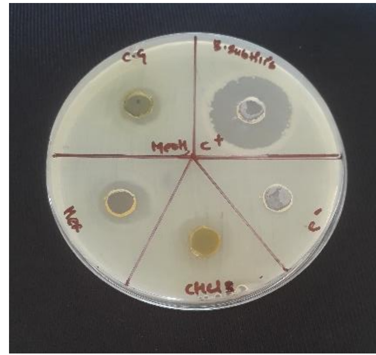
I. Test for Phenols

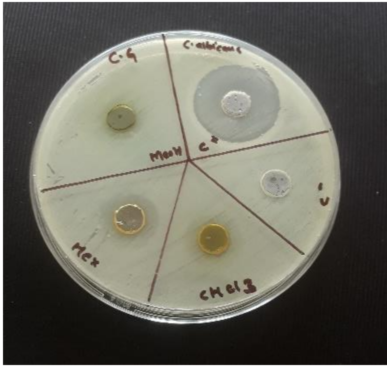
J. Test for Saponin

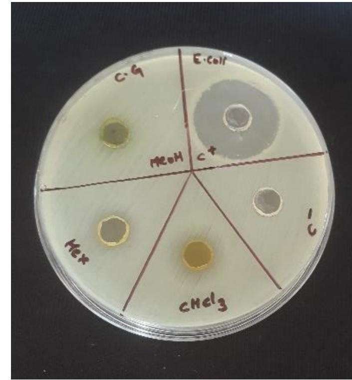
K. Test for Cardiac glycosides

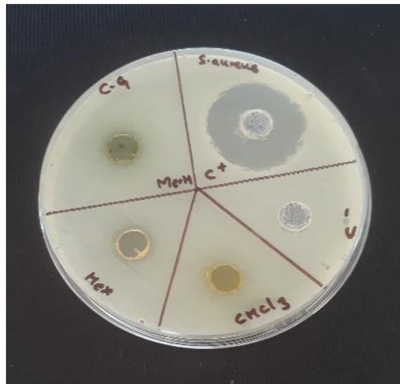












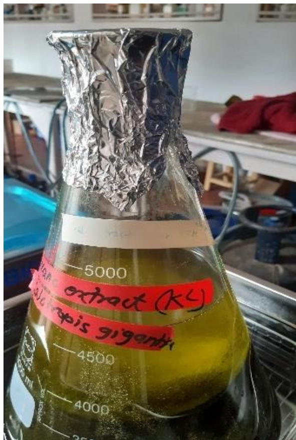
Antimicrobial activity of different extracts

Photos Taken during research work.

Collected plant material

Air-dried plant

Sample grinder



Extraction with solvent

Filtration Process

Sample powder

Solvent evaporation

Rotary Evaporator

Extracts

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प्राविधिक विशेषज्ञको प्रतिवेदन

१. नमूना परिक्षण गर्न पटाउने व्यक्ति/निकाय:- [illegible] ठमेल, काठमाडौं।

१(क) विद्यार्थीको नाम:- Ms. Soniya Bhusal (Roll no. 301/077,) & Mr. Keshar Lal Chaudhary (Roll no. 315/077)

२. प्राप्त नमूनाको विवरण:- हर्वेरियमका नमूनाहरु।

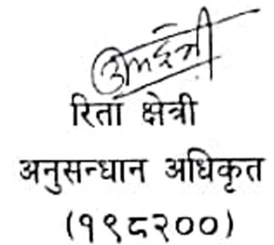
३. यस कार्यालयमा प्राप्त मिति:- २०८०/०६/१५

४. परिक्षणका आधारहरु:- (क) हर्वेरियममा भएका नमूनाहरु संगको तुलनात्मक अध्ययन (ख) सन्दर्भ सामग्रीहरुको अध्ययन ।

५. पहिचान प्रतिवेदन:- प्राप्त नमूनाको Morphological अध्ययन तथा यस राष्ट्रिय हर्वेरियम तथा बनस्पति प्रयोगशालाको हर्वेरियममा राखिएका नमूनाहरु संगको तुलनात्मक अध्ययन गर्दा उक्त नमूनाहरु निम्नानुसार भएको पहिचान हुन गएको।

S.N	Scientific Name	Family	Remarks
1	*Bauhinia variegata* L.	Fabaceae	
2	*Calotropis gigantea* (L.) W.T. Aiton	Apocynaceae	

६. परिक्षण गर्ने अधिकारी:-



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